Video Article

Isolating, Sequencing and Analyzing Extracellular MicroRNAs From Human Mesenchymal Stem Cells

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Abstract

Extracellular and circulating RNAs (exRNA) are produced by many cell types of the body and exist in numerous bodily fluids such as saliva, plasma, serum, milk and urine. One subset of these RNAs are the posttranscriptional regulators – microRNAs (miRNAs). To delineate the miRNAs produced by specific cell types, in vitro culture systems can be used to harvest and profile exRNAs derived from one subset of cells. The secreted factors of mesenchymal stem cells are implicated in alleviating numerous diseases and is used as the in vitro model system here. This paper describes the process of collection, purification of small RNA and library generation to sequence extracellular miRNAs. ExRNAs from culture media differ from cellular RNA by being low RNA input samples, which calls for optimized procedures. This protocol provides a comprehensive guide to small exRNA sequencing from culture media, showing quality control checkpoints at each step during exRNA purification and sequencing.

Introduction

Extracellular and circulating RNAs (exRNAs) are present in various bodily fluids and are resistant towards RNases[1,2]. Their high abundance, stability and ease of accessibility are attractive for clinical assessment as diagnostic and prognostic markers[3]. The mode of transport for exRNAs include extracellular vesicles (EVs), association with lipoproteins (such as high-density lipoprotein; HDL) and ribonucleoprotein complexes (such as with Argonaute2 complexes)[4].

A subset of exRNAs are microRNAs (miRNAs), which are small non-coding RNAs of about 22 nt that regulate posttranscriptional gene expression. Ex-miRNAs have been implicated in cell-cell communication and regulation of cell homeostasis[5]. For example, HDL delivers ex-miR-223 to endothelial cells to repress intercellular adhesion molecule 1 (ICAM-1) and inflammation[6]. Interestingly, miR-223 is also seen transported by extracellular vesicles from leukocytes to lung cancer cells, programming them to take on a more invasive phenotype[7]. Thus, the transcriptome of ex-miRNAs from various bodily fluids and cell culture medium will greatly improve our understanding of ex-miRNA signaling.

Small RNA sequencing (small RNA seq) is a powerful tool that can be used to understand the transcriptomics of small RNAs. Not only can different samples be compared amongst differentially expressed known RNAs, but novel small RNAs can also be detected and characterized. Consequently, it is also a robust method to identify differentially expressed miRNAs under different conditions. However, one of the hurdles of small RNA seq is the difficulty in generating small RNA seq libraries from low exRNA input fluids like cerebrospinal fluids, saliva, urine, milk, serum and culture media. The TruSeq Small RNA Library Prep protocol from Illumina requires approximately 1 µg of high quality total RNA and the NEBNext Small RNA Library Prep Set protocol from New England Biolabs requires 100 ng-1 µg of RNA[8,9]. Oftentimes, total RNA from these samples are below detection limit for conventional UV-vis spectrophotometers.

Ex-miRNAs derived from bodily fluids are potentially good prognostic and diagnostic markers. However, in order to study the functional effects or to determine the origin of specific ex-miRNAs, cell culture systems are often used instead. Mesenchymal stem cells (MSCs) have been studied extensively because their EVs have been implicated in alleviating many diseases including myocardial infarction, Alzheimer's disease and graft versus host disease[10]. Here, we demonstrate the purification of ex-miRNAs from bone marrow-derived MSCs (BMSCs) and the specific steps used to optimize small RNA library construction, sequencing and data analysis (**Figure 1**).

Protocol

NOTE: Mesenchymal stem cell growth medium (MSC media) is prepared beforehand as indicated in the **Table of Materials**.

1. Cell culture

NOTE: Human mesenchymal stem cells can be obtained from the bone marrow, adipose tissue or other sources[11]. Alternatively, hMSCs can be bought through a supplier. The BMSCs used in this protocol were derived from the bone marrow of patients and bought from a company.

1. Thaw 1 x 10^6 BMSCs into a T175 flask containing 20 mL of MSC media. Incubate the cells at 37 °C with 5% CO_2 and replace the media every 2-3 days until 80% confluency.
2. Wash the cells with 5 mL of 1x PBS and discard the PBS.
3. Detach the cells by adding 5 mL of 0.05% Trypsin-EDTA and incubating the cells for 5 min at 37 °C. Tap the sides of the flask to facilitate detachment.
4. Add 15 mL of MSC media to inactivate the trypsin, detach the cells from the surface, and pipette up and down to obtain single cell suspensions.
5. Collect the cells in a 50 mL tube and spin down for 5 min at 300 x g to pellet the cells.
6. Resuspend the cells in 1 mL of MSC media and count the cells using a hemocytometer.
   NOTE: Primary human bone marrow MSCs at 80% confluency in a T175 flask is around 2 x 10^6 cells.
7. Plate hMSCs at 2,000 cells/cm^2 in fresh MSC media in 5 T175 flasks. Grow the cells at 37 °C with 5% CO_2 and replace the media every 2-3 days until 5 flasks of 90% confluent T175 flasks are obtained.

2. EVs and RNA-associated biomolecules collection

NOTE: EV collection media is prepared beforehand (Dulbecco's Modified Eagle's Medium [DMEM] with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin [P/S]). EV collection media is normal MSC media, but prepared with commercial EV-depleted FBS (**Table of Materials**). This is to avoid bovine exRNA contamination from FBS, which normally contains exRNAs associated with EVs, ribonucleoproteins, and lipoproteins. For small RNA library preparation, exRNAs derived from 5 confluent flasks of MSCs are required to enable library construction.

1. Wash the cells 3x with 20 mL of PBS per T175 flask. Add 20 mL of EV collection media per confluent T175 flask of MSCs and incubate at 37 °C with 5% CO_2 for 48 h.
2. Collect the media and centrifuge the media for 10 min at 300 x g and 4 °C.
3. Collect the supernatant and centrifuge the media for 20 min at 2,000 x g and 4 °C.
4. Collect the supernatant and centrifuge the media for 30 min at 15,500 x g and 4 °C. Then collect the supernatant.
5. Transfer the media to ultracentrifuge tubes and pellet the exRNAs for 90 min at 100,000 x g and 4 °C. The pellet is anchored to the side of the tube, which is visible, brown and transparent.
6. Remove the supernatant, dry the inside of the tube by inverting the tube on absorbent paper and use small pieces of absorbent paper to remove the liquid inside the tube without touching the bottom of the tube. Resuspend the pellet in 200 µL of PBS by vortexing for 30 s and pipetting up and down 20x.
7. Assess the EVs and biomolecules with nanoparticle tracking analysis (NTA) (**Figure 2**).
   NOTE: The EVs and biomolecules can be assessed with nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) or transmission electron microscopy (TEM)[12].
8. Store the EVs and biomolecules at -80 °C until further downstream experiments.
   NOTE: If EVs are going to be used for functional studies, 20% glycerol must be added to protect them from rupturing. The cells can be collected using standard procedures if necessary.

3. EVs and RNA-associated biomolecules collection of differentiated cells

NOTE: EVs and RNA associated biomolecules can also be collected from the cell culture media while the cells undergo differentiation. The example depicted in the protocol describes osteoblastic differentiation and exRNA collection at day 0 and 7 of differentiation. If no differentiation is required, then skip Section 3 and go to Section 4.

1. Prepare osteoblastic differentiation media (DMEM with 10% FBS, 1% P/S, 10 mM β-glycerophosphate, 10 nM dexamethasone, 50 µM ascorbate-2-phosphate, and 10 mM 1.25-vitamin-D3) fresh every time.
2. Once MSCs are 80% confluent, change the MSC media to osteoblastic differentiation media.
3. Replenish the osteoblastic differentiation media after 2-3 days.
4. On day 5 of differentiation, remove the media and wash the cells 3x with 20 mL of PBS per T175 flask.
5. Add 20 mL of EV collection media containing 10 mM β-glycerophosphate, 10 nM dexamethasone, 50 µM ascorbate-2-phosphate and 10 mM 1.25-vitamin-D$_3$ per confluent T175 flask of MSCs. Incubate the cells at 37 °C with 5% CO_2 for 48 h to ensure continued differentiation while collecting the EVs and biomolecules.
6. Collect the media on Day 7 and proceed to isolate the EVs and biomolecules as described in steps 2.2-2.6.
7. For quality control, seed cells in 96-wells or 6-wells to assess differentiation using an alkaline phosphatase (ALP) activity assay or with quantitative polymerase chain reaction (qPCR), respectively.
   NOTE: **Figure 3** is an example showing osteogenic differentiation of the cells.

4. RNA extraction and quality control

1. Thaw samples from step 2.8 on ice. Extract RNA using an RNA isolation kit (**Table of Materials**).
2. Elute the RNA from the column provided in the RNA isolation kit (**Table of Materials**) in 100 µL of RNase-free water.
3. Concentrate the RNA through ethanol precipitation by adding 1 µL of glycogen, 10 µL of 2 M pH 5.5 sodium acetate and 250 µL of pre-chilled 99% ethanol into 100 µL of purified RNA.
4. Incubate the samples at -20 °C overnight to precipitate the RNA. Pellet the RNA by centrifuging for 20 min at 16,000 x g and 4 °C.

NOTE: The pellet is white due to co-precipitation with glycogen.

5. Remove the supernatant and wash the RNA pellet with 1 mL of 75% ethanol. Pellet the RNA again for 5 min at 16,000 x *g* and 4 °C.
6. Remove the ethanol and leave the lid of the RNA tube open for 5-10 min to air dry the RNA pellet. Resuspend the RNA pellet in 7 µL of RNase-free water.
7. Check the RNA quality and concentration using a chip-based capillary electrophoresis machine to detect the RNA according to the manufacturer's protocol prior to library construction.
   NOTE: A representative size distribution of the RNA is shown in **Figure 4**.
8. Extract cellular RNA using a commercial purification kit (**Table of Materials**) if necessary.

5. Library construction and quality control

NOTE: Small RNA libraries are constructed using commercial kits (**Table of Materials**) with adjustments due to the low RNA input. Library construction is performed on the chilled block.

1. Chill the heating block for 0.2 mL PCR tubes on ice and pipette 5 µL of the RNA from step 4.6 into 0.2 mL RNase-free PCR tubes on a chilled block.
2. Dilute 3' adaptors (1:10) in RNase-free water in a 0.2 mL RNase-free PCR tube. Add 0.5 µL of diluted adaptor and mix with 5 µL of RNA by pipetting up and down 8x and centrifuge briefly to collect all the liquid at the bottom of the tube.
3. Incubate the RNA and 3' adaptor mix at 70 °C for 2 min in a preheated thermal cycler and then place the sample back on the chilled block.
4. Add 1 µL of ligation buffer, 0.5 µL of RNase inhibitor, and 0.5 µL of T4 RNA ligase (deletion mutant) into the RNA and 3' adaptor mixture. Mix by pipetting up and down 8x and centrifuge briefly.
5. Incubate the tube at 28 °C for 1 h in the preheated thermal cycler.
6. Add 0.5 µL of stop solution into the sample tube with the tube staying in the thermal cycler, mix by pipetting up and down 8x and continue to incubate at 28 °C for 15 min.
7. Dilute 5' adaptors (1:10) in RNase-free water in a 0.2 mL RNase-free PCR tube. Add 0.5 µL of 5' adaptor into a separate RNase-free 0.2 mL PCR tube, heat the 5' adaptor at 70 °C for 2 min in the preheated thermal cycler, and then place the sample on the chilled block.
8. Add 0.5 µL of 10 nM ATP, 0.5 µL of T4 RNA ligase to the 5' adaptor tube, mix by pipetting up and down 8x and centrifuge briefly to collect all the liquids into the bottom.
   NOTE: Keep the 5' adaptor on chilled block as much as possible.
9. Add 1.5 µL of the 5' adaptor mixture to the sample from step 5.6, and mix very gently by pipetting 8x slowly. Incubate the sample at 28 °C for 1 h in the preheated thermal cycler.
10. Dilute RT primer 1:10 in RNase-free water in an RNase-free 0.2 mL PCR tube. Add 0.5 µL of diluted RT primer into the sample from step 5.9, mix very gently by pipetting up and down 8x slowly and centrifuge briefly.
11. Incubate the sample at 70 °C for 2 min in the preheated thermal cycler and then place the sample on a chilled block.
12. Add 1 µL of 5x first strand buffer, 0.5 µL of 12.5 mM dNTP mix, 0.5 µL of 100 mM dithiothreitol (DTT), 0.5 µL of RNase inhibitor, and 0.5 µL of reverse transcriptase. Mix very gently by pipetting up and down 8x slowly and centrifuge briefly.
13. Incubate the sample at 50 °C for 1 h in the preheated thermal cycler to obtain cDNA.
14. Add 4.25 µL of ultrapure water, 12.5 µL of PCR mix, 1 µL of index primer, and 1 µL of universal primer into the cDNA. Mix by pipetting up and down 8x and centrifuge briefly.
15. Place the sample in a thermal cycler and set up the cycler as follows: 98 °C for 30 s; 15 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s; and end the cycle with 72 °C for 10 min.
   NOTE: The prepared library can be stored at -20 °C for one week.
16. Purify the RNA library by separating the library on a DNA gel and cutting the gel (gel extraction) between 140 bp and 160 bp and eluting the library from the gel following the protocol given by the library construction kit.
   NOTE: In this study, the prepared library from step 5.15 is loaded onto a commercial gel (**Table of Materials**) and the library between 140 bp and 160 bp is purified out by an automated DNA size fractionator (**Table of Materials**) according to the manufacturer's protocol.
17. Concentrate and wash the library from step 5.16 using a column-based PCR purification kit (**Table of Materials**) and elute the library in 10 µL RNase-free water finally.
18. Load 1 µL of the purified library onto a DNA chip (**Table of Materials**) to check the size of the library following the manufacturer's protocol.
19. Dilute 1 µL of the purified library (1:1000) in 10 mM pH 8.0 Tris-HCl with 0.05% polysorbate 20 and quantify the library by qPCR using a commercial library quantification kit to quantify the library concentration using the DNA standards in the kit.
20. Pool equal amounts of the libraries according to the requirements of the sequencing machine and sequence on a high-throughput sequencing system.
   NOTE: The library construction of cellular RNA can be done using the same kits by following standard protocol of the kits.

6. Bioinformatics pipeline

NOTE: This is an in-house pipeline and the programs used here are listed in the **Table of Materials.**

1. Trim away low-quality reads and remove adaptor sequences from the raw reads.
2. Map the clean reads to different kinds of RNAs.
   1. Annotate tRNAs by allowing two mismatches because the heavily modified tRNA sequences cause frequent base misincorporation by reverse transcriptase.
   2. Annotate miRNAs by mapping to human miRNAs from miRBase v21 allowing zero mismatches. Since miRNAs are subject to A and U nontemplated 3'end additions, sequences that do not map are 3'trimmed of up to three A and/or T nts after which reads are mapped to human miRNAs and other miRNAs from miRBase v21 allowing zero mismatches.
   3. Annotate to other relevant small RNAs (snRNA, snoRNA, piRNA, and Y RNAs) by allowing one mismatch.
   4. Map the remaining unmapped reads to long RNA datasets (rRNA, other RNA from Rfam, and mRNA) to assess degradation.
   5. Annotate the sequences to the human genome.

6. Annotate the sequences to bacterial genomes.
7. Normalize miRNA expression using the following formula: miRNA counts / the total counts of all mapped miRNAs) x 10^6.

Representative Results

The method described in this protocol is optimized to collect exRNA from MSC culture for next generation sequencing. The overall scheme of the workflow is in **Figure 1** on the left and the respective quality control checkpoints are on the right.

The morphology of the cells on the day of collection for undifferentiated (**Figure 3A**) and differentiated (**Figure 3B**) cells are shown. Furthermore, representative normalized levels of ALP activity of cells induced for 7 days are also shown (**Figure 3C**). Here, ALP activity is approximately 2.5 times that of the uninduced cells.

Three T175 flasks of confluent MSCs yield 2-5 x 10^{10} particles/mL (**Figure 2**). Mean particle size is approximately 160-165 nm while most of the particles are 130-140 nm. There were a few smaller peaks between 200 and 500 nm, which would indicate large complexes or large aggregates.

Since RNA can be degraded quickly, adhere strictly to the conditions of RNA extraction as per the kit or protocol used. Use RNase-free conditions, in particular RNase-free water to resuspend the RNA and keep RNA on ice when possible. After extraction, the RNA can be quantified using a chip-based capillary electrophoresis machine. Representative electropherograms of the RNA after being analyzed are shown in **Figure 4**. The electropherogram of exRNAs includes a broad range of RNAs (**Figure 4A**). In contrast, cellular RNA has very distinct peaks at around 70-120, 1800, and 3800 nt, which correspond to tRNA/5S rRNA/5.8S rRNA, 18S rRNA, and 28S rRNA, respectively (**Figure 4B**). The RNA integrity number (RIN) represents the quality of the RNA. A good RIN of cellular RNA is >8 (indicating almost no RNA degradation). The algorithm on which RIN is based is the ratio between 28S and 18S. This means that RIN is not a good indicator of RNA quality of exRNAs because full-length rRNAs are usually not detectable in exRNAs.

Following RNA extraction, the miRNA libraries were constructed and the cDNA libraries were separated by gel electrophoresis. The adaptor-ligated cDNA of the miRNAs is typically between 140 and 160 nt. The quality of the library was then visualized on a chip-based capillary electrophoresis machine (**Figure 5**). **Figure 5A** and **Figure 5B** are the libraries from the cellular RNA at Day 0 and Day 7, respectively. **Figure 5C** and **Figure 5D** are the exRNA libraries at Day 0 and Day 7 respectively. All four samples had peaks at around 140 nt, which indicate successful miRNA library construction. The amount of cDNA in the libraries was quantified by qPCR using a library quantification kit. The quantification cycle (Cq) of the standards was plotted against the log of the concentration to obtain a standard curve (**Figure 6**). The equation of the standard curve was then used to calculate the concentration of the libraries (**Table 1**). In our sample, the concentration of the libraries from exRNAs was 5-8 nM, which was significantly lower than libraries from cellular RNAs (8-30 nM). The libraries were then pooled with equal amount and sent for sequencing.

After the libraries were sequenced, the low-quality reads were trimmed away with FASTX-Toolkit and the resulting quality of the library exRNAs was high and comparable to that of the libraries from the cells (**Figure 7A,B**). The sequence length of the libraries from cells had a single peak at around 22 nt (**Figure 7C**), which correlates with miRNAs. In contrast, the libraries from exRNAs were more heterogeneous and contained 3 major peaks: 22 nt, 30 nt, and 33 nt (**Figure 7D**). Similar to their cellular counterparts, the peak at 22 nt is the miRNAs. Closer inspection revealed that the peaks at 30 and 33 nt are tRNAs halves/fragments or piRNAs. The annotations of the mapped reads were then plotted. Most of the reads (65.1%) from cellular RNA mapped to human miRNAs and each of the other small RNAs accounted for a small portion of the mapped reads (**Figure 8A**). Contrastingly, only 8% of exRNAs mapped to human miRNAs (**Figure 8B**). The most abundant small RNA to which the reads mapped were tRNAs. Most of the reads were "unmatched reads" (43%). Further examination of the unmatched reads from exRNAs to global databases led to the surprising finding that most of them correspond to bacterial sequences (74% and 85% for D0 and D7 exRNA, respectively; **Table 2**). In contrast, only 0.9% of the cellular RNA was unmatched and, of those, only 10% mapped to bacteria. A comparison to the bovine genome showed less than 1% match suggesting that FBS was not a source of contamination (Table 2). Hence, exRNAs exhibit an atypical profile compared to normal cellular RNA.

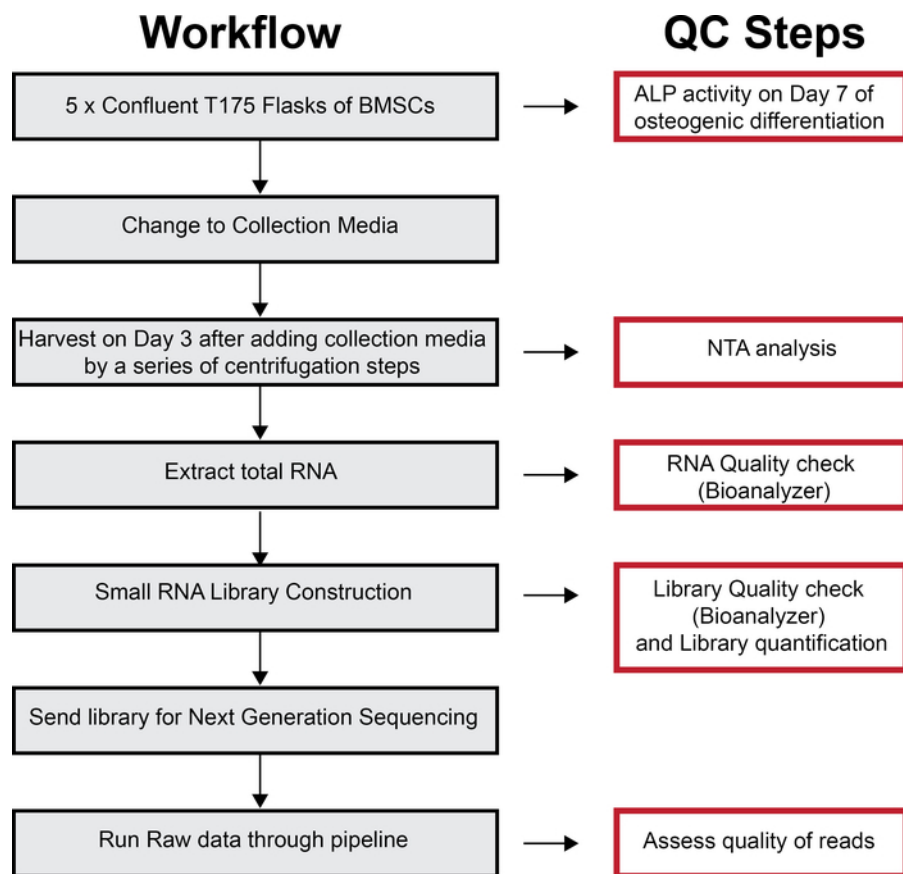## Workflow

5 x Confluent T175 Flasks of BMSCs → **ALP activity on Day 7 of osteogenic differentiation**

↓

Change to Collection Media

↓

Harvest on Day 3 after adding collection media by a series of centrifugation steps → **NTA analysis**

↓

Extract total RNA → **RNA Quality check (Bioanalyzer)**

↓

Small RNA Library Construction → **Library Quality check (Bioanalyzer) and Library quantification**

↓

Send library for Next Generation Sequencing

↓

Run Raw data through pipeline → **Assess quality of reads**

QC Steps

Figure 1: Workflow of exRNA sequencing and analysis. The entire workflow is depicted on the left in the grey boxes and quality control associated with each step is in the red boxes on the right. Please click here to view a larger version of this figure.

A. Representative NTA results from 3 x T175 flasks at D0   B. Representative NTA results from 3 x T175 flasks at D7
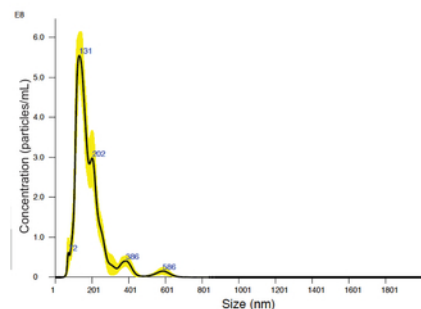
Mean:	161.7 ± 4.0 nm
Mode:	140.8 ± 6.3 nm
SD:	66.9 ± 8.4 nm
Concentration:	$5.08 \times 10^{10} \pm 4.04 \times 10^9$ particles/mL
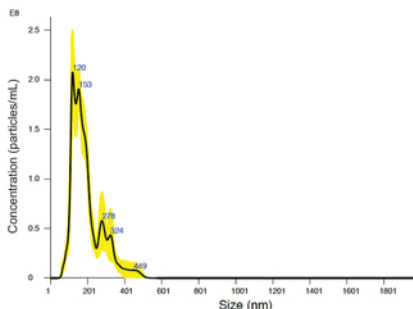
Mean:	165.9 ± 1.7 nm
Mode:	132.9 ± 6.6 nm
SD:	63.8 ± 6.5 nm
Concentration:	$2.03 \times 10^{10} \pm 1.87 \times 10^9$ particles/mL

Figure 2: Size distribution of particles secreted by the cells at day 0 and day 7 of differentiation. Representative NTA results of particles collected from 3 x T175 flasks at (**A**) Day 0 and (**B**) Day 7 of differentiation. The respective mean and mode sizes as well as the concentration of the particles are tabulated beneath the graphs. SD: standard deviation. Please click here to view a larger version of this figure.
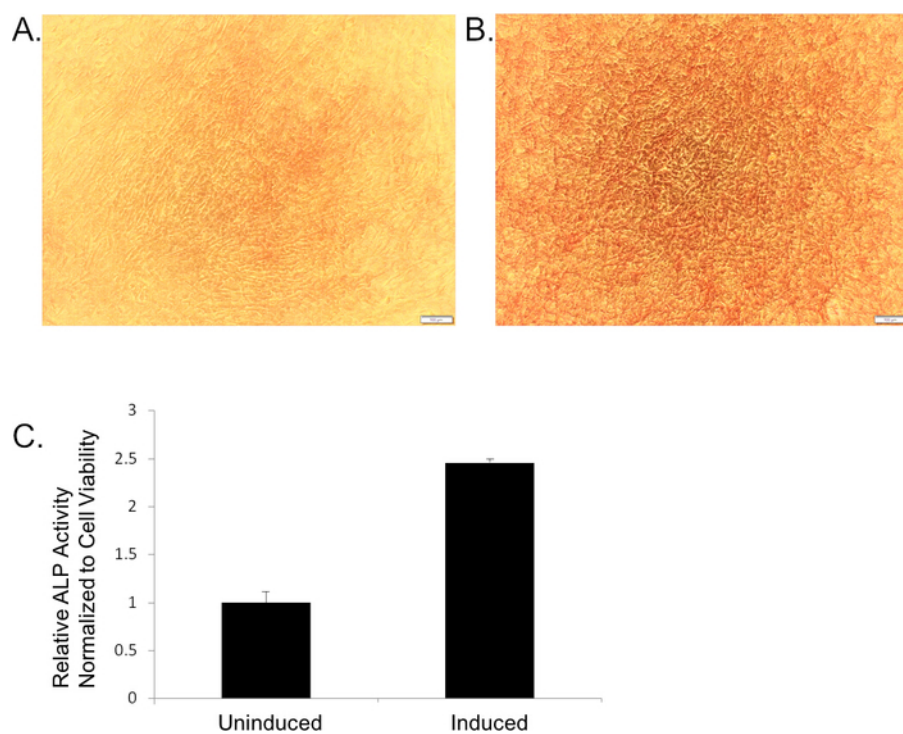
Figure 3: Cell morphology and osteogenic differentiation prior to extracellular RNA collection. (**A**) Micrograph of undifferentiated BMSCs cultured with collection media on the day of collection. (**B**) Micrograph of BMSCs differentiated for 7 days with collection media on the day of collection. Scale bars = 100 µm. (**C**) ALP Activities of the cells were normalized to their respective cell viabilities. The values plotted were that of three independent experiments. Please click here to view a larger version of this figure.
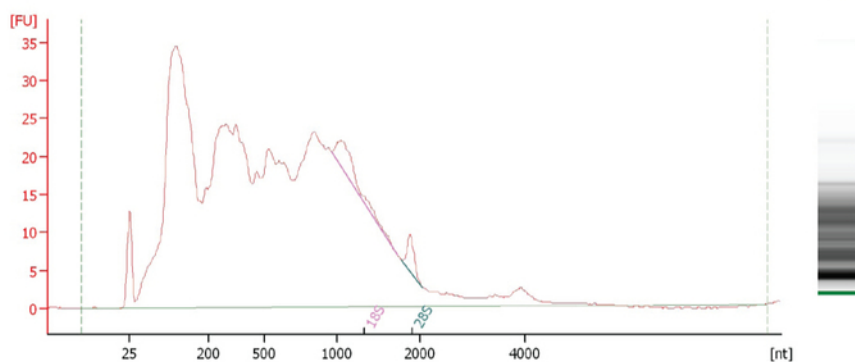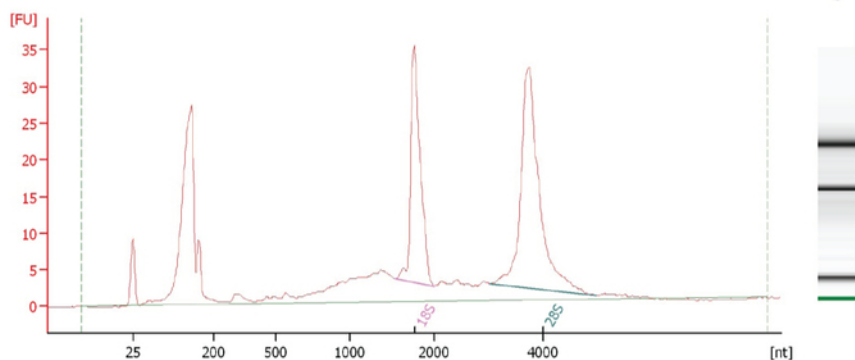
Figure 4: Representative electropherogram of RNA integrity after RNA extraction. RNA analyses of extracellular RNA (**A**) and cellular RNA (**B**). The x-axis is the length of the RNA (nt) and the y-axis is the fluorescence. The first peak is the ladder at 25 nt. Ribosomal RNAs are shown as 18S (1,800 nt) and 28S (3,800 nt). RNA integrity number (RIN) is an indicator of RNA quality based on the 18S and 28S ribosomal RNA integrity. Higher RIN numbers equal better quality. Please click here to view a larger version of this figure.
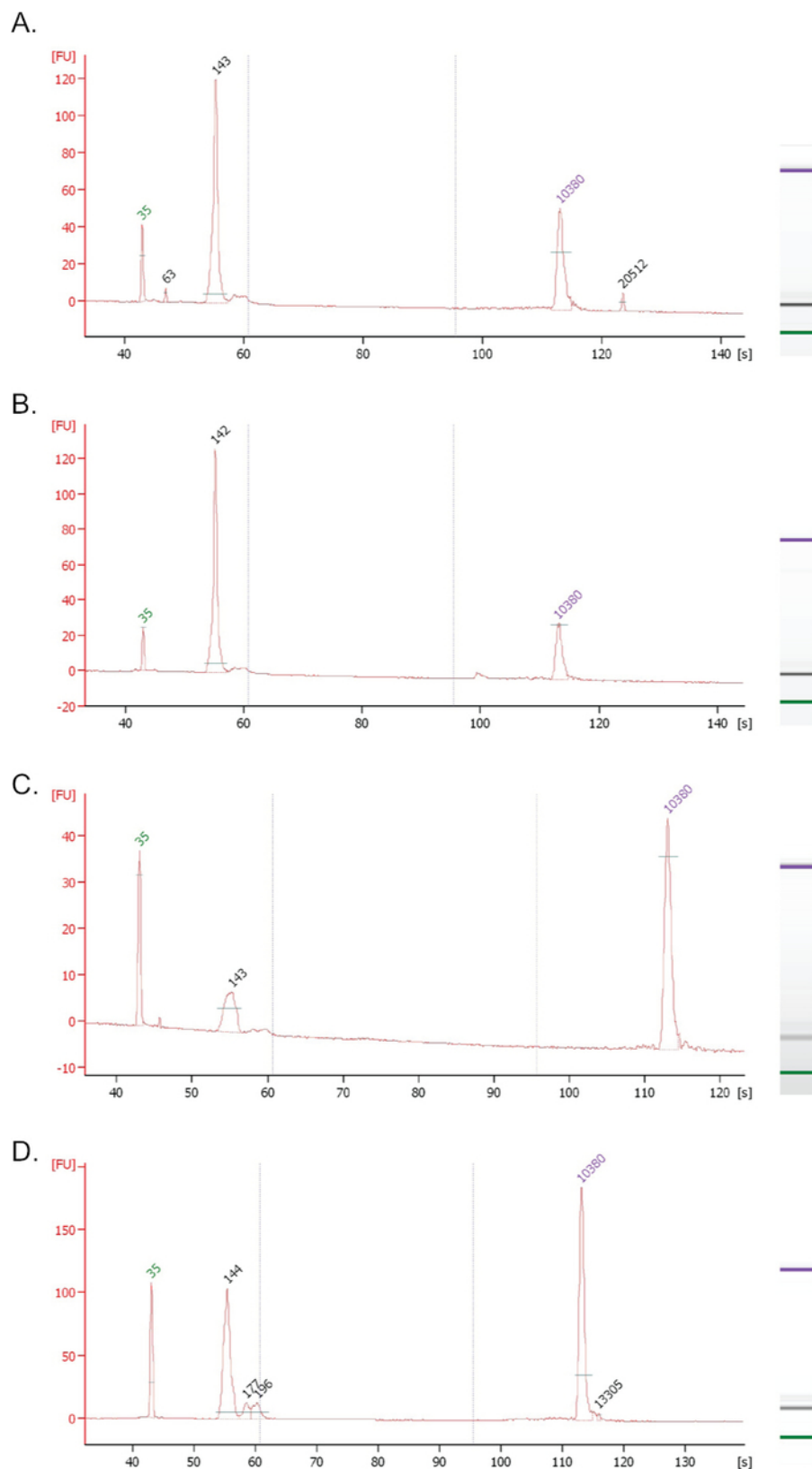
Figure 5: Representative electropherogram of the cDNA libraries after library construction. DNA analyses of the libraries from (**A**) cells at Day 0, (**B**) cells at Day 7, (**C**) extracellular RNA from Day 0, and (**D**) extracellular RNA from Day 7. Green and purple numbers are the ladders at 35 nt and 10,380 nt, respectively. Please click here to view a larger version of this figure.
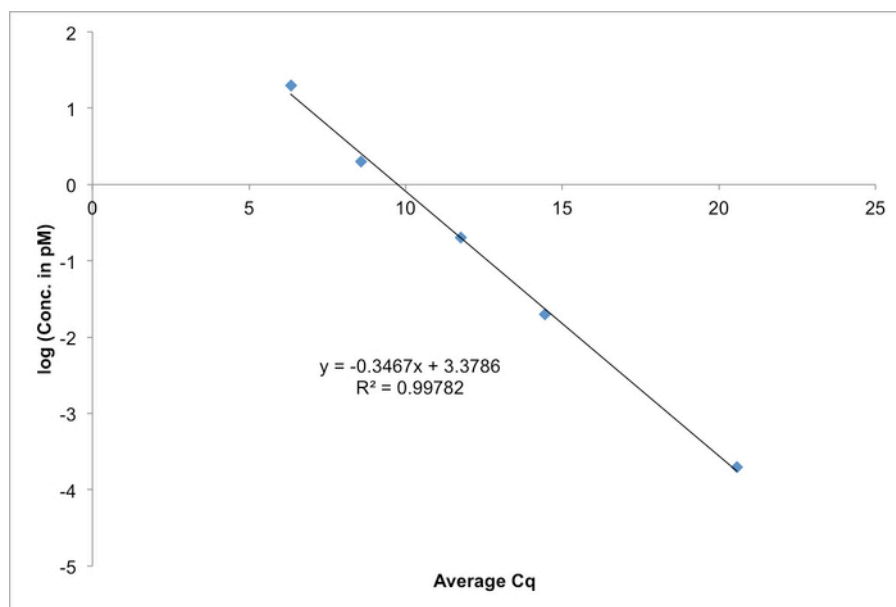
Figure 6: cDNA library quantification and calculation. Libraries were diluted between 500-1,000 times for those from exRNA and between 1,000 and 4,000 times for cell RNA. The libraries were run alongside the standards from the library quantification kit. The average Cq values of the DNA standards were plotted against the $\log_{10}$ concentration (pM) to generate a standard curve, an equation for the standard curve and an R^2 value. Please click here to view a larger version of this figure.
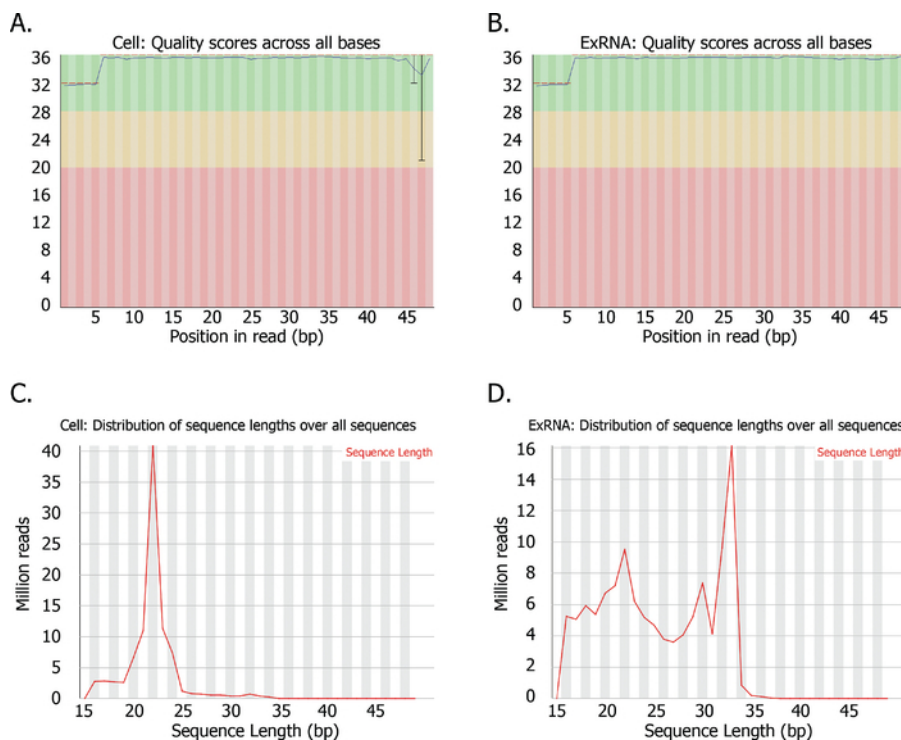

Figure 7: Quality scores and length distribution of sequences. Low quality reads and adaptor sequences were trimmed with FASTX-Toolkit for (**A**) cells and (**B**) exRNAs. The length distribution of clean reads for (**C**) cells and (**D**) exRNAs. Please click here to view a larger version of this figure.
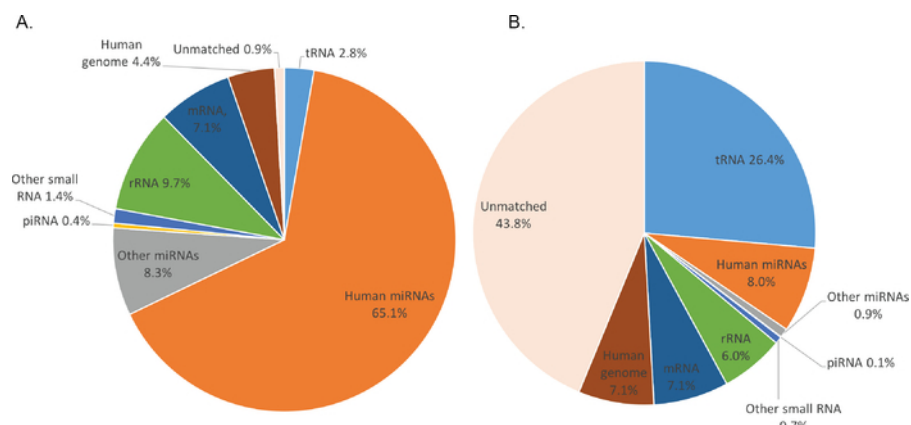
Figure 8: Annotation of small RNAs after sequencing. Annotations represent percentages of clean reads for (**A**) cells and (**B**) exRNAs. Please click here to view a larger version of this figure.

Sample	Dilution factor	Cq value	log(pM)	Concentration (pM)	Concentration mean (pM)	Concentration mean * (452/143)	Multiply by dillution factor (pM)
BMSC D0 exRNA	500	7.72	0.702	5.036	5.276	16.678	8338.993
BMSC D0 exRNA	500	7.57	0.754	5.677			
BMSC D0 exRNA	500	7.7	0.709	5.117			
BMSC D0 exRNA	1000	8.64	0.383	2.416	2.365	7.477	7476.846
BMSC D0 exRNA	1000	8.68	0.369	2.340			
BMSC D0 exRNA	1000	8.68	0.369	2.340			
BMSC D7 exRNA	500	8.52	0.425	2.659	3.221	10.182	5090.913
BMSC D7 exRNA	500	8.42	0.459	2.880			
BMSC D7 exRNA	500	7.97	0.615	4.125			
BMSC D7 exRNA	1000	8.87	0.303	2.011	1.933	6.110	6110.391
BMSC D7 exRNA	1000	8.92	0.286	1.932			
BMSC D7 exRNA	1000	8.97	0.269	1.857			
BMSC D0 cell	1000	6.86	1.000	10.005	9.810	31.007	31006.974
BMSC D0 cell	1000	6.91	0.983	9.614			
BMSC D0 cell	4000	8.68	0.369	2.340	2.398	7.581	30322.041
BMSC D0 cell	4000	8.59	0.400	2.514			
BMSC D0 cell	4000	8.68	0.369	2.340			
BMSC D7 cell	1000	8.44	0.452	2.834	2.880	9.104	9104.439
BMSC D7 cell	1000	8.43	0.456	2.857			
BMSC D7 cell	1000	8.39	0.470	2.950			
BMSC D7 cell	4000	10.36	-0.213	0.612	0.702	2.218	8872.689
BMSC D7 cell	4000	10.27	-0.182	0.658			
BMSC D7 cell	4000	9.97	-0.078	0.836			
Calculations:							
log(pM) = -0.3467 x (Sample Cq value) + 3.3786; use the standard curve and formula							
concentration (pM) = 10^log(pM)							
Size adjustment calculation = Size of DNA standard (452 bp) divided by the average fragment length (143 bp)							

Table 1: miRNA library quantification.

Sample:	# reads not mapping human	# reads mapping to bacteria	% bacterial reads	# reads mapping to cow	% bovine reads
BMSC D0 Cell	131007	9858	8%	99	0.08%
BMSC D7 Cell	169730	7935	5%	188	0.11%
BMSC D0 exRNA	6122833	4551477	74%	891	0.01%
BMSC D7 exRNA	7046691	5970086	85%	771	0.01%

Table 2: The percentage of unmatched reads that map to bacteria and bovine reads.

Discussion

Here, we describe a protocol for next generation sequencing of exRNAs that enables differential expression analyses from low input samples. Adhering to a specific protocol for EV and exRNA isolation is important because even small alterations (i.e., the ultracentrifugation step or a change in rotor type) can influence the transcriptome and miRNA levels[13,14]. Thus, regardless of how the exRNA is isolated, it is important to apply the same experimental and bioinformatic procedure to all the samples in an experiment to be able to compare the results.

RNA integrity is usually assessed on the bioanalyzer. However, since exRNAs are devoid of full length rRNA, their RIN value is very low, but this does not necessarily reflect low quality RNA samples. Additionally, the RNA concentration of the exRNAs varied greatly and is often inaccurate. Hence, instead of assessing RNA quality and quantifying the RNA on the bioanalyzer, use the same volume of media each time for further processing. Also, resuspend the extracted RNA in the same volume of resuspension buffer and use the same volume for library construction.

Small RNA library construction was optimized by reducing the amount of adaptors to a tenth of the standard protocol and using one-half of the other reagents. Reducing the adaptors not only prevents adaptor dimers, it also prevents intramolecular RNA circularization[15]. The reduction in reagents is optimized for the Illumina TruSeq Small RNA Sample Prep Kit. Should other kits be used, it is recommended to also lower adaptor and reagents accordingly, unless the kit specifies that it is tailored to low input samples. Finally, the PCR cycle for library construction was increased from 12 to 15 cycles in this protocol to account for the low concentration of exRNAs. We have found this to be the optimal adjustment without seeing amplification biases[15].

Various quality control metrics can be assessed including base quality scores and read length profile. When doing bioinformatics analyses, the base quality of the reads in cellular RNA and exRNAs were similar; however, the length distribution and the annotated origin of sequences were completely different.Most of the reads from cellular RNA mapped to human miRNAs, however, a large proportion of the exRNA reads were unmatched reads. Upon closer inspection, the unmatched reads turned out to be bacterial reads (**Table 2**). This was surprising because antibiotics were routinely used in our culture. Our result aligns well with other reports that also showed large proportion of unmatched reads when sequencing cell culture derived exRNA[16]. One of the reasons for these contaminants is that bacteria overlap in size with extracellular complexes or EVs, and hence co-purify during the ultracentrifugation step[17]. Previous publications have identified widespread contamination of certain bacteria in culture media which remain undetected under normal cell lab procedures[18]. To date, this problem has largely been ignored but should be taken into account when purifying and analyzing exRNAs.

This protocol details a complete guide to harvesting exRNAs from culture media, optimizing small RNA library preparation and processing the raw library data. This protocol specifically highlights the various quality control checkpoints throughout the process to demonstrate how low input samples (like exRNAs) can deviate from normal sample preparations so that others working with low input samples may know what to expect.

Disclosures

The authors have nothing to disclose.

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