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ExoMir™-MINI Kit Manual

Catalog #: 5148-01

GENERAL INFORMATION

Application

The ExoMir™-MINI Kit is designed for filtration-based capture of exosomes and other microparticles from small volumes of cell-free biofluids, and for extraction of RNA from the captured particles. An appropriate range of biofluid volumes to use with the kit are from about 0.1 mL – 2 mL, although volumes outside this range are not necessarily incompatible with the kit. Traditional methods for recovering exosomes and other microparticles involve centrifuging biofluid samples at increasing centrifugal force, to sequentially pellet the larger and smaller particles. To recover exosomes, ultracentrifugation of samples at relative centrifugal force of at least 100,000 x g for several hours or more is generally required.

Product Description

The ExoMir-MINI Kit uses an alternative approach for concentrating microparticles, in which they are captured by passing the samples over syringe filters. The filters are then flushed with an RNA extraction reagent to lyse the captured particles and release their contents. In the standard procedure, samples are passed over 2 filters connected in series, where the top filter has a larger pore size of approximately 200 nanometers to effectively capture larger particles such as apoptotic bodies and microvesicles, and the bottom filter has a smaller pore size of approximately 20 nanometers for capturing the smaller particles including exosomes. Examples of cell-free fluids that can be processed in this way include blood serum, plasma, urine, saliva, and eukaryotic cell culture media ("conditioned media"). After the sample has passed through the filters, they are disconnected and separately flushed with BiooPure™-MP to lyse the captured particles and release their contents. BiooPure-MP is a single-phase RNA extraction reagent containing guanidinium thiocyanate and phenol, which has been optimized to provide maximal recovery of the low-mass amounts of RNA in microparticles. Recovery of RNA is further improved by using the inert co-precipitant (linear acrylamide) included in the kit.

Kit Contents, Storage and Shelf Life

The ExoMir-MINI Kit contains sufficient components to treat and fractionate 10 samples of cell-free fluids, using 2 filters for each sample, and to extract RNA from both filters as well as from the flow-through filtrate.

The shelf life of the *ExoMir-MINI Kit* is 12 months when stored properly.

Kit Contents	Amount	Storage
ExoMir-MINI Syringe Filter Assembly	10 each of two-filter assemblies	20-25°C
PBS Diluent	30 mL	20-25°C
BiooPure™-MP RNA Isolation Reagent	30 mL	4°C
Co-precipitant (inert linear polyacrylamide) (18 µg/µL)	0.3 mL	-20 °C or 4˚C
RNase-free water (for wash solution)	12.5 mL	20-25°C
RNA Resuspension Solution (0.1 mM EDTA in nuclease-free water)	1.8 mL	20-25°C
1 mL syringe	2	20-25°C
3 mL syringe	2	20-25°C
6 mL syringe	1	20-25°C

Required Materials Not Provided

- 1-Bromo-3-Chloropropane (BCP); suggested source: Sigma Life Science (cat #B9673). Store at room temperature. Chloroform may be used as an alternative to BCP.
- Additional 3 mL and/or 5 mL Luer Lock syringes of appropriate size, for filtering additional samples
- Additional 3 mL Luer Lock syringes to flush the filters with BiooPure-MP (optional)
- Waste collection vessel to catch filtrate (for example, small plastic weigh-boat) (optional)
- 1.5 mL microfuge tubes to collect lysate and use for RNA extraction. Each prep requires 2 tubes.
- P-1000, P-200, P-10 pipettors and tips; note, small-bore tips (10 µL tips) are required for last step
- Isopropyl alcohol
- Ethanol (may be denatured with 5% isopropanol)
- Low-speed centrifuge for pelleting cells from media (optional)
- Vortex mixer
- Microcentrifuge capable of at least 14,000 x g or 12,000 rpm
- Heat block at 65°C for solubilizing RNA at end of procedure

Warnings and Precautions

BIOO strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or BIOO at **techsupport5@biooscientific.com**.

BiooPure-MP reagent contains phenol so be sure to wear lab coat, gloves, and safety glasses when using this reagent. **See MSDS available on the ExoMir product page at BiooScientific.com for more information related to precautions and hazards associated with use of the BiooPure-MP reagent.** Wear safety glasses when filtering samples and washing filters to avoid possible contact with sample or reagents.

SAMPLE PREPARATION

Low-speed centrifugation of sample to remove intact cells (optional)

To remove any contaminating cells, centrifuge sample for ~5 minutes at ~2,000 – 5,000 x g, ideally using a refrigerated centrifuge. The exact time and rcf (relative centrifugal force) to use is generally not critical, and can be adjusted according to sample type and volume. Avoid subjecting the sample to excessive centrifugal force as this may rupture any cells present in the sample. After centrifugation, remove the supernatant fluid by carefully decanting or aspirating it and save it for recovery of exosomes as described below. A small amount of supernatant fluid (~5% of the total sample volume) may be left behind to avoid contaminating the supernatant with any pelleted cells.

EXOMIR™-MINI KIT PROTOCOL

Reagent Preparation

- Prepare Wash Solution (75% ethanol) by adding 37.5 mL of ethanol to the bottle containing 12.5 mL of RNase-free water, provided with the kit, and mix thoroughly. Store at room temp.

- Label an ExoMir-MINI 2-filter stack and ensure that the top filter, labeled with a yellow dot, is securely attached to the bottom filter, labeled with a blue dot. Note, the ExoMir-MINI filters are provided as a stack of 2 connected filters, the top filter with a pore size of ~200 nm, the bottom filter with a pore size of ~ 20 nm. If only a single filter is used, to recover only larger particles using only the top filter, or to recover all particles using only the bottom filter, disconnect the filters before use.

Exosome Capture

1. **Dilute the sample.** Mix the sample with PBS provided with the kit, to give a final sample volume of at least 1 ml. Recommended dilutions for various sample volumes are shown in the Table below.

Starting sample volume	Amount of PBS to add
Less than 0.3 mL	To give final volume of 1 mL
0.3 – 0.5 mL	To give final volume of 2 mL
0.5 – 1 mL	To give final volume of 3 mL
1 – 2 mL	To give final volume of 4 – 5 mL

2. **Load sample into a syringe**
   Loaded the sample into a syringe of appropriate size, either by drawing it up into the syringe, or by removing the plunger of the syringe, then attaching the ExoMir-MINI filter stack, then pipetting or pouring the sample into the barrel of the syringe, and finally, re-inserting the plunger. Be sure to use a syringe with a Luer-lok fitting, so that it can be attached to the filter stack. Remove any air space in the syringe by inverting the assembly, removing the filter stack, depressing the plunger to remove the air space, then re-attaching the filter stack.

3. **Pass the sample through the filter stack**
   Ensure that the filters are securely attached to the syringe holding the sample. Position the assembly over a reservoir to collect the flow-through if desired. Gently depress the plunger to pass the media through the filters. The sample should flow through at a rate of ~ 1– 2 drops per second. During filtration, observe the top of the filters and tilt the syringe if needed to ensure that the sample contacts and wets the entire surface of the filters.

4. **Wash filters and remove residual fluid**
   After the sample has been filtered, load the syringe with 2 ml of 75% ethanol wash soln and flush it through the filter stack to wash the filters. Then remove the filter stack, retract the plunger of the syringe slightly, then separately re-attach each filter and gently depress the plunger to force the residual fluid out of the syringe filter. There should be a visible clearing of the residual fluid from the top chamber of the filter housing. Complete removal of residual fluid from the filter disks is important to minimize the volume of aqueous phase recovered during the RNA extraction step, which will allow the RNA precipitation step to be carried out in a single 1.5 mL tube.

5. **Remove Filters**
   After removing residual fluid, remove the filter disks from the syringe. The filter disks contain microparticles captured from the cell-free biofluid sample.

Lyse Captured Particles to Release RNA

Note: Wear safely goggles during this step!

1. Remove the plunger from a 3 mL syringe, attach the syringe barrel to a filter with captured particles, and load the syringe with 1 mL of BiooPure-MP RNA extraction reagent.

2. Position the outlet of the syringe filter over a 1.5 mL microfuge tube, then re-insert the plunger and gently depress it to slowly flush the filter with the BiooPure-MP reagent. Tilt the assembly if needed to allow the entire surface of the filter to contact the reagent. When the filter is saturated with reagent, slightly pump the plunger up and down several times to aid in complete mixing of the reagent with the trapped particles on the filter, then depress the plunger completely to recover all the BiooPure-MP lysate into the 1.5 mL tube. If the BiooPure-MP reagent passes through the filter too quickly (in less than ~4 seconds), re-apply the lysate back over the filter to ensure adequate time of contact of the trapped particles with the lysis solution. Note: The filter will remain green after flushing it with the BiooPure-MP reagent.

3. Repeat these steps to process the remaining filter disk. The preps may be stored at -20ºC at this step.

   Optional: To extract RNA from the Flow-Through (filtrate) or from the biofluid sample prior to filtration, mix 0.25 mL of sample with 1 mL BiooPure-MP, and vortex thoroughly.

RNA Extraction

1. Add 100 µL of 1-Bromo-3-Chloropropane (BCP) to the lysate. Alternatively, use 200 µL of chloroform. Vortex vigorously for ~ 10 seconds to create an emulsion of the lysate with the BCP and store the preps at room temp for ~5 minutes before proceeding to the next step. Note: If the preps were frozen prior to beginning this step, thaw at room temperature before adding the BCP.

2. Centrifuge preps for 15 minutes at 12,000 rpm, preferably at 4ºC.

3. Remove aqueous phase (the colorless top phase) to fresh 1.5 mL tubes. The volume of aqueous phase is usually ~450 – 550 µL from filter samples and ~550 – 650 µL from prefiltered or flow-through samples. Do not remove more than 0.75 mL of aqueous phase per 1.5 mL tube, since this would not allow sufficient space for isopropanol precipitation in the subsequent step. Avoid removing material from the interface or lower organic phase; however, the aqueous phase can be removed almost completely at this step since the amount of material at the interface is typically low compared to extractions from solid tissue.

4. Add 3 µL of the co-precipitant provided with the kit to the aqueous phase and vortex thoroughly, and then store the prep at room temp for ~5 minutes. It is important to vortex thoroughly (~5 – 10 seconds) to completely disperse the co-precipitant. Storing for a few minutes at room temp before adding the isopropanol improves recovery of RNA. It is recommended to pop-spin the prep (spin briefly in microfuge) to remove liquid from around rim of tube before opening it in the next step.

5. Add isopropanol equal or slightly greater than the volume of aqueous phase and vortex thoroughly. This can generally be approximated as 0.55 mL of isopropanol.

6. Store prep at -20ºC for at least 1 hour to precipitate the RNA. Note, this is a stopping place; storage can be extended to overnight or longer if desired.

7. To recover the RNA, centrifuge the prep for 15 - 20 minutes at maximum speed (at least 12,000 rpm) in a microcentrifuge, preferably at 4ºC.

8. Use a P-1000 to remove supernatant fluid down to level of ~30 µL. Do not aspirate all the fluid, as this runs the risk of losing the pellet of RNA. Small pellets are usually but not always visible at this step. Decanting the supernatant fluid instead of aspirating at this step runs the risk of losing the RNA pellet.

9. Wash the pellet with 0.9 mL of Wash Solution (75% ethanol). Vortex briefly but thoroughly enough to dislodge the pellet and ensure that the Wash Solution comes into contact with the entire inner surface of the tube.

10. Centrifuge for 10 minutes at maximum speed (at least 12,000 x g), preferably at 4ºC.

11. Carefully remove supernatant down to level of ~ 10- 20 µL. To minimize chance of aspirating the pellet, exercise care when removing the bottom portion of supernatant that lies directly above the pellet.

12. Re-spin tubes for ~ 5 seconds (the speed is not critical) to collect all residual fluid at bottom of tube. Orient tubes with hinges facing outward.

13. Use a fine-bore pipet tip (e.g. a P-10 tip) to completely remove all residual fluid from the bottom of the tube, taking care to avoid disturbing or aspirating the pellet. A small white pellet should be visible.

14. Resuspend pellet in ~ 50 µL of RNA Resuspension Solution provided with the kit. After adding the solvent, vortex briefly and pop-spin to recover fluid at bottom of tube. The RNA may be resuspended in as little as 25 µL to maximize the concentration.

15. Incubate the prep in 65ºC heat block for ~ 5 minutes to completely solubilize the RNA, then re-vortex and pop-spin. Store the RNA at -20ºC.

ADDITIONAL INFORMATION

Quantitation/Analysis of RNA from filters and Flow Through

The mass amount of RNA recovered from the exosomes and other microparticles in small volumes of cell-free fluids is too low to visualize by ethidium staining on agarose gels. The RNA can sometimes be detected by capillary electrophoresis on the Agilent Bioanalyzer, depending on the amount and type of sample. The RNA lacks the typical 18S and 28S ribosomal RNA peaks seen in RNA extracted from cells and tissues. Since microparticles are derived from the cytoplasmic cell compartment, they are not expected to contain appreciable amounts of genomic DNA.

The best way to confirm recovery of RNA from microparticles is by reverse transcription-polymerase chain reaction (RT-PCR) or by RT followed by quantitative PCR (RT-qPCR. A protocol for RT-qPCR detection of microRNAs in mouse serum based on the method described by Balcells et al (BMC Biotechnology 2011, 11:70) is described below.

Overview of method: PolyA polymerase and M-MLV Reverse Transcriptase are used to simultaneously add polyA tracts to the ends of RNAs in the sample, and convert the RNA templates to cDNA, thereby producing a cDNA library. This step uses a universal RT primer. The reactions are then diluted and used as templates for qPCR with microRNA-specific PCR primers.

Poly Adenylation/Reverse Transcription step

1. Assemble first 6 components as Master Mix as shown in Table below, scaling up for the number of RNA samples. Aliquot 4.2 µL of Master Mix to each 0.5 mL reaction tube, then add 2.5 µL of sample RNA to each tube. Total reaction volume = 10 µL.

Component	Volume per 10 µL Reaction	Source
10X PolyA Polymerase Buffer containing ATP	1 µL	New England Biolabs (make stock by mixing 150 uL of 10X PolyA buffer + 50 uL 10 mM ATP)
4dNTP mix, 1 mM each	1 µL	Bioo Scientific cat #370601, diluted to 1 mM in 7.5 mM Tris pH 7.5 / 0.1 mM EDTA
Universal RT primer, 10 uM	1 µL	IDT; see sequence in Table below
MML-V Reverse Transcriptase, 200 units/µL	0.5 µL	New England Biolabs
Poly A Polymerase from E.coli	0.2 µL	New England Biolabs (product includes 10X buffer and 10 mM ATP)
Nuclease free water	0.5 µL	High quality distilled deionized water; autoclaving not generally required
Sample RNA	5.8 µL	from ExoMir-MINI prep

2. Incubate reactions in thermalcycler or heat block **for one hour at 42ºC, then for 5 minutes at 85ºC** to inactivate enzymes. Pop-spin to collect all liquid at bottom of tube.

3. Dilute reactions by adding 90 uL of nuclease-free water. Vortex and pop-spin, then proceed to qPCR step or store at -20 ºC.

qPCR step (20 µL each reaction):

Component	Volume per 20 µL Reaction	Source
RT reaction	3 µL	Above reaction
Forward + Reverse Primers (2.5 uM concentration of each)	2 µL	See Table below for primer sequences
Nuclease-free water	5 µL	High quality distilled deionized water
2X qPCR MasterMix with SYBR Green	10 µL	Affymetrix, Syd Labs, BioRad, or other vendor

Typical profile: (modified as needed according to thermalcycler used and manufacturer's directions for the specific 2X SYBR MasterMix used)

1. hold 3 min at 95 ºC.

2. Cycle 40X: 15 sec at 95 ºC / 30 sec at 58 ºC.

Melt curve analysis: temperature interval 60 ºC - 95 ºC, in 0.5 ºC increments, with 5 sec dwell time at each temp.

miR	Accn# and Sequence	Forward Primer	Reverse Primer
mmu-miR-146a-5p	MIMAT0000158 UGAGAACUGAAUUCCAUGGGUU	5'-CGCACTGAGAACTGAATTCCATG Tm = 56.2	5'-AGCAGGTCCAGTTTTTTTTTTTTTTTTAACC Tm = 56.4
mmu-miR-195-5p	MIMAT0000225 UAGCAGCACAGAAAUAUUGGC	5'-CACCGAATAGCAGCACAGAAATATTG Tm = 56.4	5'-AGCAGGTCCAGTTTTTTTTTTTTTTTGC Tm = 56.8
mmu-miR-30e-5p	MIMAT0000248 UGUAAACAUCCUUGACUGGAAG	5'-AGACACTTGTAAACATCCTTGACTGG Tm = 56.9	5'-GGCACCTGGAGTTTTTTTTTTTTTTTCTT Tm = 56.5
mmu-miR-744-5p	MIMAT0004187 UGCGGGGCUAGGGCUAACAGCA	5'-TGCGGGGCTAGGGCTAAC Tm = 60.1	5'-GACCGACAGGCAGTTTTTTTTTTTTTTTTTGCT Tm = 59.9

Note, these microRNAs were reported by Mi et al to be useful as endogenous reference microRNAs for mouse serum ("Identification of Mouse Serum miRNA Endogenous References by Global Gene Expression Profiles", Mi Q-S et al. PLoS ONE vol 7 Issue 2 e31278, Feb 2012).

TROUBLESHOOTING

Filter clogs during sample filtration step, or filtration requires applying excessive force to the syringe

Recommended Actions:

- Carry out optional low-speed centrifugation to remove intact cells as described in Protocol.
- If filters still clog, re-spin sample at higher rcf and longer time, e.g. 15 min at 12,000 rpm, to pellet impurities such as fibrin clots.
- Reduce sample volume.
- If sample was blood plasma, consider using serum instead of plasma. To use serum, collect blood without anticoagulant and allow blood to clot, then centrifuge to fractionate sample into cellular components and non-cellular serum, and remove serum for use. Serum is depleted of blood clotting factors, which may help to avoid clogging. Serum samples that are turbid usually indicate presence of high concentration of lipids, and these may not be suitable for filtration.
- Prefilter sample using filters with larger pore size (700 nanometers). These are available from Bioo Scientific as a separate product (please contact techsupport5@biooscientific.com).
- Be sure to remove air space between the plunger and the filter stack
- Sometimes clogging is reduced by using a smaller syringe (e.g. 1 mL instead of 3 mL) to filter the sample, although this may require sequential loading of multiple aliquots of the sample into the smaller syringe.

Volume of Aqueous Phase Exceeds 0.75 mL

(Volume of aqueous phase at removed exceeds 0.75 mL, so that the RNA cannot be precipitated in single 1.5 mL tube)

Recommended Action: Completely remove residual fluid from the syringe filter disks before flushing with BiooPure-MP. Traces of fluid that may remain in the filter disk can be removed by tapping the outlet port onto paper towels. Precipitation of the RNA in 2 mL tubes is not recommended because the shape of the tube does not taper to a point, so the RNA/co-precipitant does not form a visible pellet; also, removing the last traces of supernatant fluid from 2 mL tubes without disturbing the RNA is difficult.

Microparticle RNA is contaminated with cellular RNA

(The microparticle RNA is contaminated with cellular RNA, which can be seen if an Agilent Bioanalyzer trace shows presence of peaks of large ribosomal RNAs)

Recommended Action: Carry out optional low-speed spin to remove cells. Contaminating high molecular weight cellular RNA is generally not present, because most cell-free fluids have high levels of endogenous RNase that will degrade unprotected extracellular RNA.

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Product	Catalog Number
ExoMir™ Kit	5145-01
ExoMir™ PLUS Kit	5145-01

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Product	Catalog Number
D-pop™ Kit	5157-01

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