XMIR™ Exosome RNA Packaging

Cat #s XMIR/AXMIR/XMIRXPIxxx

User Manual

Store at -20°C upon arrival

Contents

I. Introduction

A. Exosome Overview

Exosomes are nanosized membrane vesicles secreted by most cell types *in vivo* and *in vitro*. They are produced by the inward budding of multivesicular bodies (MVBs) and subsequently released from the cell into the microenvironment following the fusion of MVBs with the plasma membrane. Exosomes are extracellular nanoshuttles that facilitate communication between cells and organs and are found in various biofluids including blood, urine, amniotic fluid, breast milk, malignant ascites fluid, and cerebrospinal fluid (CSF). Exosomes contain distinct subsets of RNAs and proteins depending upon the cell type from which they are secreted, making them useful for biomarker discovery. Additionally, their natural function as cell to cell communication vehicles makes them attractive for use as therapeutic shuttles to deliver biological molecules or drugs to target disease cells. The RNA content varies, depending upon the cell from which the exosomes was secreted. The mechanism of how specific RNA sequences are selectively packaged into exosomes is an intensive area of investigation. SBI has evaluated numerous exosome Next-Generation Sequencing (NGS) data sets and has identified a specific RNA sequence tag that targets a small RNA to be packaged into exosomes for secretion. The ***"XMotif"*** RNA sequence tag has been incorporated into the miRNA and anti-miRNA oligos for the XMIR/AXMIR products and has been built into the XMIRXpress cloning and expression lentivectors.

B. Uses of XMIR and AXMIR RNA Oligos

The XMIR and AXMIR kits allow for cell-mediated generation of ready to use exosomes packed with a miRNA (XMIR) or anti-miRNA (AXMIR) of choice. These exosomes can then be used to efficiently knock down native targets in recipient cells or be used to study biological pathways by which functional exosomal miRNA cargo is delivered to target cells. This technology is especially attractive as a means to study the efficacy of exosome mediated delivery of potential therapeutic miRNAs or anti-miRNAs to

disease cells. Recent studies into miRNA populations within exosomes and the use of exosomes as shuttles to deliver miRNAs and anti-miRNAs can be found in the reference section.

To generate XMIRs and AXMIRS, an oligo is designed to fuse the XMotif sequence to a miRNA or anti-miRNA sequence, which results in exosomal loading of the RNA oligo. The oligo is first transfected into a culture of the exosome generating cell type of choice. After 24 hours, exosomes packed with the XMIR or AXMIR are precipitated using ExoQuick-TC. After resuspension in PBS, the XMIR or AXMIR-loaded exosomes are ready to be added to target cells. Any miRNA, anti-miRNA or even siRNA sequence can also be used in a transfection-ready oligo for use in the XMIR/AXMIR system.

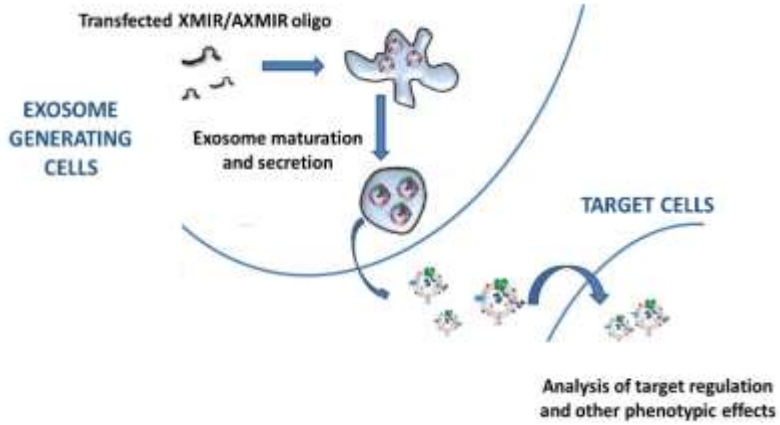


Fig. 1: How XMIR/AXMIR exosome RNA packaging works

II. XMIR and AXMIR RNA Oligo Kits

A. List of Materials:

XMIR and AXMIR Kits (cat# XMIR-xxx or AXMIR-xxx):

- **XMIR/AXMIR RNA oligo at 10 uM, 400 ul (10 reactions per well for 6-well dish)**
- **Optional: Positive Control Texas-Red conjugated RNA oligo with XMotif at 10 uM, 60 ul (cat# XMIR-POS, 10 reactions per well for 6-well dish)**

XMIRXPress Lentivectors (cat# XMIRXP-xxx or XMIRXP-Vect):

- **XMIRXpress pre-made miRNA expression lentivector construct (10 ug plasmid DNA)**

- or -

- **XMIRXpress cloning lentivector, pre-linearized (cat# XMIRXP-Vect, 10 cloning reactions)**

ExoQuick-TC and Exo-FBS are not provided in the XMIR/AXMIR kits and can be purchased separately. The following ExoQuick-TC products are recommended for exosome concentration prior to addition to target cells.

Description	Size	Catalog #
ExoQuick-TC for Tissue Culture Media and Urine (10 ml)	10 reactions	**EXOTC10A-1**
ExoQuick-TC for Tissue Culture Media and Urine (50 ml)	50 reactions	**EXOTC50A-1**
Exosome-depleted FBS Media Supplement	50 mL	**EXO-FBS-50A-1**

IMPORTANT NOTE: Be sure to culture your exosome producer cell lines in media that does not contain standard FBS. There are high levels of cow exosomes present in FBS. Instead, use SBI's Exo-FBS Exosome-depleted FBS Media Supplement (cat#EXO-FBS-50A-1) in place of standard FBS media supplements.

B. XMIR and AXMIR Transfection Protocol:

Transfection of Exosome Generating Cells:

1. Seed exosome generating cells in culture dish of choice to reach 60-70% confluency after 24 hours using media compatible with the cells and be sure to use SBI's **Exosome-depleted FBS Media Supplement** in place of standard FBS because standard FBS contains high levels of cow exosomes. Return cells to incubator.

2. 24 hours later, mix XMIR or AXMIR oligo with transfection reagent of choice and follow appropriate protocol to achieve transfection of target cells. The positive control oligo can be used in a co-transfection experiment at a final concentration of 20 nM. Target cell delivery can be visualized and quantified using fluorescence microscopy and qPCR, respectively (more details below) as follows:1) The final concentration of XMIR or AXMIR oligo should fall somewhere within the range of 20 nM – 100 nM. Because the number of exosomes generated differs between cell lines and the amount of delivered miRNA or anti miRNA required to see an effect in target cells varies, we recommend testing a series of oligo concentrations for exosomal loading and target cell effects the first time a particular exosome generating cell and/or XMIR or AXMIR oligo is used. For more details on optimizing oligo concentrations, see frequently asked questions.
    a. An example transfection setup using SBI's PureFection (cat# LV750A-1) in a 6-well plate of cells at about 70-80% confluency.
        5 ul PureFection reagent
        \+ 30 ul XMIR/AXMIR RNA oligo (10uM)
        200 ul serum-free media

b. Mix together by brief vortexing, incubate at room temperature for 15 minutes.
c. Add entire volume to 6-well of cells in a total volume of 3 ml media, this makes the final concentration of XMIR/AXMIR RNA oligo in the media at 100 nM.
d. The XMIR/AXMIR RNA oligo amount can be scaled up or down as appropriate for your experimental conditions.

3. Return cells to incubator and allow for exosome production for 24 hours.

4. Plate target cells in culture dish of choice to be at an appropriate confluency for downstream phenotypic assay in 24 hours.

Isolation of XMIR/AXMIR Exosomes and Addition to Target Cells:

1. 24 hours post-transfection, remove cell culture media and place in 15 mL or 50 mL centrifuge tube.

2. Add ExoQuick-TC at 1:5 the volume of cell culture media.

3. Mix by inversion and incubate at 4°C overnight.

4. Spin centrifuge tubes at 3,000 x g for 30 minutes at room temperature or 4°C (temperature does not affect exosome yield). Discard supernatant and resuspend exosome containing pellet in 100 uL PBS.

5. Measure exosome yield using A280 on Nanodrop. Adjust concentration to 1 ug/uL.

6. Add exosomes to cell culture dish containing target cells. For an assay in a 6 well plate format, 50 ug exosomes is sufficient to see effects of both XMIRs and AXMIRs on native protein targets (see sample data Figure 3). The

number of exosomes required in culture dishes of other size can be scaled up or down proportionally to the difference in total cell number relative to one well of a 6 well plate. For more details, see frequently asked questions.

7. Perform desired assay in target cells to analyze effects of exosome mediated delivery of miRNA or anti-miRNA.

Validation of target cell delivery using positive control (cat# XMIR-POS):

The inclusion of the positive control oligo allows for confirmation of target cell delivery in two ways: (1) visualization of target cells on a fluorescent microscope, or (2) qPCR.

1. Visualization of target cells on a fluorescence microscope: As quickly as 2 hours after exosomes are added to target cells, delivery can be visualized using a standard RFP filter set on fluorescence microscope (see sample data Figure 4 for fluorescence analysis of target cell delivery).

2. qPCR: 24 hours post-transfection after exosomes are added to target cells, RNA can be extracted from the cell lysate and analyzed by qRT-PCR using our SeraMir kit **(catalog #RA820A-1),** (see sample data Figure 5. for qPCR analysis of target cell delivery). Our positive control oligo can be detected using the spike-in forward primer, and your XMIR or AXMIR can be detected using a forward primer specific to its sequence.

C. Sample XMIR and AXMIR Data

XMIRs are packaged into exosomes:

XMIR oligos for miR-1 and miR-122 were transfected at a 20 nM final concentration to test exosomal loading in HEK-293 cells. Transfection was accomplished using PureFection (catalog #LV750A-1), and exosomes were purified 24 hours later using ExoQuick-TC. RNA was extracted from exosomes, cDNA was synthesized, and qPCR was performed using the SeraMir kit **(catalog #RA820A-1),** Data were analyzed using an Applied Biosystems 7900HT qPCR instrument and relative exosome abundance levels were calculated using the delta-delta Ct method using exosomal miR-16 as a reference control.

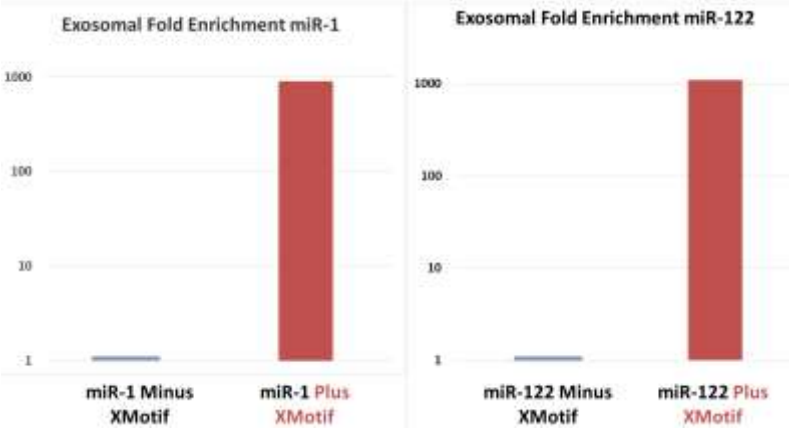


Fig. 2: Addition of XMotif to miRNA sequences results in a 1000-fold increase in exosomal loading.

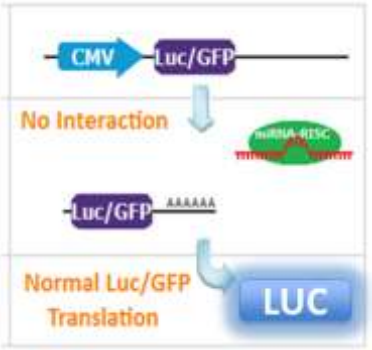
XMIRs delivered to cells by exosomes are bioactive:

The XMIR-1 and XMIR-122 loaded exosomes were added to reporter HEK-293 cells previously transfected with a luciferase gene linked to the 3' UTR for MEF2A, a known miR-1 target, or RIMS1, a known miR-122 target, respectively. After 24 hours, luciferase assays were performed to determine bioactivity of the XMIR miRNAs delivered to target cells via exosomes. Notably, exosomes from cells transfected with 20 nM miR-1 oligo caused maximum luciferase down regulation, whereas exosomes from

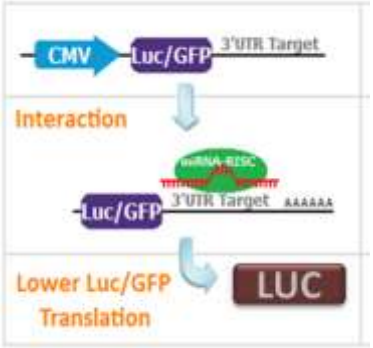
cells transfected with 100 nM miR-122 caused maximum luciferase down regulation, illustrating the need to optimize conditions for each XMIR/AXMIR oligo. The degree of knockdown XMIR-1 exosomes displayed on the MEF2A luciferase reporter is similar to that seen for transfections of miRNA oligos using a similar reporter assay (see reference 1), indicating that exosome mediated delivery of miRNAs occurs at maximal efficiency.

A.

No miRNA binding = high luciferase levels.



Successful miRNA binding = lowered luciferase levels.



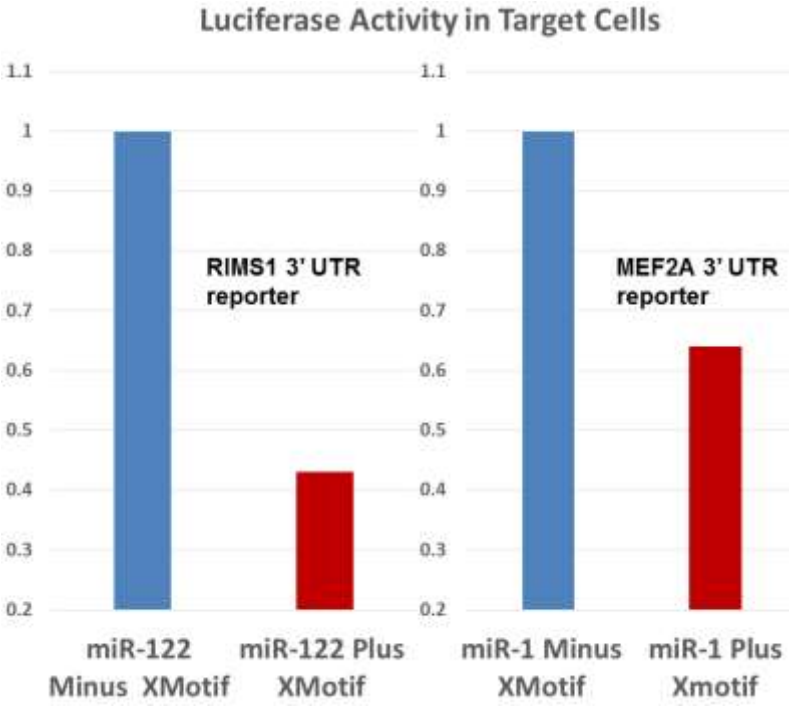


Fig. 3: A) How miRNAs influence Luciferase 3' UTR reporters in cells. B) Addition of exosomes from cells transfected with indicated X-Motif or non X-Motif oligos to target cells expressing a luciferase reporter linked to a specific 3' UTR (either RIMS1 for miR-122, or MEF2A for miR-1) resulted in down regulation of luciferase expression. Plasmids and luciferase reagents were obtained from Switch Gear Genomics, catalog numbers S810945, S807542, and LS010.

XMIRs and AXMIRs affect endogenous protein levels:

Exosomes from HEK-293 cells transfected with a miRNA-21 XMIR oligo (XMIR-21) or an anti-miRNA-21 AXMIR oligo (AXMIR-21) and then were added to naïve HEK-293 cells in culture. After 24 hours, total cell lysates were taken and Western blots for PDCD4, a known miR-21 target, were performed. GAPDH protein levels

detected in the Westerns were used as a loading control and reference signal for band intensity quantitation analysis.

Western blot analysis of PDCD4 levels in HEK-293 Cells treated with XMIR/AXMIR-loaded exosomes.

Figure 3A. Western blot image

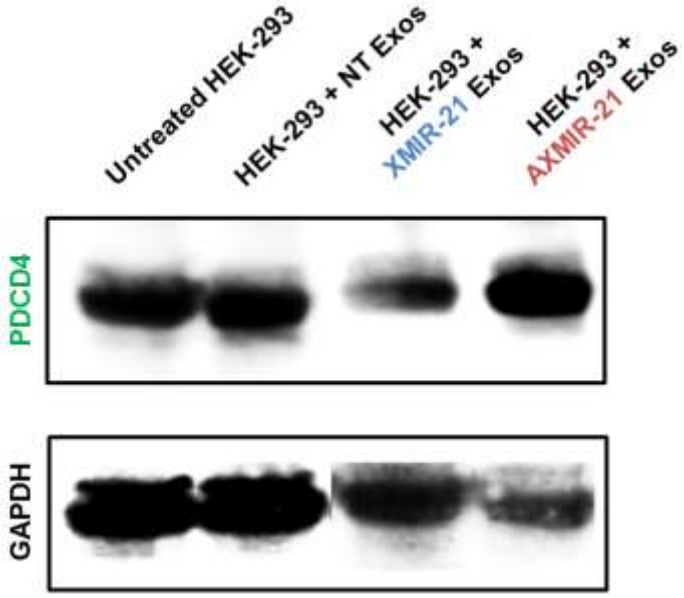


Figure 3B. Densitometric analysis of western blot

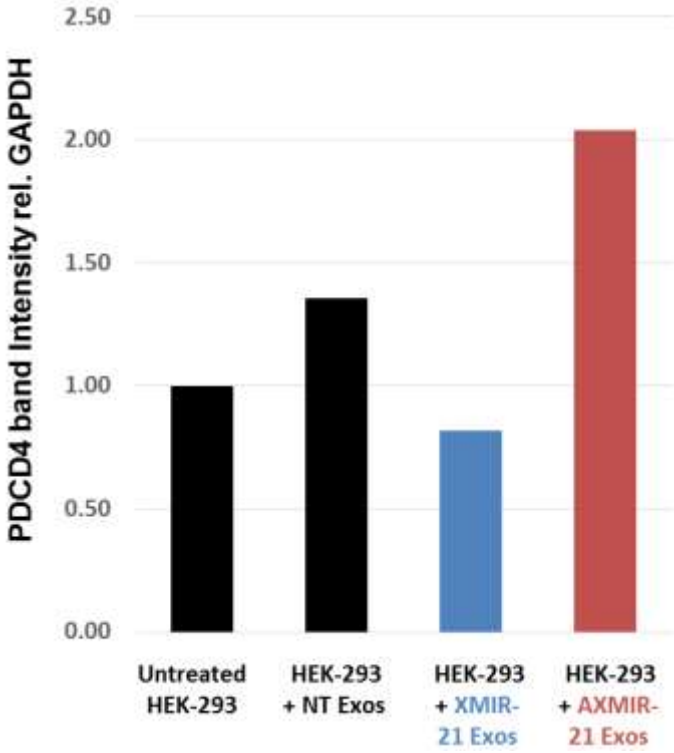


Fig. 3: A) western blot analysis of PDCD4 and GAPDH from HEK293 cells treated with XMIR-21 or AXMIR-21 loaded exosomes. B) Quantitative analysis of band intensities from the Western blot shown in Panel A. Addition of exosomes from cells transfected with XMIR-21 resulted in down regulation of endogenous levels of PDCD4, and addition of exosomes from cells transfected with AXMIR-21 resulted in increased levels of PDCD4. These results confirm that XMIRs act on endogenous targets and that AXMIRs effectively act as miRNA inhibitors in cells when delivered via exosomes. Notably, exosomes from cells transfected with 20 nM XMIR-21 oligo caused maximum PDCD4 down regulation, whereas exosomes from cells transfected with 100 nM AXMIR-21 increased PDCD4 levels, illustrating the need to optimize conditions for each XMIR/AXMIR oligo (PDCD4

antibody obtained from Cell Signaling Technologies, catalog # 9535, and GAPDH antibody obtained from Abcam, catalog # ab9485).

Positive control is delivered to target cells:

HEK-293 cells were transfected with the positive control oligo. 24 hours later, exosomes were isolated using ExoQuick-TC and added to naïve HEK293 target cells. After 4 hours, cells were imaged on a Leica DMI300B fluorescence microscope using a standard RFP filter set to visualize the Texas-Red signal conjugated to the XMIR positive control oligo.

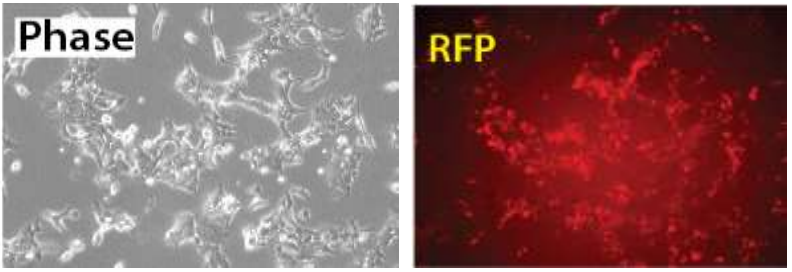


Fig. 4: Cells transfected with positive control XMIR oligo miRNA are loaded into exosomes and their cargo is delivered to target cells as visualized by fluorescence microscopy.

HEK-293 cells were subsequently lysed and total cellular RNA was extracted, cDNA synthesis performed, and qPCR analysis using SBI's QuantiMir kit **(catalog #RA420A-1),** on an Applied Biosystems 7900HT qPCR machine using the spike-in forward primer to detect abundance of the positive control oligo. Abundance was normalized to endogenous reference miR-16 levels and calculated using the delta-delta Ct method.

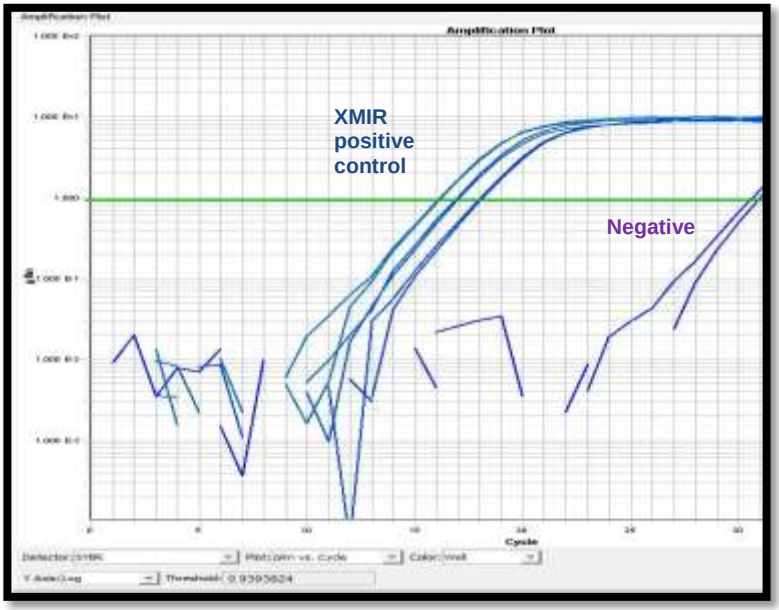


Fig. 5: miRNA cargo of exosomes from cells transfected with positive control oligo is delivered to target cells as quantitated by qPCR.

D. XMIRXpress Lentivectors

The XMIRXpress lentivectors are based on the same XMotif exosomal targeting RNA tag utilized in the XMIR/AXMIR synthetic oligos. There are a number of pre-made XMIR-Express miRNA expression constructs available and SBI will design and build a custom XMIRXpress lentivector construct for any particular miRNA or anti-miRNA of your choice for the same list price as the pre-made constructs. The lentivectors all feature an EF1a-GFP-Puro selection cassette and a downstream H1 promoter expressing the XMIR+XMotif cassette.

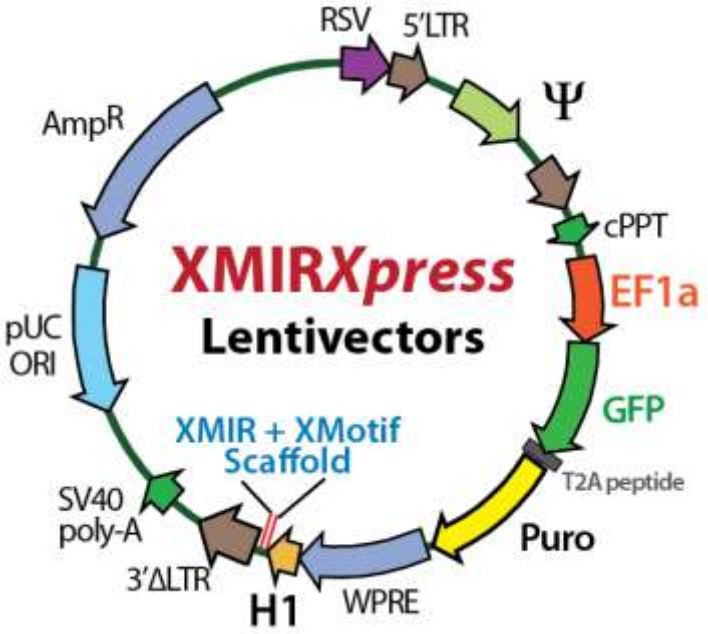


Fig. 6: Lentivector plasmid map of pre-made XMIRXpress miRNA constructs. The XMIRXpress lentivectors feature an upstream EF1a-GFP-Puro selection cassette for easy stable cell line generation using packaged lentivirus from the construct. The XMIR+XMotif cassette is expressed form the downstream H1 promoter.

For the data in Fig. 7, the premade XMIR-29b lentivector construct was transfected into HEK293 cells cultured in DMEM media with SBI's Exosome-depleted FBS Media Supplement in place of standard FBS because standard FBS contains high levels of cow exosomes. The exosomes were collected after 48 hours. The exosomal RNA was purified and converted into qPCR-compatible cDNA (as described in Section C.). Relative amounts of XMIR-29b packaged into exosomes was quantitated by qPCR and miR-16

used as a reference exosome control signal. The delta-delta Ct calculation for XMIR-1 is shown in the bar graph.

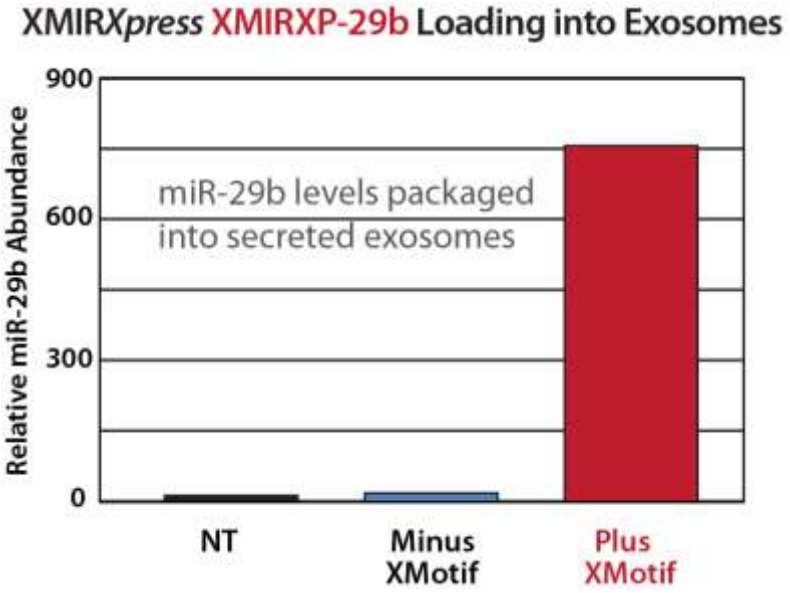


Fig. 7: Relative levels of XMIRXpress cat# XMIRXP-29b lentivector expression and packaging into secreted exosomes.

SBI also provides a cloning and expression XMIRXpress lentivector plasmid (cat# XMIRXP-VECT) to allow you to clone any miRNA or anti-miRNA of your choice simply and it will automatically be fused to the XMotif sequence upon ligation. The XMIRXP-VECT plasmid is provided in a linearized form for rapid cloning. Simply design two DNA oligos for the top and bottom strand of the miRNA hairpin as detailed below, anneal the top and bottom oligos and ligate directly into the XMIRXP-VECT plasmid.

A.

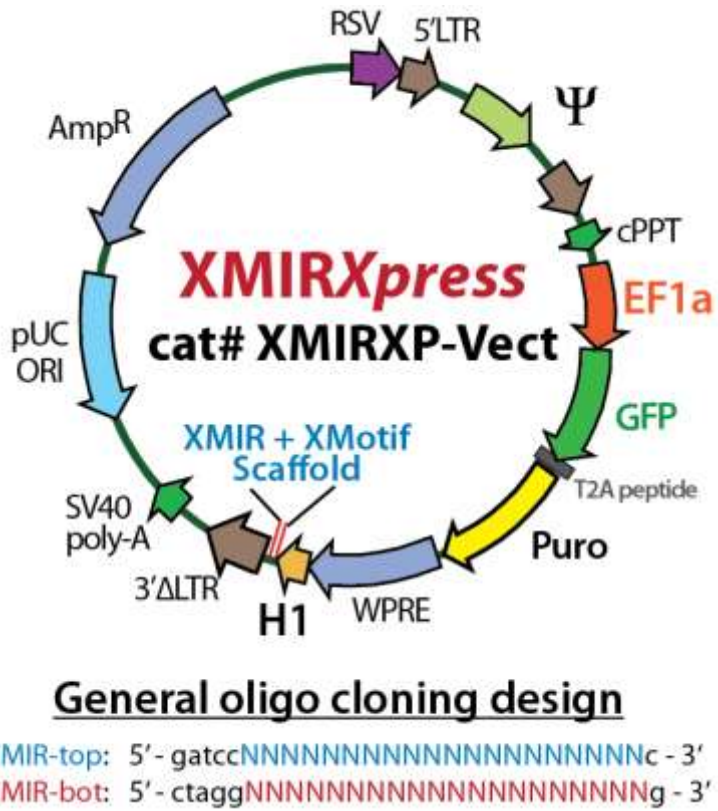
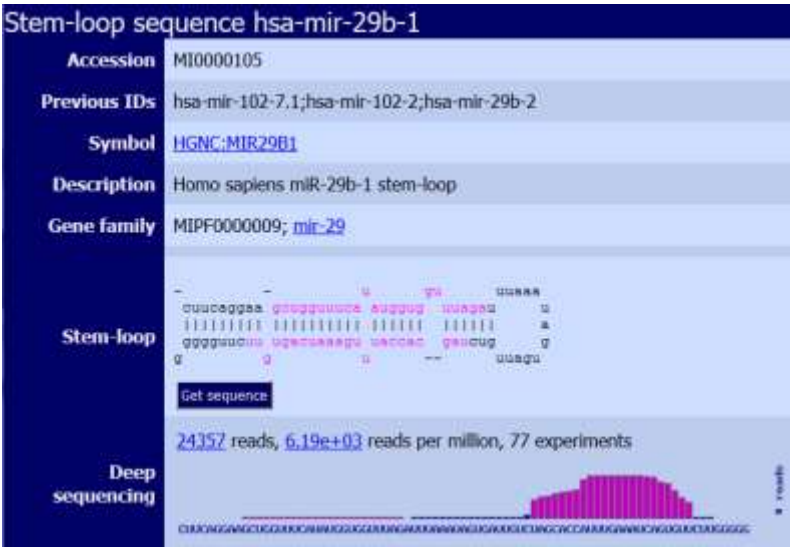


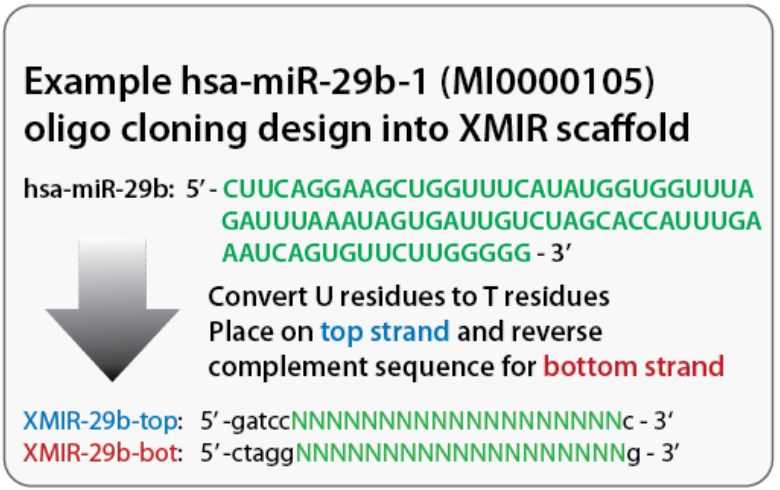
Fig. 8: A) Lentivector plasmid map of XMIRXpress miRNA cloning lentivector and how to design oligos to clone into XMIR scaffold. The XMIRXpress lentivectors feature an upstream EF1a-GFP-Puro selection cassette for easy stable cell line generation using packaged lentivirus from the construct. The XMIR+XMotif cassette is expressed form the downstream H1 promoter. The cat# XMIRXP-VECT plasmid is provided in linear form for direct cloning of miRNAs of interest into the XMIRXpress scaffold cassette. The

NNNNNNN sequence corresponds to the miRNA hairpin which is typically is about 70 nucleotides in length

B) An example of cloning hsa-miR-29b-1 (MI0000105) is shown below. First, look up miRNA of interest using miRBase (www.miRBase.org) and identify what miRNA sequence you want to express as an XMIR/XMotif fusion.

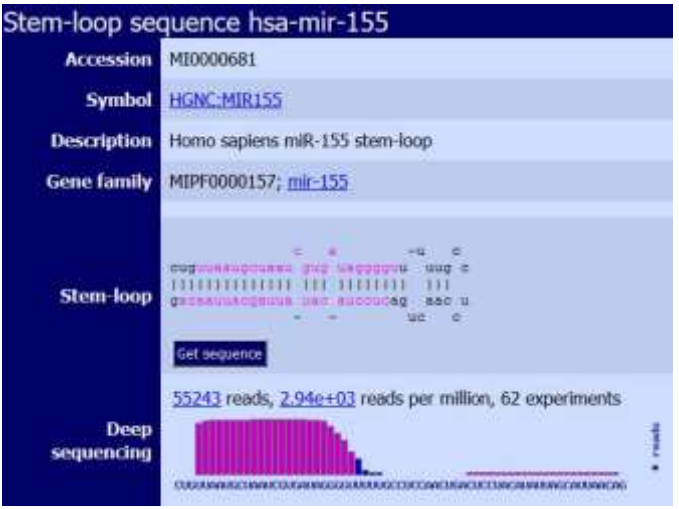
B. If desired miRNA is a -3p in the hairpin





C. If desired miRNA is a -5p in the hairpin

An example of cloning hsa-miR-155-5p (MI0000681) is shown below. First, look up the miRNA of interest using miRBase (www.miRBase.org) and identify what miRNA sequence you want to express as an XMIR/XMotif fusion. If the mature miRNA to be tagged for exosome packaging is on the -5p side of the miRNA precursor like miR-155, the stem loop needs to be switched such that it now is placed on the -3p side for optimal XMIR motif tagging. The original miR-155-5p is shown in red and then its placement for the Arm switched version for XMIR tagging is shown below the original miRNA precursor. The loop and adjacent sequences shown in black remain unchanged.



>hsa-mir-155 MI0000681
CUGUUAAUGCUAAUCGUGAUAGGGGUUUUUGCCUCCAACUGACUCCUAC
AUAUUAGCAUUAACAG

>Arm switched hsa-mir-155 MI0000681
GACUCCUACAUAUUAGCAUUAACAGUUUGCCUCCAACUCUGUUAAUGCU
AAUCGUGAUAGGGGUU

Oligo Annealing and Ligation Instructions:

1. Anneal the two single stranded DNA oligos:
   Dilute each oligo to a concentration of 20 uM. Combine 1 uL of each with 18 uL of TE buffer to achieve a final concentration of 1 uM each oligo. Heat at 95°C for two minutes, then allow to cool to room temperature for 20 minutes.
2. Ligate of annealed oligo into XMIRXpress lentivector:
   Combine 1 uL of linearized vector with 1 uL of annealed oligos. Add ligation buffer and DNA ligase of choice and ligate as per the conditions required by your ligase.
3. Transform ligation reaction into competent cells, shake in 1 mL LB + ampicillin at 37°C for 1 hour, then plate 250 uL onto an LB + ampicillin plate and grow overnight at 37°C.

4. To confirm the sequence of XMIRXpress clones, use the H1 forward primer for sequencing your XMIR expression cassette.
   H1 fwd: 5' - TGCATGTCGCTATGTGTTCTGGGA - 3'

The XMIRXpress lentivectors can be used in transfection experiments with cells to test the exosomal loading of the XMIR and can also be packaged into lentivirus to easily create stable XMIR producer cell lines constitutively secreting exosomes packed with XMIRs.

XMIR Exosome Producer Cell Factories

To create stable cell lines stably expressing XMIRs that will be loaded into secreted exosomes, package the XMIRXpress lentivector plasmid into virus using SBI's lentivirus packaging systems. Visit www.systembio.com/lenti for product information on the kits to perform virus packaging. Please refer to SBI's official Guide to Lentiviral Packaging for details on making high titer virus preparations. SBI also offers custom lentivirus packaging as a service.

Lentivirus packaging, concentration, titering and transduction reagents from SBI:

LentiStarter™ Kit
New to Lentiviral Technology? Try SBI's LentiStarter™ 2.0 Kit that enables optimal lentiviral Packaging, Concentration and Transduction in one convenient starter sample kit (pPACKH1, PEG-it and TransDux)

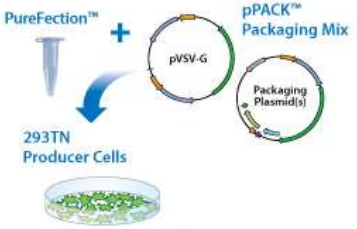
LentiSuite™
Complete system that enables optimal lentiviral Packaging, Concentration and Titering in one convenient toolset

Virus Packaging Systems
pPACK™ packaging plasmid mix for optimized lentivirus production
Producer Cell Line
SBI's 293TN cell line produces high titer lentivirus
Virus Concentration and Transduction
Easily concentrate Lentiviruses with **PEG-it™** virus precipitation solution. Efficiently transduce your target cells with **TransDux™**

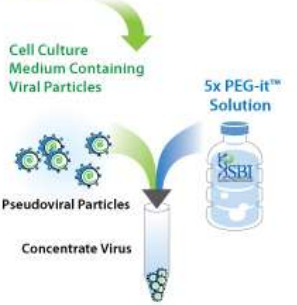
How to Make High Titer Lentivirus

1. Make Your Lentivector Clone (cDNA, shRNA, reporter, microRNA, etc.).

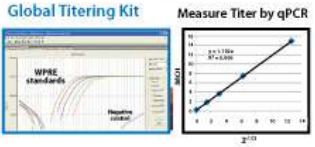
2. To make lentivirus, co-transfect your lentivector plus pPACK packaging mix using PureFection into 293TN packaging cell line.



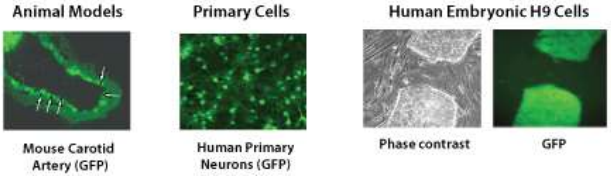
3. Wait 48-72 hours, collect supernatant and combine with PEG-it virus concentration solution. Next day, remove supernatant and resuspend pellet in sterile PBS .



4. Check Titer using Global UltraRapid Titer Kit using standard cell line (ex. 293 cells).



5. Combine the appropriate amount of virus with TransDux and infect your target cells.



E. Additional Materials Required

1. ExoQuick-TC (Cat# EXOTC10A-1) to isolate exosomes
2. Exosome-generating cells in culture and target cell lines
3. Exo-FBS, exosome-depleted FBS supplement (Cat# EXO-FBS-50A-1)
4. Transfection reagent of choice for RNAs or plasmids
5. Lentivirus packaging, concentration and transduction reagents for XMIRXpress lentivector constructs

F. Related Products

SBI offers a number of exosome research products. You can review them here: http://www.systembio.com/exosomes

- ExoQuick exosome isolation reagents
- Exo-FBS exosome-depleted media supplement
- Detect and quantitate exosomes with Antibodies and ELISAs
- Purify exosome RNA and profile by qPCR with SeraMir
- Discover novel exoRNA biomarkers with Next-Gen sequencing services

F. Shipping and Storage Conditions for Kit

The XMIR/AXMIR kits are shipped on **dry ice, the XMIRXpress constructs and cloning lentivectors are shipped on blue ice (-20°C)** and all products should be stored at -20°C upon arrival. Avoid freeze-thawing the reagents. Shelf life of the product is 1 year after receipt if stored in -20°C.

III. Frequently Asked Questions

Q. How do I optimize amount of XMIR/AXMIR oligo to use?

The first time an XMIR or AXMIR oligo is used, we recommend transfecting at 20 nM, 50 nM, and 100 nM final concentration, then adding each isolated exosome population independently to target cells to assay for bioactivity. The concentration of XMIR or AXMIR resulting in the desired level of downstream effects should be used in all subsequent experiments.

Q. How long and in what condition should I store exosomes after isolation from exosome generating cell line?

After exosomes are isolated with Exo-QuickTC, the pellet can be stored at -80°C for 1 year. After resuspension in PBS, it can be stored at 4°C for 2 weeks or -20°C for 3 months.

Q. How many exosomes should I add to my target cells?

50 ug of exosomes (as determined by A280 on NanoDrop) is sufficient to see effects of both XMIRs and AXMIRs on native protein targets (see Figure 3). The number of exosomes required in culture dishes of other size can be scaled up or down proportionally to the difference in total cell number relative to one well of a 6 well plate.

Example: HEK-293 cells
6 well seeding density: 400,000 cells
24 well seeding density: 100,000 cells
100,000/400,000 = ¼ number of cells
50 ug exosomes x ¼ = 12.5 ug exosomes for use in 24 well plate format

IV. References

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V. Technical Support

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

http://www.systembio.com

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