Chapter 5

Surface Acoustic Wave Lysis and Ion-Exchange Membrane Quantification of Exosomal MicroRNA

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Abstract

MicroRNA detection and quantification are commonly explored techniques for diagnostic and prognostic predictions. Typically, microRNAs are extracted and purified from a biological source, converted into complementary DNA (cDNA), and amplified using real time polymerase chain reaction (RT-PCR). The number of RT-PCR cycles required to reach the threshold of detection provides a relative quantification of the target microRNA when this data is normalized to the quantity of a control microRNA. This methodology has several drawbacks, including the need to artificially amplify the target microRNA for detection as well as quantification errors that can occur due to expression level differences of the control microRNAs for normalization in various sample sources. Here, we provide a technique to quantify actual concentrations of target microRNAs directly from any biological source without the requirement of these additional steps. In addition, we describe an alternative approach for obtaining exosomal microRNAs directly from biological samples without the use of harsh detergents and RNA isolation.

Key words MicroRNA, RNA, Detection, Quantification, Exosomes, SAW, Microfluidics, Ion-exchange, Membrane, Biosensor

1 Introduction

One of the many challenges facing clinicians and scientists today is the lack of early detection and prognostic biomarkers for many diseases. MicroRNAs isolated from patient serum samples have the potential to be promising biomarkers because they can be obtained noninvasively and are stable in circulation. However, the process of detecting them can be lengthy and can require larger sample volumes than is sometimes available from routine procedures. In order to detect microRNAs using RT-PCR, the RNA must be isolated from a sample, converted into cDNA, and a specific cDNA is amplified by DNA polymerase, which increases the abundance of the cDNA to reach the minimum threshold of detection. When normalized to a control microRNA, the cycles needed to reach this limit of

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detection are used to quantify relative levels of target micoRNA in the original sample. Ideally, direct quantification of the target microRNA would be done from a patient sample since the additional RNA isolation and RT-PCR steps leave room for human error and sample loss. We developed a way to quantify small concentrations of microRNA (down to 2 pM) without the need for these steps [1, 2, 10]. A microRNA probe is attached to a positively charged ion-exchange membrane where it can bind to its complimentary microRNA. When the proper current is applied, anions flow through the pores of the membrane, but this flow is disrupted by higher amounts of bound microRNAs, creating a measurable shift in voltage. The ion-exchange membrane can be reused by dehybridizing the probes on the membrane from their complementary miRNA before adding a new sample [2, 3]. This allows us to incrementally add higher known concentrations of a commercially available microRNA to the same membrane to create a calibration curve linking the amount of voltage shift to target microRNA concentration. Thus, the shift in voltage is used to directly detect and quantify the amount of target microRNA in a sample without the need for reverse transcription or amplification. If normalization to a control microRNA is needed, the steps below can be ran side-by-side using two different biosensors/membranes, one with the control probe and one with the target-microRNA probe (one probe is used per membrane). Most microRNAs in circulation are contained in lipid vesicles known as exosomes [4]. Exosomes can by lysed by detergents to release the RNA within, but we found these detergents to be disruptive to both the probe-sensor and the RNA-probe binding. Instead, microRNAs in exosomes can be released by exposing exosome-containing samples to surface acoustic waves, which results in about a 40% exosome lysis rate (which can be optimized by the user by changing variables such as power used) [1]. Surface acoustic waves (SAWs) are Raleigh waves that can be generated on the surface of a piezoelectric substrate to induce fluid flow in microfluidic channels. When an electric current is applied to a transducer consisting of interdigitated electrodes on the surface of a piezoelectric substrate, electromechanical couple generates a SAW that travels along the surface of the substrate. Although the amplitude of the displacement is small (~nanometers), the energy contained in the wave is high, and when the wave encounters a fluid film or drop on the substrate, it will refract and induce convective motion in the fluid [5, 6]. Here we use a standard electrode-width controlled (EWC) single-phase unidirectional SAW transducer (SPUDT) design to generate SAWs with a wavelength of 132 μm [1, 7–10]. When conditioned cell media flows through a microchannel on the surface of the SAW device, the induced acoustic and electric forces lyse the exosomes, releasing their RNA into the media. The media can then be directly channeled onto the probe-functionalized ion-exchange membrane,

after creation of a calibration curve, to determine the concentration of target microRNA in the cell media sample. Conditions may also be optimized to use patient serum samples as the biological source of microRNA-containing exosomes. This technique required little volume (roughly 100 μL media) and did not require the RNA isolation, RNA to cDNA conversion, and PCR amplification steps required to quantify microRNA with RT-PCR [1].

2 Materials

2.1 Biosensor Materials

1. Benzophenone-3,3′,4,4′-tetracarboxylic acid powder.
2. Sodium hydroxide.
3. pH strips.
4. AMH5E-HD RALEX® ion-exchange membrane.
5. Electro-Cure 500 Light.
6. Loctite® 3492 UV curing glue.
7. (Poly)vinyl alcohol.
8. TAP Quik-Cast Polyurethane Resin.
9. Acrylic sheet (Poly(methyl methacrylate) or PMMA).
10. DREMEL® 4000 drill with 2 mm drill bit.
11. Tygon tubing (inside diameter 2 mm).
12. Gamry™ potentiostat.
13. MES buffer: 0.1 M MES, pH 5.5.
14. 0.1× PBS buffer: 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4, 0.18 mM KH_2PO_4, pH 7.4.
15. EDC powder (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride).
16. ssDNA probe complementary to RNA target.
17. Artificial target RNA to create a calibration curve.

2.2 SAW-Incorporated Microfluidics Device Materials

1. Oerlinkon Evaporation System.
2. Lithium niobate piezoelectric wafer (Precision Micro-Optics).
3. L-Edit software.
4. Photoresist spinner.
5. S1813 photoresist.
6. Hot plate.
7. Karl Suss MJB3 Mask Aligner.
8. DRYTEK plasma etcher.
9. Acetone.

10. 300 μm thick polycarbonate sheets.
11. Graphtec Cutting Pro.
12. Glass slides.
13. Agarose powder.
14. ACRIFIX® 1R 0192 UV curing glue.
15. Centrifuge and ultracentrifuge.
16. Agilent 33250A generator.
17. E&I 325LA RF power amplifier.
18. Microcentrifuge tubes.
19. Exosome-free FBS.
20. 200 nm pore size filters.

3 Methods

3.1 RNA Probe Biosensor Fabrication

3.1.1 Membrane Carboxylation

1. Add 3 mg of benzophenone-3,3′,4,4′-tetracarboxylic acid powder, to 30 μL of DI water. Add NaOH to reach pH between 6 and 8. Keep this mixture in dark or in aluminum foil (*see* **Note 1**).
2. Cut the ion-exchange membrane into a piece just large enough to cover a 0.25 mm^2 opening in the biosensor. With a permanent marker, label one side of the membrane as the back side.
3. Soak membrane piece in benzophenone solution for 30 min.
4. Expose the front side of the membrane to UV light from Electro-Cure 500 for 10 min.
5. With forceps, carefully transfer the membrane into DI water to rinse, and then allow to dry.
6. Add benzophenone solution to the front of the membrane piece, covering the surface. Incubate with benzophenone solution in the dark for 15 min.
7. Repeat **steps 4–6** until all of the benzophenone solution has been consumed, and keep membrane piece in DI water until continuing with the membrane biosensor fabrication steps (below).

3.1.2 Membrane Biosensor Fabrication

1. Allow carboxylated membrane to dry then put small drop of polyvinyl alcohol (PVA) in the central post in the bottom piece of the primary mold (*see* **Note 2**). Put the back surface of the membrane on the polyvinyl alcohol and secure the membrane in place with the top piece of the primary mold.
2. Mix polyurethane resin (casting resin A and B 1:1 ratio) for 1 min, load into syringe, and pour into a hole in the top of the primary mold (*see* **Note 3**). Allow to solidify for 30 min to 1 h.

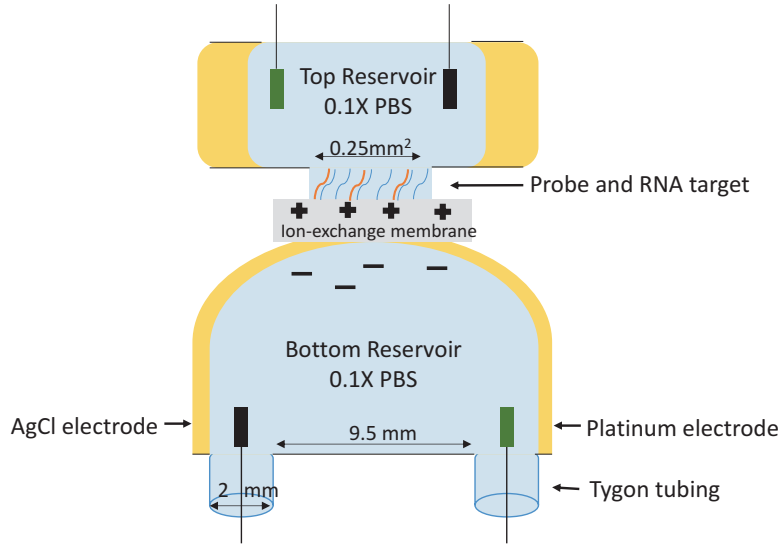

Fig. 1 Biosensor assembly. The ion-exchange membrane is sandwiched between two polyurethane molds with the functionalized probe on top. Both chambers are filled with 0.1× PBS, and the electrodes of the Gamry™ Potentiostat are connected to the inside of the *top chamber* and the inside of the *bottom chamber* through the tygon tubing

3. Obtain a piece of acrylic sheet. Drill two holes (that electrodes will later go into) using 2 mm drill bit. The distance between the two holes should be such that they fit just inside the bottom reservoir of the biosensor (*see* Fig. 1).
4. Cut two pieces of tygon tubing to ~4 mm in length. Glue these to the acrylic sheet using UV curing glue so the inner diameter of tubing is aligned with the holes (*see* **Note 4**). UV treat with Electro-Cure 500 for 10 min to cure.
5. When the polyurethane resin from **step 2** has solidified, detach from mold. This is the biosensor. Attach the acrylic sheet to the biosensor with UV curing glue. Treat with UV light for 10 min with ElectroCure 500. Let cool at room temperature for 10 min (*see* **Note 5**; *see* Fig. 1).
6. Fill the top and bottom reservoir of the biosensor with 0.1× PBS. Let soak overnight.
7. User can take raw current–voltage characteristic measurements at this step using the Gamry™ potentiostat (as a reference prior to probe functionalization and RNA sample exposure; *see* Subheading 3 below).

3.1.3 Gamry™ 500 Potentiostat Current–Voltage Characteristic (CVC) Measurements

1. Wash the top and bottom reservoirs of the biosensor with 0.1× PBS then fill the top and bottom reservoirs with 0.1× PBS while making sure not to accumulate air bubbles (bottom reservoir is filled with 0.1× PBS through tygon tubes).

2. Insert platinum electrode and silver chloride reference electrode of Gamry™ potentiostat into the tygon tubes of the bottom reservoir (*see* Fig. 1).
3. Insert platinum electrode and silver chloride reference electrode into top reservoir, making sure that the two electrodes do not touch each other (*see* **Note 6**).
4. Start up the Gamry™ Framework software application. Under the "Experiments" menu, select "D – Electrochemical Energy," then "6 Polarization Curve" from the submenu.
5. When pop-up options occur, make sure "Working Connection" is set to negative, the scan rate is set to "5×10^{-7}A/s," and the sample period to "0.5 s."
6. The Gamry™ will begin to take the current–voltage characteristic (CVC) curve. Take about three to five measurements until each CVC curve has stabilized and looks approximately the same (*see* **Note 7**). The user should take measurements before and after probe functionalization. Once the probe is functionalized the user can then add biological sample to the top reservoir prior to CVC measurements.

3.1.4 Probe Functionalization and Target RNA Detection

1. Fill top reservoir of biosensor with pH 12 sodium hydroxide for at least 4 h (overnight permissible).
2. Remove sodium hydroxide and wash top reservoir three times with 0.1 M MES buffer, pH 5.5. Fill top reservoir with MES buffer and allow membrane to soak for 30 min.
3. Bring EDC powder to room temperature. Combine 19 mg EDC powder with 250 μL of MES buffer, and mix thoroughly. EDC powder should fully dissolve into the solution.
4. Aspirate MES buffer from top reservoir, and add EDC buffer solution. Let the membrane soak in EDC solution for 30 min.
5. Mix probe with 0.1× PBS to obtain a probe concentration of at least 10 μM. Aspirate EDC buffer from top reservoir. Place 40 μL of probe-containing buffer in top reservoir, on top of membrane, and allow overnight incubation (*see* **Note 8**).
6. After overnight incubation, current–voltage characteristic curves may be taken using the Gamry™ Potentiostat to determine if probe was successfully functionalized (*see* **Note 9**; *see* Fig. 2).
7. Once probe is functionalized onto ion transport membrane, wash top reservoir 4× with 0.1× PBS, then add the target RNA (complementary to functionalized probe) sample directly to the membrane. Wash top reservoir/membrane again with 4× PBS to wash away unbound RNA and other biological debris. We recommend starting off with commercially available, known concentrations of your target miRNA of interest before

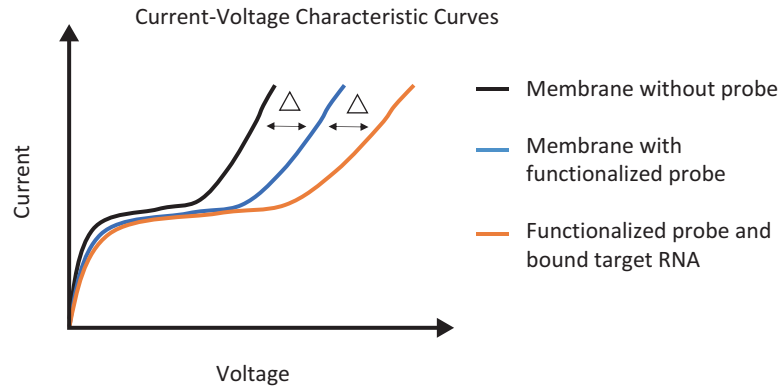

Fig. 2 Current–voltage characteristic curves. The addition of probe and hybridized RNA to the probes creates an increase in the voltage shift, which is measured for incremental known concentrations of target miRNA to establish a calibration curve prior to the addition of the biological sample

adding your biological sample (conditioned cell media sample with exosomes exposed to SAW lysis in our case). This will allow you to obtain voltage data points at known concentrations of target RNA to create a calibration curve using the same membrane.

8. Take CVC measurements as described above. We recommend adding incrementally higher concentrations of RNA target on the same membrane and taking CVC measurements post each concentration to establish successful binding of target RNA to probe (*see* **Note 10**).
9. In order to quantify target RNA concentration of multiple RNA samples using the same membrane piece, the membrane must be washed with 0.1× PBS, pH 13 (buffered with NaOH) to dehybridize the target RNA from the probes before adding the next RNA sample to the membrane. Wash membrane with 0.1× PBS to reestablish pH. Take CVC measurements to affirm that the voltage shift is back to that of membrane and probe alone. Now another RNA sample may be added to the membrane. Repeat **steps 7–9** as necessary to measure target RNA concentrations of separate biological samples using the same membrane.

3.2 SAW Microfluidics Platform Fabrication

3.2.1 Surface Acoustic Wave (SAW) Device Fabrication

1. Use Oerlinkon Evaporator to deposit 20 nm of titanium followed by 200 nm aluminum onto a 128° Y-cut lithium niobate piezoelectric wafer (101.6 mm × 101.6 mm).
2. Dice wafer into six to eight pieces large enough to accompany each individual SAW device (each SAW device is 16 mm × 40 mm).
3. Make a SAW mask using the L-Edit software to design an interdigitated transducer consisting of 20 pairs of interdigitated electrodes. Each electrode leg should be 1/8 of

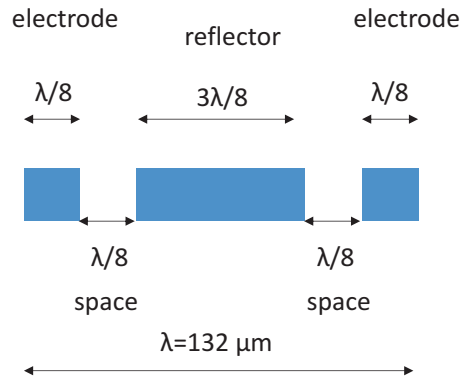

Fig. 3 Mask design. An example of one electrode pair is shown as designed in L-Edit for the etching of the interdigitated electrodes of the SAW device. The device uses the standard SPUDT design

the designed SAW wavelength (λ) and the spacing between electrodes should also be 1/8 of the designed SAW wavelength. In our case, the SAW wavelength was λ = 132 μm (*see* Fig. 3, *see* **Note 11**).

4. Spin coat the lithium niobate wafer with S1813 photoresist at 2500 RPM for 30 s.
5. Place wafer on 90 °C hot plate for 1 min; then place wafer on plastic container and allow to cool for 5 min.
6. Expose in Karl Suss MJB3 Mask Aligner using SAW mask for 14 s, then develop for 1 min or until the SAW pattern is clearly visible.
7. Place lithium niobate piece in DRYTEK plasma etcher, and apply the descum process for 1 min.
8. Use the reactive ion etch (RIE) process to etch away the areas without photoresist in order to expose the titanium–aluminum interdigitated electrodes. An etch time of 5 min and 15 s is usually sufficient. This will need to be optimized by checking under a microscope to validate that the pattern appears correctly. Then wipe the substrate with acetone to remove any residual photoresist.

3.2.2 Microfluidic Device Fabrication

1. Obtain a 300 μm thick polycarbonate sheet and attach it to Graphtec Cutting Pro to cut a channel into the sheet with height of 300 μm and width of 2000 μm. With another polycarbonate sheet cut the holes for the inlet and outlet ports of the microfluidic device (*see* Fig. 4).
2. Clamp two of the three polycarbonate sheets one on top of the other while being sandwiched between glass slides to prevent scratching with binder clips. Place in 180 °C oven for 15 min.

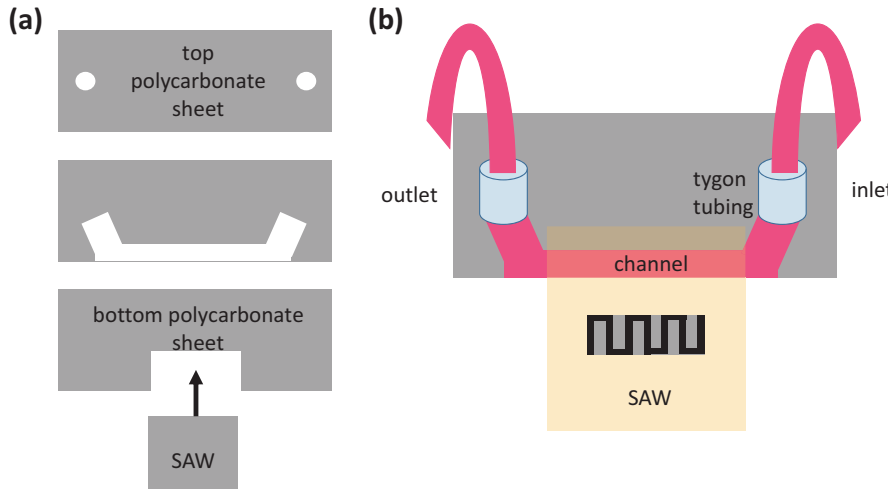

Fig. 4 Integrated microfluidics device design. (**a**) The three polycarbonate sheets are welded together to create an inlet and outlet hole in the *top* where the biological sample enters and exits, the channel in the *middle* which will come into contact with the SAW device, and the *bottom* polycarbonate sheet which is cut to form to the attached SAW device. (**b**) Depiction of what the integrated device looks like after addition of the cell media sample and tygon tubing

3. Repeat this process to add the third polycarbonate sheet to the two adhered sheets. The user should now have one polycarbonate sheet (consisting of three combined sheets) which has inlet and outlet holes in the top, which connect to the beginning and end of the interior channel.
4. To attach the SAW device to the microfluidics polycarbonate device beneath the channel, first seal the bottom of the channel (*see* **Note 12**).
5. Heat up 12% agarose powder in DI water in microwave until it is in liquid form and add it to the channel through the outlet or inlet using a syringe. Allow it to cool and solidify.
6. Once the agarose gel is solidified, remove the sealant on the bottom of the channel and attach the SAW device just beneath the bottom of the channel with UV curing glue (Loctite® 3492). Cure in Electro-Cure 500 for 10 min.
7. Heat the entire device in 80 °C oven for 10 min to liquefy the agarose gel. Aspirate out the liquid agarose from the channel.
8. Tightly seal the channel to the SAW device with ACRIFIX® 1R 0192 UV curing glue to prevent leakage of biological sample from the channel. Cure in Electro-Cure 500 for 20 min.
9. Obtain 4 mm long tygon tube pieces that have the same inner diameter as the inlet and outlet holes and seal them over top of the inlet and outlet holes using the Loctite® 3492 UV curing glue and cure for 5 min.

10. Finally, tightly seal the tube pieces to the microfluidics device by applying the outside circumference of each tube with ACRIFIX®1R 0192 UV curing glue and curing for 20 min.

3.2.3 Exosome Lysis via Surface Acoustic Wave Exposure

1. To detect a specific miRNA or RNA contained in exosomes collected from cell culture media we suggest culturing cells in exosome-free media to start.
2. Collect the media from the cells. Wash the cells with 1× PBS and add it to the collected cell media.
3. Spin down any cells and large cell debris. We typically spin down the media at 500 × *g* for 5 min, collect the supernatant, spin down the supernatant at 16,500 × *g* in an ultracentrifuge for 20 min, and collect the supernatant. The supernatant at this point can also be filtered using a 200 nm pore size filter, but this step may result in some exosome sample loss.
4. This cell media supernatant can now be used and be pumped through the inlet of the microfluidics-SAW device by surface acoustic waves activated by Agilent 33250A generator along with E&I 325LA RF power amplifier (*see* **Note 13**).
5. Collect the media exposed to the SAWs from the outlet, allowing the media to drip into a microcentrifuge tube. This media is now ready to be placed onto the probe-functionalized ion-exchange membrane of the biosensor in order to detect the target RNA within (*see* **Note 14**).

4 Notes

1. pH strips may be used to verify the pH. It is recommended to start by adding 9 μL of concentrated NaOH solution and then add 1–2 μL as needed to reach the desired pH.
2. The bottom and top reservoirs are created by pouring polyurethane into a primary mold. This primary mold was created using CAD designs and 3D printing [2]. After polyurethane reservoirs have solidified the membrane should be sandwiched between the two reservoirs and exposed by an approximately 0.25 mm^2 opening between the two reservoirs (*see* Fig. 1).
3. We mix resin in a weigh boat while vigorously stirring and avoiding accumulation of air bubbles.
4. Use a syringe needle to poke a hole through the tubing to make sure you did not just glue the tubes shut before you cure the glue with UV light. Also make sure the electrodes you will use will fit through the tubing. If not, adjust the tubing diameter and acrylic sheet hole diameters accordingly.

5. Make sure the acrylic sheet holes and tubing fit just within the diameter of the bottom reservoir (*see* Fig. 1). If too close together, air bubbles often accumulate in the bottom reservoir upon adding 0.1× PBS that will skew data. If outside the diameter of the bottom reservoir, the electrodes will not come into contact with solution and measuring the current–voltage characteristic will be impossible. We also typically glue the outside of the bottom reservoir where it comes into contact with the acrylic sheet and UV cure again to make sure the biosensor is securely attached to the acrylic sheet.
6. To secure the biosensor in place while adding the electrodes to the top reservoir we usually tape the biosensor to a flat platform. To help ensure that the two electrodes do not touch we hold the reference electrode in place with a ring stand.
7. If the system jumps erratically or if the data points appear in red, it could be due to an air bubble in the bottom reservoir. Replacing the 0.1× PBS in the bottom reservoir to eliminate air bubbles may fix the problem (*see* **Note 5**). Otherwise, the user may repeat **steps 4–6** to take additional measurements.
8. For our purposes we used an amine-coupled ssDNA oligonucleotide probe complementary to microRNA 550 which was purchased from Life Technologies.
9. Successful functionalization of the probe onto the membrane can be determined by measuring the current–voltage characteristic (*see* Fig. 2) or by IR spectroscopy.
10. The more target RNA which binds to the probe, the larger the shift in voltage should be, until the membrane has become saturated, with all probes bound to their targets. If no shift in voltage occurs after addition of more RNA, there may be many dilemmas such as RNA degradation or that the starting concentration was too high and membrane became saturated. A few microliters of conditioned cell culture media should be sufficient (detection down to ~2 pM target RNA). Ensure that probe was successfully functionalized (*see* **Note 9**). Try dehybridizing membrane from target RNA (*see* **Note 10**) then adding a more dilute RNA sample. Always keep RNA sample at −80 °C when not in use and on ice when in use.
11. We used standard protocols to produce electrode width controlled (EWC) single phase unidirectional SAW transducer (SPUDT).
12. We found scotch tape to be sufficient for this task.
13. The operating frequency should be 28.3 MHz and the samples we used were pumped through the device at a rate of 250 μL/h with the SAW device operating at 1 W of power. Media within the channel was exposed to the SAW portion for approximately 30 s.

14. Only 100 μL of media exposed to the SAW device was needed to transfer onto the biosensor membrane and detect target miRNA. If wanted, exosome lysis can be validated by examining exosomes via transmission electron microscopy (exosomes should look jagged and broken apart), quantifying the exosomes using a NanoSight, or by quantifying the amount of target RNA prior to and after SAW exposure using the biosensor.

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