Tumor exosomes: from loading to release

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Chapter 5

Analysis of Viral MicroRNA Exchange via Exosomes In Vitro and In Vivo

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Abstract

The isolation and analysis of microRNAs (miRNAs) contained in microvesicles and in particular nano-sized exosomes has become an increasingly important tool to understand their widespread impact in various fundamental and interactive cellular processes. Fundamental studies regarding exosome biogenesis and miRNA sorting may ultimately unravel their potency as a promising class of highly specific disease biomarkers. Here we describe the methods and protocols used in our laboratory to isolate and purify exosomes, how we extract the (small) RNA content, how to analyze copy numbers, and finally how to measure exosome-mediated transfer of these molecules into recipient cells. Our techniques have been optimized for the detection of Epstein–Barr virus (EBV)-encoded miRNAs that are loaded into exosomes. We discuss how a focus on EBV-miRNA detection may yield important new clues into exosome-mediated cross talk by B cells in humans.

Key words Exosomes, Viral miRNA, Genetic transfer, Cell–cell communication, Biomarker

1 Introduction

Horizontal transfer of noncoding regulatory microRNAs (miRNAs) is recently emerging as an evolutionary conserved pathway for cell–cell communication although little in vivo evidence has been provided yet [1]. Multiple in vitro studies have now shown that mature "functional" miRNAs can be secreted via nanometer-sized vesicles that are derived from endosomal compartments and usually termed collectively as "exosomes." Exosomes as well as other microvesicles of varying size and origin (that are not discussed here) are secreted by many cell types, including stem cells, immune cells, blood cells, placental cells, and in particular cancer cells [2–6]. It is possible, if not likely, that nonautonomous miRNAs upon transfer to

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recipient cells play an important role in all biological processes that require intricate cell–cell communication. One biological advantage could be that exosome-mediated delivery of miRNAs is a powerful mechanism to influence the physiology of target cells, by directly reducing the stability and thus preventing translation of specific target messenger (m)RNAs into functional proteins. Such intricate communications include, but are not restricted to, the development and function of the immune system and tissue homeostasis [7] but may also promote oncogenic transformation events upon deregulation or virus infection [8, 9]. Indeed viruses exploit the vesicle-dependent miRNA-secretion system by loading their own sets of (viral) miRNAs in exosomes to affect mRNA-levels in recipient cells [10]. Alternatively, viral infection may also affect the composition of cellular miRNAs that are secreted by the infected cell, thus indirectly altering cell–cell communication [11]. Epstein–Barr virus (EBV) is a common human (γ-herpes) virus that encodes viral miRNAs [12] of at least 44 distinct mature sequences [13]. Interestingly, the viral miRNA expression pattern differs between the various latency stages of the virus life cycle, and is deregulated in tumors. Using a principal component analysis on the complete miRNA expression profiles, the viral miRNAs were sufficient to distinguish four EBV-associated tumor types, highlighting their association with oncogenic processes in vivo [14]. Apart from EBV being a large DNA virus, recently the bovine leukemia virus (BLV), a retrovirus, was reported to encode for viral miRNA species as well. Both these DNA and RNA viruses encode for miRNAs that contribute to B-cell tumorigenesis stressing the importance of further unraveling their function in vivo [15, 16].

The ability to distinguish virus- from host cellular miRNAs by PCR techniques makes EBV a valuable highly specific tool to study the effects of miRNAs in human oncogenesis and to study their presumed nonautonomous function in (noninfected) recipient cells in vitro and in vivo. Indeed exploiting the strict B-cell tropism of EBV in circulation, we were able to provide evidence for horizontal genetic transfer between human cells in part due to the unique sequence of EBV miRNAs [10]. Here we describe in detail the methodology used to detect viral miRNAs in exosomes and how detection of viral miRNAs in infected and noninfected cells can be used to "track" miRNA exchange in vivo. We propose that this methodology could be extrapolated to other viral infection models and thus provide a unique model to study the transfer of genetic material between infected and noninfected cells in animals and/or humans.

Apart from studying virus-derived miRNA exchange between cells in vivo, it is evident that secretion of nucleic acids including particular miRNAs, the expression of which is dramatically altered in disease, can be used as surrogate biomarkers [6, 17, 18]. MiRNA-containing vesicles can be isolated from a variety of

biological fluids including serum, plasma, cerebrospinal fluid, saliva, urine, and milk. Although some secreted miRNAs in the peripheral blood may bind to soluble proteins such as Ago2 [19, 20], Gallo et al. reported that the majority of circulating miRNAs in serum and saliva is in fact associated with microvesicles and exosomes [21]. Isolating the circulating RNA that is protected from degradation by external RNAses in vesicles may have considerable advantages over isolating total RNA in whole serum. It is likely that vesicle-associated RNA is enriched for RNAs with biomarker potential compared to circulating RNAs that are derived from passive leakage or dying cells which may not be associated with the disease process.

2 Materials

2.1 Transfection of Epithelial Cells for Exosome Isolation

1. DMEM with 4.5 g/l glucose w/o L-glutamine, 5 % FBS (exosome-depleted; *see* Subheading 3), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine.
2. PBS (8.2 g/l NaCl, 3.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.3 g/l $NaH_2PO_4 \cdot 2H_2O$, pH 7.4).
3. Lipofectamine 2000 reagent (Life Technologies) (*see* **Note 1**).
4. Plasmid DNA (10 μg).
5. 10 ml tube.
6. T75 tissue culture flask.

2.2 Exosome Isolation by Differential Centrifugation

1. RPMI-1640 with 25 mM HEPES, 5 % FBS (exosome-depleted), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine.
2. PBS (8.2 g/l NaCl, 3.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.3 g/l $NaH_2PO_4 \cdot 2H_2O$, pH 7.4).
3. Trypan blue.
4. Ultra-Clear Centrifuge Tubes (Beckman Coulter, Inc).
5. Ultracentrifuge with, e.g., SW32Ti rotor (Beckman Coulter, Inc.).

2.3 Labeling of Exosomes with PKH67 Green Fluorescent Linker Dye

1. PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma-Aldrich) (*see* **Note 2**).
2. 0.1 % BSA/PBS.
3. Ultra-Clear Centrifuge Tubes (Beckman Coulter, Inc).
4. Ultracentrifuge with, e.g., SW32Ti rotor (Beckman Coulter, Inc.).
5. PBS (8.2 g/l NaCl, 3.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.3 g/l $NaH_2PO_4 \cdot 2H_2O$, pH 7.4).

2.4 Exosome Transfer Using a 1.0 μm Transwell Coculture System

1. RPMI-1640 with 25 mM HEPES, 5 % FBS (exosome-depleted), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine.
2. Trypan blue.
3. PBS (8.2 g/l NaCl, 3.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.3 g/l $NaH_2PO_4 \cdot 2H_2O$, pH 7.4).
4. ThinCerts TC Inserts, 24 well, 1.0 μm pore size, transparent (Greiner bio-one) (*see* **Note 3**).

2.5 RNase A Treatment

1. Ribonuclease A from bovine pancreas.
2. Nuclease-free water.

2.6 Separation B Cell/Non-B Cell Fraction from PBMCs

1. 0.1 % BSA/PBS (cold).
2. CD19 (Pan B) Dynabeads (Dynal Biotech) (*see* **Note 4**).
3. Magnetic device (e.g., from Dynal Biotech).
4. TRIzol Reagent (Life Technologies) (*see* **Note 5**).

2.7 RNA Isolation

1. TRIzol Reagent (Life Technologies) (*see* **Note 5**).
2. Chloroform.
3. Glycogen (special quality for molecular biology, free from nucleic acids, proteases, DNases, and RNases).
4. Isopropyl alcohol.
5. 75 % Ethanol.
6. Nuclease-free water.

2.8 cDNA Synthesis

1. TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems; No. 4366596) (*see* **Note 6**), containing 10× Reverse Transcriptase buffer, dNTP Mix (100 mM), RNase Inhibitor (10 U/μl), MultiScribe Reverse Transcriptase (50 U/μl).
2. 10× Multiplex stem-loop RT Primer Mix, containing RT primers for 40 EBV miRNAs (Applied Biosystems) (*see* **Notes 7** and **8**) (125 nM of each primer).
3. Nuclease-free water.

2.9 miRNA PCR

1. TaqMan Universal PCR MasterMix, No AmpErase UNG (Applied Biosystems; No. 4324018) (*see* **Note 6**).
2. 10× MiRNA specific forward primer (Applied Biosystems) (*see* **Note 8**) (15 μM).
3. 10× Universal reverse primer (Applied Biosystems) (*see* **Note 8**) (7 μM).
4. 10× MiRNA specific probe (Applied Biosystems) (*see* **Note 8**) (2 μM, keep dark).
5. Water.
6. Real-time PCR system, e.g., TaqMan ABI 7500 Fast.

3 Methods

3.1 Transfection of Epithelial Cells for Exosome Isolation

1. Culture epithelial cell line(s) (e.g., HEK293, HeLa) in DMEM, supplemented with 10 % FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 mM glutamine at a density of 5.0×10^6 cells/T75.
2. Culture cells for 24 h at 37 °C and 5 % CO_2.
3. Add 10 μg plasmid DNA to 2 ml of DMEM (w/o FBS, penicillin G, streptomycin sulfate, and glutamine) in a 10 ml tube.
4. Vortex vigorously for 10 s.
5. Add 30 μl of Lipofectamine 2000 reagent to the mixture.
6. Vortex vigorously for 10 s.
7. Incubate mixture for 30 min at room temperature.
8. Meanwhile, remove medium from the flask and wash cells with PBS.
9. Add 8 ml of DMEM supplemented with 5 % exosome-depleted FBS (*see* **Note 9**), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 mM glutamine [exo-free DMEM +/+] to your flask.
10. Add the 2 ml of DNA/Lipofectamine 2000 mixture.
11. Incubate for 4 h at 37 °C and 5 % CO_2.
12. Replace transfection medium with 15 ml exo-free DMEM +/+.
13. After 24 h collect medium and proceed with **step 5** of the "Exosome isolation by differential centrifugation" protocol.
14. Optionally, add 15 ml of fresh exo-free DMEM +/+ for an additional 24 h of culturing and exosome harvesting.

3.2 Exosome Isolation by Differential Centrifugation

1. Routinely culture EBV-positive lymphoblastoid cell lines (LCLs) in RPMI-1640, supplemented with 10 % FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 mM glutamine at a density of 0.5×10^6 cells/ml.
2. Wash LCLs with PBS and resuspend cells at a density of 0.5×10^6 cells/ml in RPMI-1640, supplemented with 5 % exosome-depleted FBS (*see* **Note 9**), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 mM glutamine. A typical exosome culture consists of 100×10^6 cells/200 ml RPMI-1640.
3. Culture cells for 48 h at 37 °C and 5 % CO_2.
4. Resuspend cells by careful repetitive pipetting to gain a single-cell suspension.
5. Check viability by trypan blue exclusion. If the viability is at least 95 % continue with exosome isolation (*see* **Note 10**).

6. Centrifuge 2× at 500 × *g* for 10 min and 2× at 2,000 × *g* for 15 min in a tabletop centrifuge to remove cells and cellular debris.
7. After each step carefully remove (exosome containing) supernatant to a fresh tube leaving few ml behind.
8. Centrifuge 2× at 10,000 × *g* for 30 min, slow brake, at 4 °C in Ultra-Clear centrifuge tubes in an SW32Ti rotor (Beckman Coulter, Inc.).
9. After each step carefully remove (exosome containing) supernatant to a fresh Ultra-Clear tube leaving few ml behind.
10. Centrifuge 1× at 70,000 × *g* for 1 h, slow brake, at 4 °C in Ultra-Clear centrifuge tubes in an SW32Ti rotor (Beckman Coulter, Inc.).
11. Carefully remove exosome-depleted supernatant.
12. Pool exosome-containing pellets with additional PBS.
13. Centrifuge 1× at 70,000 × *g* for 1 h, no brake, at 4 °C in Ultra-Clear centrifuge tubes in an SW32Ti rotor (Beckman Coulter, Inc.).
14. Carefully remove the supernatant without disturbing the exosome pellet by pipetting.
15. Resuspend exosome pellet in 200 µl of PBS.
16. Use immediately or store at −80 °C (*see* **Note 11**).

3.3 Labeling of LCL-Released Exosomes with PKH67 Green Fluorescent Linker Dye

1. For labeling a complete exosome prep (200 µl, *see* "Exosome isolation by differential centrifugation" protocol) add 180 µl of diluent C.
2. Dilute 1 µl of PKH67 dye (*see* **Note 12**) in 50 µl of diluent C.
3. Add 20 µl of the diluted PKH67 dye to the diluted exosome prep and mix by pipetting.
4. Incubate at room temperature for 3 min.
5. Add 0.1 % BSA/PBS.
6. Centrifuge 1× at 70,000 × *g* for 1 h, no brake, at 4 °C in Ultra-Clear centrifuge tubes in an SW32Ti rotor (Beckman Coulter, Inc.).
7. Carefully remove the supernatant without disturbing the exosome pellet by pipetting.
8. Resuspend exosome pellet in 200 µl of PBS.
9. Use immediately or store at −80 °C.

3.4 Exosome Transfer Using a 1.0 µm Transwell Coculture System

1. Routinely culture EBV-positive LCLs in RPMI-1640, supplemented with 10 % FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 2 mM glutamine at a density of 0.5×10^6 cells/ml.

2. Check viability by trypan blue exclusion. If the viability is at least 95 % continue with coculture experiment.
3. Wash cells with PBS.
4. Plate recipient cells of choice (e.g., HEK293 cells, HeLa cells, iMoDC) in the lower compartment in 1 ml complete culture medium.
5. Plate 5×10^5 LCLs in 350 µl of RPMI-1640 in the upper compartment of a 24-well 1.0 µm (*see* **Note 13**) transwell. Be sure not to touch the porous membrane (optional: label cells with PKH67 green fluorescent linker dye according to manufacturers' protocol to visualize exosome transfer).
6. Incubate at 37 °C and 5 % CO_2 for up to 72 h.
7. Carefully remove upper compartment.
8. Harvest recipient cells.
9. Wash cells 2× with PBS to remove exosomes that are not taken up.
10. Add 1 ml of TRIzol to cell pellet.
11. Incubate for 10–20 min at room temperature, store at −80 °C, or proceed with RNA isolation.

3.5 RNase A Treatment

1. Add RNase A to purified exosomes at a final concentration of 400 ng/µl.
2. Incubate at 37 °C for 1 h.
3. Add TRIzol Reagent to a final volume of 1 ml (if >200 µl exosome prep is used split sample for RNA isolation).
4. Incubate for 10–20 min at room temperature, store at −80 °C, or proceed with RNA isolation.

3.6 Separation B Cell/Non-B Cell Fraction from PBMCs

1. Use freshly isolated PBMCs or thaw PBMCs by carefully adding 10 ml of cold 0.1 % BSA/PBS (use 10 ml tubes).
2. Centrifuge at $260 \times g$ for 5 min at 4 °C.
3. Resuspend cell pellet in 2 ml of cold 0.1 % BSA/PBS.
4. Count viable cells using a hemocytometer.
5. Add cold 0.1 % BSA/PBS up to 10 ml.
6. Centrifuge at $260 \times g$ for 5 min at 4 °C.
7. Resuspend cell pellet in 250 µl (for up to 2×10^6 total cells) of cold 0.1 % BSA/PBS (use 1.5 ml screw cap tubes).
8. Add 10 µl of CD19 Dynabeads (for up to 2×10^6 total cells).
9. Rotate for 30 min at 4 °C (for depletion protocol).
10. Place tubes in the magnetic device for 2 min.
11. Carefully remove supernatant (= non-B cell fraction).

12. Centrifuge at 260 × *g* for 5 min.
13. Add 800 µl of TRIzol Reagent to the cell pellet.
14. Wash Dynabeads (= CD19-positive B cell fraction) with 1 ml 0.1 % BSA/PBS.
15. Place tubes in the magnetic device for 2 min.
16. Carefully remove supernatant.
17. Add 800 µl TRIzol Reagent to the B cell fraction attached to the Dynabeads (it is not necessary to remove the beads before RNA isolation).
18. Store both B cell and non-B cell fraction at −80 °C or proceed with RNA isolation (*see* **Note 14**).

3.7 RNA Isolation

1. Add 0.2 ml of chloroform per 1 ml of TRIzol Reagent.
2. Shake tubes vigorously for 15 s.
3. Incubate at room temperature for 2 to 3 min.
4. Centrifuge at 12,000 × *g* for 15 min at 4 °C.
5. Transfer the aqueous phase to a new tube.
6. Add 5 µl of glycogen (= 100 µg) if low amounts of RNA are expected (i.e., exosomes).
7. Add 0.5 ml of isopropyl alcohol per 1 ml of TRIzol Reagent.
8. Incubate at room temperature for 10 min.
9. Centrifuge at 12,000 × *g* for 10 min at 4 °C.
10. Wash pellet once with 1 ml of 75 % ethanol.
11. Mix the sample by vortexing (briefly).
12. Centrifuge at 7,500 × *g* for 5 min at 4 °C.
13. Briefly dry the RNA pellet.
14. Dissolve RNA in 10 µl of nuclease-free water.
15. Determine RNA quantity and purity with nanodrop and/or BioAnalyzer.

3.8 cDNA Synthesis

1. Prepare master mix: 5.66 µl of nuclease-free water, 1.50 µl of 10× RT buffer, 0.15 µl of dNTP (100 mM each), 0.19 µl of RNase inhibitor (10 U/µl), and 1.00 µl RT of enzyme (50 U/µl).
2. Add 1.50 µl of 10× Multiplex stem-loop RT Primer Mix (*see* **Note 8**) (final concentration for each primer 12.5 nM).
3. Dilute 10–100 ng RNA in 5 µl of nuclease-free water.
4. Add 10 µl of master mix (including stem-loop RT primer) to the diluted RNA.
5. Incubate for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C.
6. Dilute cDNA if necessary.

3.9 miRNA PCR

1. Prepare master mix: 5 μl of 2× TaqMan Universal PCR MasterMix, 1 μl of cDNA, 1 μl of 10× miRNA-specific forward primer (*see* **Note 8**) (final concentration 1.5 μM), 1 μl of 10× universal reverse primer (*see* **Note 8**) (final concentration 0.7 μM), 1 μl of 10× miRNA-specific probe (*see* **Note 8**) (final concentration 0.2 μM, keep dark), and 1 μl of water.
2. Incubate samples (TaqMan ABI 7500 Fast) for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.
3. Measure each sample in duplicate and include positive and negative controls.

4 Notes

1. Lipofectamine 2000 can be replaced by any other transfection reagent that is optimized for the plasmid type and cell line of interest.
2. Other kits for membrane labeling may be used, but need to be optimized for staining the purified exosomes. This is important as overstaining may reduce the integrity of the exosomes. The concentration of dye, amount of exosomes, and incubation time are paramount for staining exosomes purified from different cell types.
3. Transparent tissue culture inserts of other companies or different size wells may be used. However, the pore size of 1.0 μm in case of LCL studies is crucial (*see* **Note 13**).
4. CD19 Dynabeads may be replaced by magnetic beads from other companies, but need to be optimized for B cell depletion from PBMCs (*see* **Note 14**).
5. Other RNA isolation reagents or kits may work; however TRIzol Reagent gives high, reproducible yield and purity, as measured by Agilent 2100 Bioanalyzer. Importantly, TRIzol gives high copynumbers for all EBV-miRNAs tested using Taqman stem-loop RT-PCR.
6. Other miRNA RT kit or PCR MasterMix may be used but should be tested for compatibility with the PCR system used.
7. Sequences of EBV-miRNAs: Sequences are taken from Sanger miRNA Registry (http://microrna.sanger.ac.uk) [13] (Table 1).
8. Sequences of EBV-miRNA primers and probes used (Table 2).
9. Deplete FBS from exosomes by centrifugation for 16 h at 70,000 × *g* at 4 °C, and take the fluid phase without disturbing the pellet.

Table 1
Sequences of EBV-miRNAs

EBV-miR	Mature sequence
BHRF1-1	UAACCUGAUCAGCCCCGGAGUU
BHRF1-2	UAUCUUUUGCGGCAGAAAUUGA
BHRF1-2*	AAAUUCUGUUGCAGCAGAUAGC
BHRF1-3	UAACGGGAAGUGUGUAAGCACA
BART1-5p	UCUUAGUGGAAGUGACGUGCUGUG
BART1-3p	UAGCACCGCUAUCCACUAUGUC
BART1-3p +1	UAGCACCGCUAUCCACUAUGUCU
BART2-5p	UAUUUUCUGCAUUCGCCCUUGC
BART2-3p	AAGGAGCGAUUUGGAGAAAAUAAA
BART3	CGCACCACUAGUCACCAGGUGU
BART3*	ACCUAGUGUUAGUGUUGUGCU
BART4	GACCUGAUGCUGCUGGUGUGCU
BART5	CAAGGUGAAUAUAGCUGCCCAUCG
BART6-5p	UAAGGUUGGUCCAAUCCAUAGG
BART6-3p	CGGGGAUCGGACUAGCCUUAGA
BART7	CAUCAUAGUCCAGUGUCCAGGG
BART7*	CCUGGACCUUGACUAUGAAACA
BART8	UACGGUUUCCUAGAUUGUACAG
BART8*	GUCACAAUCUAUGGGGUCGUAGA
BART9	UAACACUUCAUGGGUCCCGUAGU
BART9*	UACUGGACCCUGAAUUGGAAAC
BART10	UACAUAACCAUGGAGUUGGCUGU
BART10*	GCCACCUCUUUGGUUCUGUACA
BART11-5p	UCAGACAGUUUGGUGCGCUAGUUG
BART11-3p	ACGCACACCAGGCUGACUGCC
BART12	UCCUGUGGUGUUUGGUGUGGUU
BART13	UGUAACUUGCCAGGGACGGCUGA
BART13*	AACCGGCUCGUGGCUCGUACAG
BART14	UAAAUGCUGCAGUAGUAGGGAU
BART14*	UACCCUACGCUGCCGAUUUACA
BART15	GUCAGUGGUUUUGUUUCCUUGA

(continued)

Table 1
(continued)

EBV-miR	Mature sequence
BART16	UUAGAUAGAGUGGGUGUGUGCUCU
BART17-5p	UAAGAGGACGCAGGCAUACAAG
BART17-3p	UGUAUGCCUGGUGUCCCCUUAGU
BART18-5p	UCAAGUUCGCACUUCCUAUACA
BART18-3p	UAUCGGAAGUUUGGGCUUCGUC
BART19-5p	ACAUUCCCCGCAAACAUGACAUG
BART19-3p	UUUUGUUUGCUUGGGAAUGCU
BART20-5p	UAGCAGGCAUGUCUUCAUUCC
BART20-3p	CAUGAAGGCACAGCCUGUUACC
BART22	UUACAAAGUCAUGGUCUAGUAGU

*It indicates the less-often occurring sequence as opposed to the dominant sequence in the opposite strand
Sequences are taken from Sanger miRNA Registry (http://microrna.sanger.ac.uk) [13]

10. Avoid contaminating nano-particles from dead cells as much as possible. Confirm purity with EM.
11. The exosome isolation protocol by differential ultracentrifugation was verified by sucrose cushioning with 1.23 and 1.06 g/ml layers (these densities were chosen based on continuous sucrose gradient studies [22, 23]). Both protocols—with or without additional sucrose purification—showed comparable results in proteomic, RT-PCR, and electron microscopy analysis.
12. PKH67 dye and subsequent steps should be kept in the dark.
13. The pore size of 1.0 μm is crucial for analysis of LCL exosome transfer using a transwell coculture system. Using a larger pore size of 3.0 μm allows passage of LCL cells through the membrane causing contamination of the recipient cells. A smaller pore size of 0.4 μm can be used as well but impairs efficient transfer of exosomes [10, 22]. It is therefore recommended to use a membrane with 0.4 μm as a negative control that precludes exosome transfer. Although exosomes are generally 100 nm or smaller in size, it is unlikely that exosomes operate alone [22].
14. Check the purity of B and non-B cell fraction by FACS analysis. Specifically the non-B cell fraction should be depleted from B cells (<3 % B cell contamination) to be able to explore transfer of viral miRNAs produced by EBV-infected to noninfected cells.

Table 2
Sequences of EBV-miRNA primers and probes used

EBV-miR	Oligo	Sequence
BHRF1-1	RT	CTCAACTGGT GTCGTGGAGTCGGCAATTCAGTTGAGAACTCCGG
	Forward	ACACTCCAGCTGGGTAACCTGATCAGCCCC
	Probe	TTCAGTTGAGAACTCCGG
BHRF1-2	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCAATTT
	Forward	ACACTCCAGCTGGGTATCTTTTGCGGCAGA
	Probe	TTCAGTTGAGTTCAATTT
BHRF1-2*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTATCTG
	Forward	ACACTCCAGCTGGGAAATTCTGTTGCAGCA
	Probe	TTCAGTTGAGGCTATCTG
BHRF1-3	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTGTGCTT
	Forward	ACACTCCAGCTGGGTAACGGGAAGTGTGTA
	Probe	TTCAGTTGAGGTGTGCTT
BART1-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAGCACG
	Forward	ACACTCCAGCTGGGTCTTAGTGGAAGTGAC
	Probe	TTCAGTTGAGACAGCACG
BART1-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGACATAG
	Forward	ACACTCCAGCTGGGTAGCACCGCTATCCAC
	Probe	TTCAGTTGAGAGACATAG
BART2-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCAAGGGC
	Forward	ACACTCCAGCTGGGTATTTTCTGCATTCGC
	Probe	TTCAGTTGAGGCAAGGGC
BART2-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTTATTTT
	Forward	ACACTCCAGCTGGGAAGGAGCGATTTGGAG
	Probe	TTCAGTTGAGTTTATTTT
BART3	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCACAAC
	Forward	ACACTCCAGCTGGGAACCTAGTGTTAGTGT
	Probe	TTCAGTTGAGAGCACAAC
BART3*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACACCTGG
	Forward	ACACTCCAGCTGGGCGCACCACTAGTCACC
	Probe	TTCAGTTGAGACACCTGG

(continued)

Table 2
(continued)

EBV-miR	Oligo	Sequence
BART4	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCACACC
	Forward	ACACTCCAGCTGGGGACCTGATGCTGCTGG
	Probe	TTCAGTTGAGAGCACACC
BART5	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGATGGGC
	Forward	ACACTCCAGCTGGGCAAGGTGAATATAGCT
	Probe	TTCAGTTGAGCGATGGGC
BART6-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCTATGGA
	Forward	ACACTCCAGCTGGGTAAGGTTGGTCCAATC
	Probe	TTCAGTTGAGCCTATGGA
BART6-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCTAAGGC
	Forward	ACACTCCAGCTGGGCGGGGATCGGACTAGC
	Probe	TTCAGTTGAGTCTAAGGC
BART7	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCCTGGAC
	Forward	ACACTCCAGCTGGGCATCATAGTCCAGTGT
	Probe	TTCAGTTGAG CCCTGGAC
BART7*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTTTCAT
	Forward	ACACTCCAGCTGGGCCTGGACCTTGACTAT
	Probe	TTCAGTTGAGTGTTTCAT
BART8	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTGTACAA
	Forward	ACACTCCAGCTGGGTACGGTTTCCTAGATT
	Probe	TTCAGTTGAGCTGTACAA
BART8*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACGACC
	Forward	ACACTCCAGCTGGGGTCACAATCTATGGGG
	Probe	TTCAGTTGAGCTACGACC
BART9	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACGGGA
	Forward	ACACTCCAGCTGGGTAACACTTCATGGGTC
	Probe	TTCAGTTGAGCTACGGGA
BART9*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTTTCCAA
	Forward	ACACTCCAGCTGGGTACTGGACCCTGAATT
	Probe	TTCAGTTGAGGTTTCCAA

(continued)

Table 2
(continued)

EBV-miR	Oligo	Sequence
BART10	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAGCCAA
	Forward	ACACTCCAGCTGGGACATAACCATGGAGTT
	Probe	TTCAGTTGAGACAGCCAA
BART10*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTACAGA
	Forward	ACACTCCAGCTGGGGCCACCTCTTTGGTTC
	Probe	TTCAGTTGAGTGTACAGA
BART11-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAACTAGC
	Forward	ACACTCCAGCTGGGTCAGACAGTTTGGTGC
	Probe	TTCAGTTGAGCAACTAGC
BART11-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGCAGTCA
	Forward	ACACTCCAGCTGGGACGCACACCAGGCTGA
	Probe	TTCAGTTGAGGGCAGTCA
BART12	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAACCACA
	Forward	ACACTCCAGCTGGGTCCTGTGGTGTTTGGT
	Probe	TTCAGTTGAGAAACCACA
BART13	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAGCCGT
	Forward	ACACTCCAGCTGGGTGTAACTTGCCAGGGA
	Probe	TTCAGTTGAGTCAGCCGT
BART13*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG CTGTACGA
	Forward	ACACTCCAGCTGGGAACCGGCTCGTGGCTC
	Probe	TTCAGTTGAGCTGTACGA
BART14	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTAAATC
	Forward	ACACTCCAGCTGGGTACCCTACGCTGCCGA
	Probe	TTCAGTTGAGTGTAAATC
BART14*	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATCCCTAC
	Forward	ACACTCCAGCTGGGTAAATGCTGCAGTAGT
	Probe	TTCAGTTGAGATCCCTAC
BART15	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAAGGAA
	Forward	ACACTCCAGCTGGGGTCAGTGGTTTTGTTT
	Probe	TTCAGTTGAGTCAAGGAA

(continued)

Table 2
(continued)

EBV-miR	Oligo	Sequence
BART16	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGAGCACA
	Forward	ACACTCCAGCTGGGTTAGATAGAGTGGGTG
	Probe	TTCAGTTGAGAGAGCACA
BART17-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTTGTATG
	Forward	ACACTCCAGCTGGGTAAGAGGACGCAGGCA
	Probe	TTCAGTTGAGCTTGTATG
BART17-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG ACTAAGGG
	Forward	ACACTCCAGCTGGGTGTATGCCTGGTGTCC
	Probe	TTCAGTTGAGACTAAGGG
BART18-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTATAGG
	Forward	ACACTCCAGCTGGGTCAAGTTCGCACTTCC
	Probe	TTCAGTTGAGTGTATAGG
BART18-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG GACGAAGC
	Forward	ACACTCCAGCTGGGTATCGGAAGTTTGGGC
	Probe	TTCAGTTGAGGACGAAGC
BART19-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCATGTCAT
	Forward	ACACTCCAGCTGGGACATTCCCCGCAAACA
	Probe	TTCAGTTGAGCATGTCAT
BART19-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCATTCC
	Forward	ACACTCCAGCTGGGTTTTGTTTGCTTGGGA
	Probe	TTCAGTTGAGAGCATTCC
BART20-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGAATGAA
	Forward	ACACTCCAGCTGGGTAGCAGGCATGTCTTC
	Probe	TTCAGTTGAGGGAATGAA
BART20-3p	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGTAACAG
	Forward	ACACTCCAGCTGGGCATGAAGGCACAGCCT
	Probe	TTCAGTTGAGGGTAACAG
BART22	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTACTAG
	Forward	ACACTCCAGCTGGGTTACAAAGTCATGGTC
Universal R	Probe	TTCAGTTGAGACTACTAG
	Reverse	CTCAACTGGTGTCGTGGAGT CGGCA

*It indicates the less-often occurring sequence as opposed to the dominant sequence in the opposite strand

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