Exo-FBS™ Exosome-depleted FBS

Cat #s EXO-FBS/Hlxxx

User Manual

Store at -20°C upon arrival

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 4-2015-09-01)

Contents

- ExoQuick exosome isolation methods are a patented technology. Antes, T. et al. Methods for Microvesicle Isolation and Selective Removal. Patent No.: US 9,005,888 B2

- The process of manufacturing of Exo-FBS is a patented method in Patent No.: US 9,005,888 B2.

List of Components

Cat#	Description	Size
EXO-FBS-50A-1	Exosome-depleted FBS Media Supplement	50 ml
EXO-FBS-250A-1	Exosome-depleted FBS Media Supplement	250 ml
EXO-FBSHI-50A-1	Exosome-depleted, Heat Inactivated FBS Media Supplement	50 ml
EXO-FBSHI-250A-1	Exosome-depleted, Heat Inactivated FBS Media Supplement	250 ml

The bottles of Exo-FBS are sterile and shipped frozen. Thaw the frozen Exo-FBS overnight at 4°C to use the next day. Store at 4°C for additional use, do not freeze again.

Add the same amount of Exo-FBS as you would standard FBS as a supplement to DMEM (or other media). This is typically 10% in DMEM.

Heat inactivated FBS media supplement is treated at 65°C for 15 minutes before bovine exosome removal.

Exo-FBS™ Exosome –depleted FBS

A. Overview

Exosomes are 40-120 nm membrane vesicles secreted by most cell types in vivo and in vitro. Exosomes are found in blood, urine, amniotic fluid, malignant ascite fluids, urine and in media from cells in culture. Exosomes contain distinct subsets of microRNAs depending upon the cell type from which they are secreted. Standard growth medium for most cells in culture require fetal bovine serum (FBS) as a growth supplement to DMEM. FBS is derived from bovine (cow) serum and contains a high abundance of cow exosome vesicles. These exosomes can interfere or cause significant background issues when studying the exosomes secreted from your cells of interest in standard culture conditions.

SBI has developed an exosome-depleted FBS growth supplement called Exo-FBS that has been stripped of bovine exosomes. Exo-FBS supports equivalent growth of many types of cells in culture, is devoid of cow CD63 positive exosomes and does not have any measurable bovine microRNAs. Perform your studies on cellular secreted exosomes in culture without the worry of contaminating cow exosomes in your experiments. ***No ultracentrifugation required.***

- Exosome-sized vesicles removed
- FBS that has been stripped of CD63-positive cow exosomes
- No detectable cow microRNAs
- Same cellular growth rates supported as standard FBS

B. Media preparation with Exo-FBS

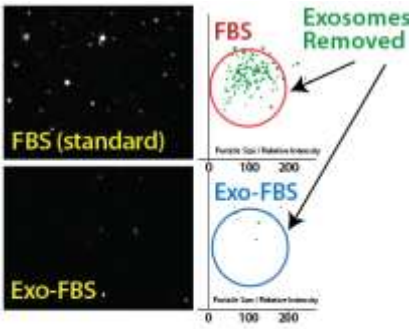
Thaw Exo-FBS overnight at 5°C (refrigerated). Next day, combine 50 ml of Exo-FBS and 5 ml PenStrep stock (10,000 U/mL Pen, 10 mg/mL Strep) in 500 ml DMEM, RPMI or other base media.

We recommend culturing the cells in Exo-FBS/Exo-FBSHI media for a minimum of 3 days (or when it reaches ~80% confluency) prior to collection of exosomes to ensure sufficient exosomes are isolated. This will require optimizing seeding densities for a given cell line to meet these conditions.

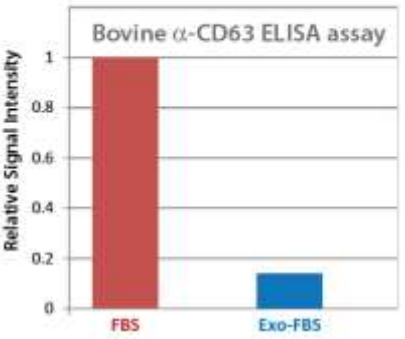
C. Exosomes removed from FBS

Standard FBS media supplements are treated to remove bovine exosomes**.** The resulting Exo-FBS is tested for exosome microvesicle removal by NanoSight analysis and bovine anti-CD63 ELISA assays.

NanoSight particle analysis of FBS and Exo-FBS
Standard FBS and Exo-FBS samples were diluted 1:1000 and then analyzed for particle size and abundance using a NanoSight LM10 instrument. The standard FBS sample shows a significant amount of exosome-sized microvesicles where the Exo-FBS exosome-depleted FBS sample has a drastic reduction in exosome particles.



Exo-FBS is depleted of bovine CD63 exosomes
The tetraspanin CD63 protein is a common marker for exosomes. We utilized a bovine-specific anti-CD63 antibody to develop an Enzyme Linked Immunosorbent Assay (ELISA). Equal volumes (50 ul) of either standard FBS or Exo-FBS depleted media supplement were used in this ELISA assay. Amounts of CD63-positive bovine exosomes dramatically reduced. The results are graphed and normalized to the signal level of standard



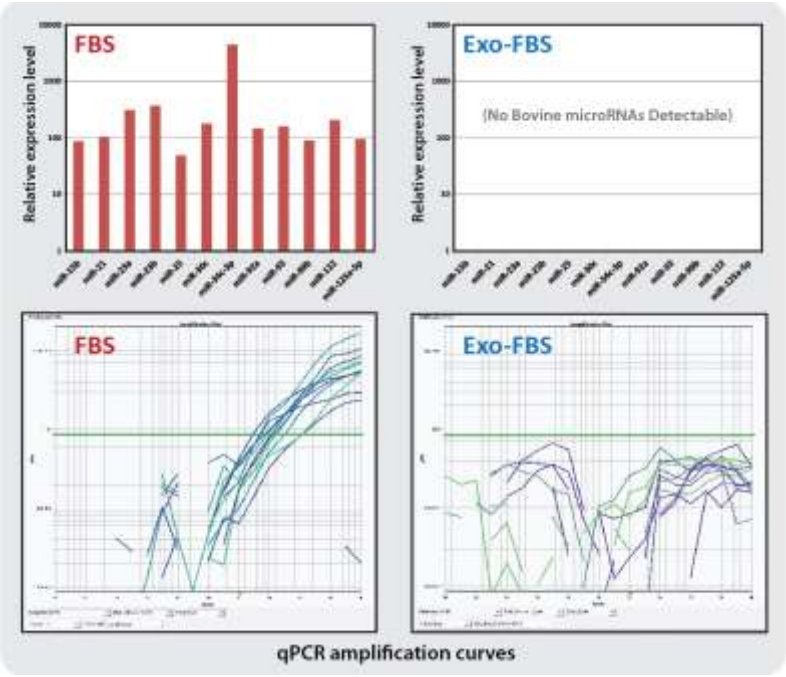
FBS.

D. Bovine MicroRNAs removed from FBS

Bovine microRNAs are absent in Exo-FBS

Standard FBS and Exo-FBS media supplements (4 ml) were treated with Trizol extraction methods to recover exosome RNAs. RNA was converted to cDNA and 72 individual bovine microRNAs were measured by qPCR using SBI's QuantiMir system. Of the 72 microRNAs tested, 12 yielded amplification curves in the FBS sample but not in the Exo-FBS sample.

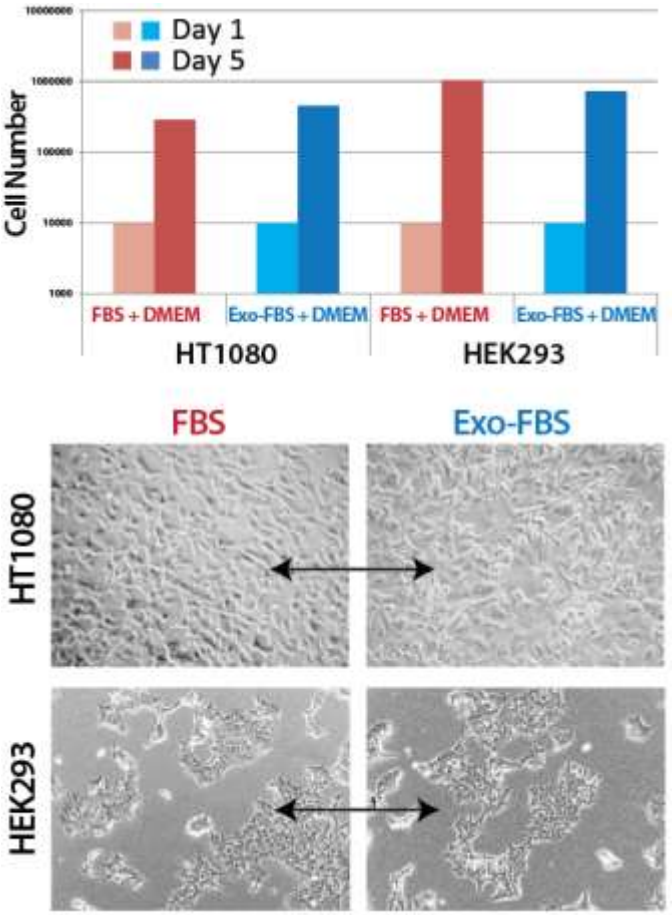
Bovine microRNAs present in FBS are no longer detectable in Exo-FBS - ***No more cow microRNAs!***



qPCR amplification curves

E. Exo-FBS supports robust growth rates

Exo-FBS media supports robust growth of cells in culture equal to standard FBS media.
Complete DMEM medium either with 10% standard FBS or with 10% Exo-FBS supplement. HT1080 fibrosarcoma cells and HEK293 cells were seeded at 10,000 cells and then cultured under standard conditions at 37°C with 5% CO2 for 5 days in the medium indicated. The cells were imaged for growth numbers and morphologies. Equivalent growth and morphologies were observed for FBS and Exo-FBS media tested.



ADDITIONAL KITS FROM SBI FOR EXOSOME ISOLATION AND RNA ANALYSIS

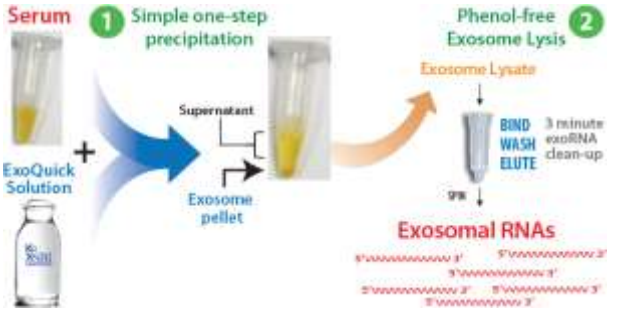
The ExoQuick™ and ExoQuick-TC™ exosome isolation kits are shipped at room temperature or on blue ice and should be stored at +4°C upon receipt. Properly stored kits are stable for 1 year from the date received. The reaction size is based on using 5 ml of tissue culture media or urine for exosome isolation. Examples of precipitating exosomes from various Biofluids can be seen in the Table below. For best recovery for both RNA and Protein analysis, we recommend starting with 10 ml sample.

Biofluid	Sample volume	ExoQuick-TC volume
Urine	5 ml	1 ml
Spinal fluid	5 ml	1 ml
Culture media	5 ml	1 ml
For best RNA and Protein recovery (10ml sample)		
Urine	10 ml	2 ml
Spinal fluid	10 ml	2 ml
Culture media	10 ml	2 ml

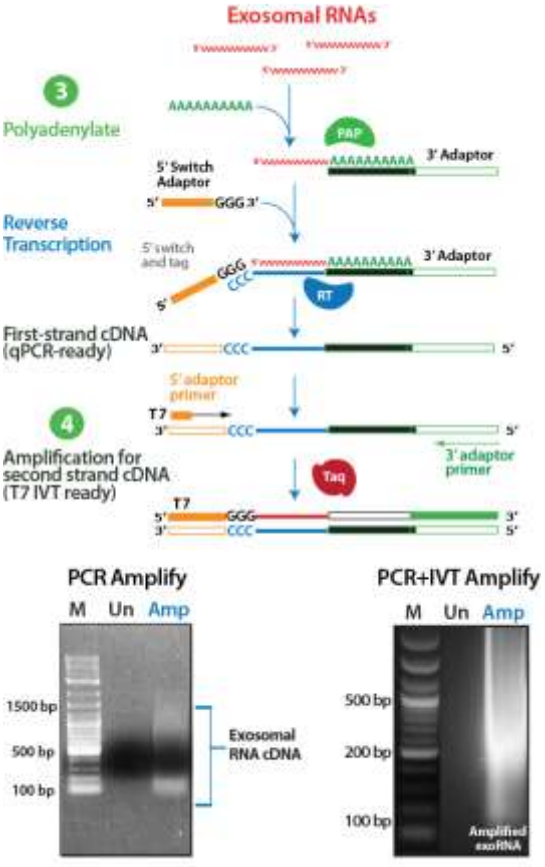
F. How to study exosome RNA from media and serum

PROTOCOL AT A GLIMPSE

Precipitate serum exosomes and purify exoRNAs



Tail exoRNAs and synthesize double-tagged cDNA


Exosomal RNAs
3
Polyadenylate
AAAAAAAAAA
PAP
3' Adaptor
5' Switch Adaptor
5' GGG 3'
Reverse Transcription
5' switch and tag
GGG
CCC
3' Adaptor
RT
First-strand cDNA (qPCR-ready)
3' CCC 5'
5' adaptor primer
T7
4
Amplification for second strand cDNA (T7 IVT ready)
3' adaptor primer
Taq
T7
5' GGG 3'
3' CCC 5'
PCR Amplify
M Un Amp
1500 bp
500 bp
100 bp
Exosomal RNA cDNA
PCR+IVT Amplify
M Un Amp
500 bp
200 bp
100 bp
Amplified exoRNA

Protocol

I. EXOSOME RNA ISOLATION PROTOCOL FROM 500µl SERUM or 5ml Media

**** Collect biofluid and centrifuge at 3000 × g for 15 minutes to remove cells and cell debris.***

Exosome Isolation and Lysis

1. Thaw serum sample on ice
2. Combine 500µl serum + 120 µl **ExoQuick**
   Or: 1 ml **ExoQuick-TC** with 5 ml Media
3. Mix well by inversion three times
4. Place at 4ºC for 30 minutes (serum) or 6h-overnight(urine or media)
5. Centrifuge at 13,000 rpm for 2 minutes
6. Remove supernatant, keep exosome pellet
7. Add 350 µl **LYSIS Buffer** to exosome pellet and vortex 15 seconds
8. Place at room temperature for 5 minutes (to allow complete lysis)
   *--- optional--- add 5*µl *of SeraMir control RNA spike-in (cat#RA805A-1)*

exoRNA Purification

9. Add 200µl of 100% **Ethanol**, vortex 10 seconds
10. Assemble spin column and collection tube
11. Transfer all (600µl) to spin column
12. Centrifuge at 13,000 rpm for 1 minute (check to see that all flowed through, otherwise spin longer)
13. Discard flow-through and place spin column back into collection tube
14. Add 400µl **WASH Buffer**
15. Centrifuge at 13,000 rpm for 1 minute
16. Repeat steps 13 to 15 once again (total of 2 Washes)
17. Discard flow-through and centrifuge at 13,000 rpm for 2 minutes to dry (IMPORTANT !)

exoRNA Elution

18. Discard collection tube and assemble spin column with a fresh, RNase-free 1.5ml elution tube (not provided)
19. Add 30µl **ELUTION Buffer** directly to membrane in spin column
20. Centrifuge at 2,000 rpm for 2 minutes (loads buffer in membrane)
21. Increase speed to 13,000 rpm and centrifuge for 1 minute (elutes exoRNAs)
22. You should have recovered 30-40µl exosome RNA

The yield of RNA from isolated exosomes is different depending on the starting biofluid or the type of cells that were grown in

culture. Different cell types secrete varying levels of exosomes. For serum, the level of RNA isolated from 500 µl is usually in the 500ng range and can be measured using an Agilent Bioanalyzer or a NanoDrop Spectrophotometer. The recovery from cell media varies depending the cell type and growth confluency.

II. EXOSOME RNA cDNA SYNTHESIS

1 Poly A reaction

Add:	Per reaction
exoRNA	5 µl (eluted from spin column)
5X polyA Buffer	2 µl
$MnCl_2$ (25 mM)	1 µl
ATP (5 mM)	1.5 µl
polyA polymerase	0.5 µl

Incubate at 37ºC for 30 minutes

2 Adaptor Anneal Reaction

Add 0.5 µl SeraMir 3' Adaptor Oligo
Incubate at 60ºC for 5 minutes
Incubate at Room temperature 2 minutes
Place on ICE

3 RT Reaction

Add:	Per reaction
polyA exoRNA	(10 µl from above)
5X RT Master Mix	4 µl
5' SeraMir Switch Oligo	1 µl
Reverse Transcriptase	1 µl
Water	4 µl
	20 µl TOTAL

Incubate at 42ºC for 30 minutes
Incubate at 95ºC for 10 minutes
HOLD at 15ºC

qPCR PROFILING OF exo-cDNA

(cat# RA805A-1 SeraMir Spike-in RNA qPCR assay and #RA810A-1 SeraMir Exosome RNA 384 microRNA qPCR Profiler)

To test your exo-cDNA, we recommend performing a qPCR assay for the RA805A-1 Spike-in RNA control or proceed to the 384 well SeraMir Profiler setup (qPCR array contains the Spike-in qPCR assay).

For 96-well plates:

Add:	Per well
exo-cDNA	0.5 µl from above
2X SYBR Master Mix *	15 µl
5' SeraMir Spike-in assay primer	1 µl
SeraMir 3' Reverse qPCR primer	0.5 µl
Water	13 µl
	30 µl TOTAL

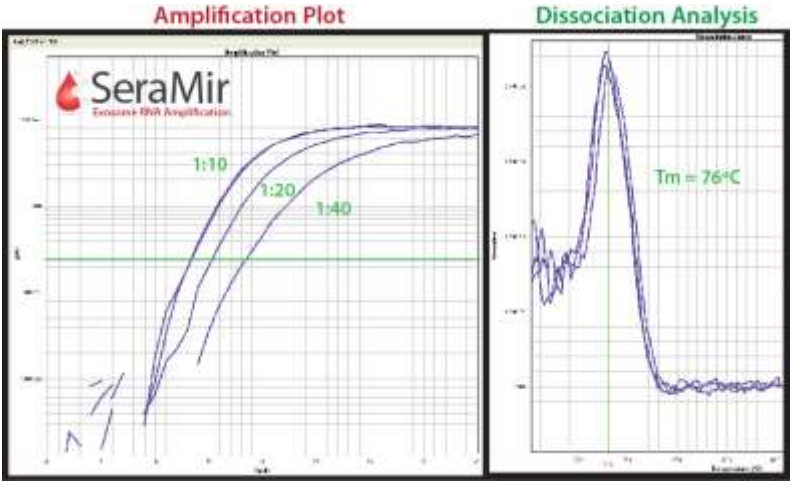
* SBI recommends Fermentas 2X Maxima SYBR Green/ROX qPCR Master Mix, cat# K0221.

For 384-well plates:

Add:	Per well
exo-cDNA	1 µl (of 1:50 dilution)
2X SYBR Master Mix *	3 µl
5' SeraMir Spike-in assay primer	0.2 µl
SeraMir 3' Reverse qPCR primer	0.1 µl
Water	1.7 µl
	6 µl TOTAL

* SBI recommends Fermentas 2X Maxima SYBR Green/ROX qPCR Master Mix, cat# K0221

Sample qPCR data for the SeraMir Spike-in RNA



If 5 µl of the SeraMir Spike-in RNA was used during the exoRNAs isolation, then you should expect to observe a Ct of about 15 to 20.

Setup of the 384 well SeraMir Profiler (cat# RA810A-1)

Mastermix qPCR Reaction Set up for ONE entire 384-well qPCR plate

To determine the expression profile for your miRNAs under study, mix the following for 1 entire 384 qPCR plate:

For 1 entire plate :

+	1150 µl	**2X SYBR Green*** qPCR Mastermix buffer
	39 µl	**3' SeraMir Reverse Primer (10µM)**
	5 µl	**User-synthesized SeraMir cDNA**
	1090 µl	**Nuclease-free water**
	2284 µl	Total

Aliquot 5µl of Mastermix *into every well* in your 384-well qPCR Plate

*** SBI has tested and recommends SYBR Green Master mix from three vendors:**
1. Fermentas 2X Maxima SYBR Green/ROX qPCR Master Mix, cat# K0221
2. Power SYBR Master Mix® (Cat numbers 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems
3. SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat numbers 11760-100, 11760-500, and 11760-02K)

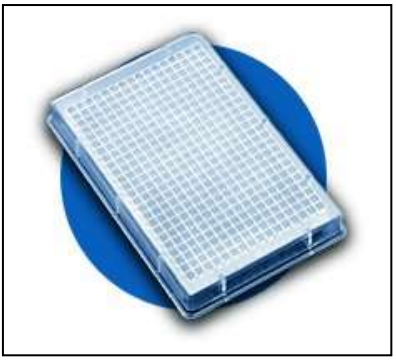
Resuspend the MicroRNA assay Primers with 22µl water in each well before use. Spin briefly to collect contents at bottom of wells.

Then :

Load 1µl per well of each of the Primers from the Primer Stock plate into your qPCR plate (well A1 into qPCR plate A1, etc.)

Once reagents are loaded into the wells, cover the plate with an optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

*** Use a Multichannel pipette to load the qPCR plate with MasterMix and Primers:** Pour the Mastermix into a reservoir trough and use a 8 or 12 channel pipette to load the entire 384-well qPCR plate with the Mastermix. Then load the primers from the primer plate to the qPCR plate using a separate multichannel pipette.



2. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7900HT Real-time PCR System but can also apply to any other 384-well system. The details of the thermal cycling conditions used in testing at SBI are below. A screenshot from SBI's 7900HT Real-time instrument setup is shown below also. Default conditions are used throughout.

Create a detector:

1. Create a new Detector
2. Name the Detector (any name will do)
3. Select Reporter Dye as "SYBR Green"
4. Select Quencher Dye as (none)

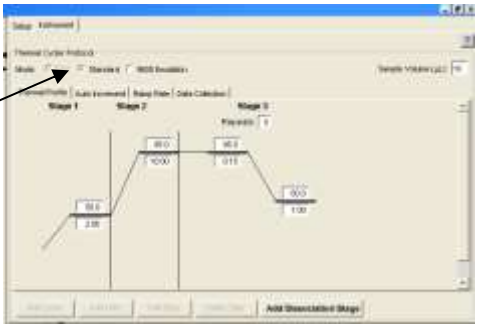
Instrument setup:

qPCR cycling and data accumulation conditions:

Standard Protocol

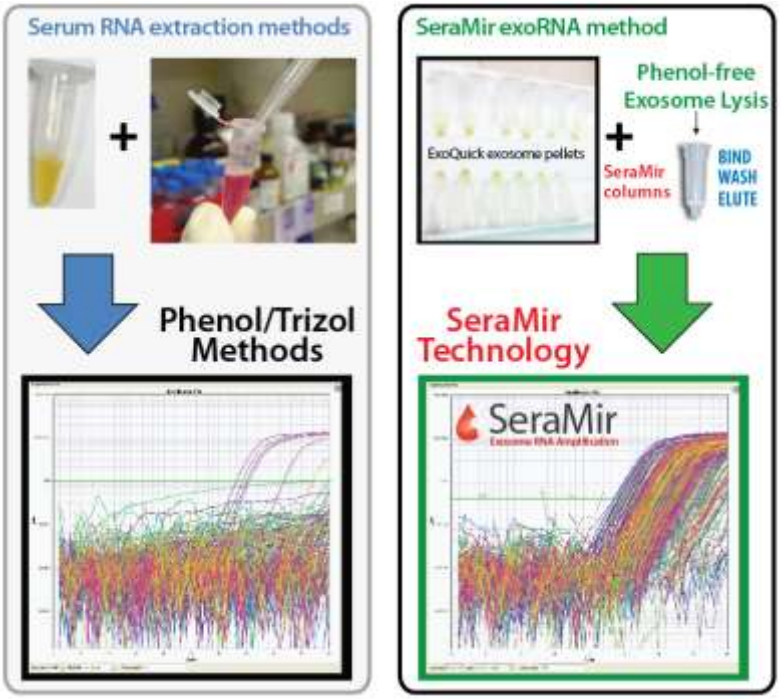
1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of Stage 3), data read at 60°C 1 min. Step.



An additional recommendation is to include a **Dissociation Stage** after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

Sample 384 well SeraMir Profiler Data



The results are clear – obtain more data with SeraMir.

Serum RNA prepared by conventional Trizol versus the SeraMir kit. Profiling of 380 Human microRNAs across the SeraMir 384 Profiler (cat# RA810A-1). The phenol-free exosome lysis step coupled to the small RNA binding columns isolates exoRNAs with much higher purity than Trizol/Phenol based methods. The SeraMir exoRNAs are compatible with downstream polyadenylation and reverse trancription reactions for amplification and accurate qPCR profiling.

EXOSOME cDNA AMPLIFICATION

The first-strand exoRNAs cDNA made in STEP II. Can be amplified to make double-stranded exo-cDNA compatible with T7 in vitro transcription reactions to make amplified “sense” RNAs that work with most microRNA microarrays and can be adapted to use with NextGen sequencing preparation protocols.

Add:	Per reaction
exoRNA amplified cDNA	(2 µl from above)
2X PCR Master Mix	12.5 µl
5' SeraMir PCR Primer Mix	1 µl
Water	9.5 µl
	25 µl TOTAL

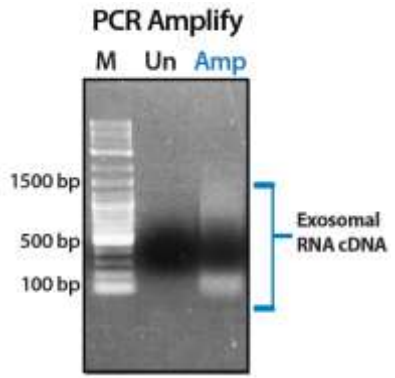
Place the reactions in a thermal cycler, and cycle using the following program:

- 95°C for 5 min
- 95°C for 25 sec
- 60°C for 20 sec
- 72°C for 30 sec

35 Cycles

- 72°C for 30 sec
- 15°C hold

Visualize the PCR products on a 1.5% agarose gel, load 5 µl per well.



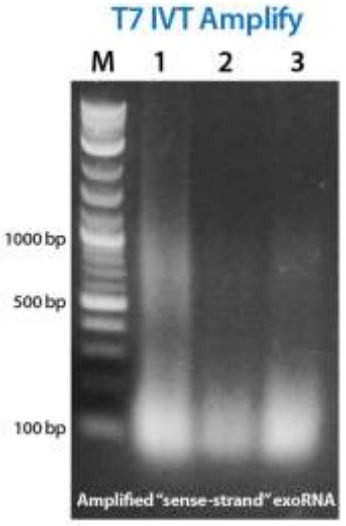
EXOSOME SENSE RNA AMPLIFICATION

SBI recommends using Epicentre's AmpliScribe™ T7-Flash™ Transcription Kit, catalog# ASF3257.

	4.3 µl RNase-Free water
	2 µl Amplified exo-cDNA (from STEP IV.)
	2 µl AmpliScribe T7-Flash 10X Reaction Buffer
	1.8 µl 100 mM ATP
+	1.8 µl 100 mM CTP
	1.8 µl 100 mM GTP
	1.8 µl 100 mM UTP
	2 µl 100 mM DTT
	0.5 µl RiboGuard RNase Inhibitor
	2 µl AmpliScribe T7-Flash Enzyme Solution
	20 µl Total reaction volume

Incubate at 45ºC for 45 minutes.

Visualize the PCR products on a 1.5% agarose gel, load 5 µl per well.



The RNA sizes will range from 80 bases to as long as 1kb. The SeraMir adaptors add 62 bases to the sizes of the exoRNAs, thus a base T7 IVT product corresponds to an exoRNAs insert sequence of about 20 bases.

G. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

System Biosciences (SBI)
265 North Whisman Rd.
Mountain View, CA 94043

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

II. Licensing and Warranty Statement

Limited Use License

Use of the Exo-FBS™ and SeraMir™ Exosome RNA Amplification Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

- ExoQuick exosome isolation methods are a patented technology. Antes, T. et al. Methods for Microvesicle Isolation and Selective Removal. Patent No.: US 9,005,888 B2
- The process of manufacturing of Exo-FBS is a patented method in Patent No.: US 9,005,888 B2.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.

This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein.

Limited Warranty

SBI warrants that the Product meets the specifications described in this manual. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or

implied, including the merchantability or fitness of the Product for a particular purpose.
SBI is committed to providing our customers with high-quality products. If you should have any questions or concerns about any SBI products, please contact us at (888) 266-5066.