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# Human Milk MicroRNA and Total RNA Differ Depending on Milk Fractionation

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## ABSTRACT

MicroRNA have been recently discovered in human milk signifying potentially important functions for both the lactating breast and the infant. Whilst human milk microRNA have started to be explored, little data exist on the evaluation of sample processing, and analysis to ensure that a full spectrum of microRNA can be obtained. Human milk comprises three main fractions: cells, skim milk, and lipids. Typically, the skim milk fraction has been measured in isolation despite evidence that the lipid fraction may contain more microRNA. This study aimed to standardize isolation of microRNA and total RNA from all three fractions of human milk to determine the most appropriate sampling and analysis procedure for future studies. Three different methods from eight commercially available kits were tested for their efficacy in extracting total RNA and microRNA from the lipid, skim, and cell fractions of human milk. Each fraction yielded different concentrations of RNA and microRNA, with the highest quantities found in the cell and lipid fractions, and the lowest in skim milk. The column-based phenol-free method was the most efficient extraction method for all three milk fractions. Two microRNAs were expressed and validated in the three milk fractions by qPCR using the three recommended extraction kits for each fraction. High expression levels were identified in the skim and lipid milk factions for these microRNAs. These results suggest that careful consideration of both the human milk sample preparation and extraction protocols should be made prior to embarking upon research in this area. J. Cell. Biochem. 116: 2397–2407, 2015. 

**KEY WORDS:** BREASTMILK; HUMAN MILK; RNA; microRNA; CELLS; LIPIDS; FAT; FAT GLOBULE; SKIM MILK; MILK FRACTIONS

Human milk (breastmilk) is a complex secretion of the mammary gland that is the main source of nutrition, immune protection, and developmental programming for the infant [Hassiotou and Geddes, 2013]. In addition to being a balanced food for infants containing water, minerals, vitamins, proteins, carbohydrates, and lipids, human milk is a potent source of immunomodulatory factors [Hanson et al., 1997; Kramer, 2010]. These include bioactive molecules, such as immunoglobulins and lactoferrin [Lonnerdal, 2003; Hassiotou et al., 2013], and immune cells amongst stem cells, progenitor cells, and epithelial cells that constitute a cellular hierarchy in human milk [Hassiotou et al., 2012]. Further to these components, milk is a rich source of RNAs, and microRNAs [Lemay et al., 2013]. The latter have been recently discovered in human milk [Kosaka et al., 2010; Weber et al., 2010; Zhou et al., 2012; Munch et al., 2013] and in the milk of other mammalian species [Chen et al., 2010; Gu et al., 2012; Bai et al., 2013; Izumi et al., 2014], suggesting that they may play crucial roles both in the lactating mammary gland and for the breastfed infant [Zhou et al., 2012; Munch et al., 2013].

MicroRNAs are small non-coding RNA molecules found in plants and animals [Ambros, 2004]. First discovered in 1993 in *Caenorhabditis elegans* [Lee et al., 1993; Wightman et al., 1993], they are considered to be crucial regulators of gene expression at the post-transcriptional level by attaching to messenger RNA (mRNA) to either

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inhibit protein translation and/or induce mRNA degradation [He and Hannon, 2004; Pritchard et al., 2012]. They are thus involved in a number of developmental and physiological processes, including cellular differentiation, apoptosis, proliferation, immune response, and maintenance of cell and tissue identity [Bartel, 2004]. Deregulation of microRNAs is associated with aberrant cell functions leading to cancers [Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006] and other diseases [Lu et al., 2008]. MicroRNA exhibit diversified expression patterns, with some of them being specific to certain organs, such as miR-122, which is primarily found in the liver [Lagos-Quintana et al., 2002], or miR-1, which predominantly exists in the mammalian heart [Lee and Ambros, 2001]. The number of new microRNA molecules discovered is increasing, with over 2,000 having been identified in humans thus far (http://www.mirbase.org). In addition to cells and tissues, microRNA are present in body fluids, such as plasma [Sourvinou et al., 2013] urine, saliva, and tears [Cortez et al., 2011] as well as abundantly in milk [Weber et al., 2010; Munch et al., 2013]. The few studies that have examined microRNA in human milk have largely focused on its skimmed fraction. In 2010, Kosaka et al. reported 281 novel microRNA in skim milk [Kosaka et al., 2010], while Weber et al. found 429 microRNA in mature skim milk and 368 in skimmed colostrum [Weber et al., 2010]. In addition to skim milk microRNAs, human milk contains microRNA packaged in vesicles. These have been examined more recently, with 452 pre-microRNA detected in human milk exosomes [Zhou et al., 2012]. Exosomes are small membrane vesicles secreted from mammalian cells that protect molecules and proteins, which are then transported into the extracellular environment participating in cell-cell communication [Admyre et al., 2007]. Human milk exosomes are rich in microRNA and immune-associated proteins, particularly these that have been isolated from skim milk [Admyre et al., 2007]. So far, 59 immune-related microRNA have been described within human milk exosomes [Zhou et al., 2012]. Further to those, other particles in human milk also contain microRNA. Recently, Munch et al. showed that the milk fat globule encompasses novel microRNAs [Munch et al., 2013]. Similar to adults [Baier et al., 2014], most likely human milk carries these microRNAs to the infant. Although some hypotheses were raised that oral microRNAs do not survive in the human gastrointestinal tract [Dickinson et al., 2013; Witwer and Hirschi, 2014], milk microRNAs are protected within fat globules, exosomes, or cells, and therefore are likely to be transferred intact across to the infant′s blood. Moreover, human milk is known to contain maternal cells [Hassiotou et al., 2013], which are transferred to the infant [Hassiotou et al., 2014]. These cells are rich in microRNA [Alsaweed et al., 2015]. Exogenous microRNAs have been previously described to play functional roles in adults [Zhang et al., 2012; Baier et al., 2014], and therefore it is likely that cellular microRNAs from human milk contribute regulatory functions in the infant.

The properties and regulation of microRNA in the different cell types present in human milk as well as in the milk fat globule, skim milk, and exosomes remain unknown. Early evidence suggests that certain human milk microRNA support the immune system of the infant, especially in the first six months of life, such as the highly abundant in milk miR-155, which regulates T and B cells and has a role in the innate immune response [Kosaka et al., 2010]. Prior to investigating the properties and roles of human milk microRNA in either the mammary gland or the infant, it is critical to establish appropriate methodology that allows consistent isolation and quantification of these molecules, similar to that of plasma [Sourvinou et al., 2013], serum [Farina et al., 2014], and blood cells [Hammerle-Fickinger et al., 2010; Monleau et al., 2014]. However, in this study, 8 extraction kits from different were used

Given that only the skim milk fraction of human milk has been widely investigated, it is not known what contribution the lipid or cell fractions make to the total microRNA population of human milk.

Considering that handling and isolation protocols play critical roles in the reliable quantification of microRNA in plasma [Sourvinou et al., 2013], it is logical to expect that special protocols, and handling procedures may apply to human milk. In addition, different methods have been utilized, contributing to the wide variation of total RNA, and microRNA published in human milk studies. In this study, we determined whether the microRNA content differed between the skim, lipid, and cell fractions of human milk and investigated the efficacy (yield and quality) of microRNA extraction for eight commercially available kits in each milk fraction. This provided valuable insight into the abundance and content of microRNA in different human milk fractions, setting the basis for profiling, and functional microRNA studies in human milk.

## MATERIALS AND METHODS

### HUMAN MILK COLLECTION

The study was approved by the Human Research Ethics Committee of The University of Western Australia. All participants provided informed written consent. Fresh human milk samples (n = 49) were collected from n = 29 breastfeeding mothers on 1–4 occasions under sterile conditions. Symphony pumps (Medela AG, Switzerland) were used for Human milk expression and sample volumes ranged 14–135 mL. All participants and their infants were healthy at the time of milk collection, with current smoking, and medication use being exclusion criteria for participation. Lactation stages at sample collection ranged from 3 to 158 weeks.

### HUMAN MILK FRACTIONATION

All human milk samples were fractionated immediately after expression by centrifugation at 720*g* for 20 min. Three fractions were obtained from each sample (cells, lipids, and skim milk), and were transferred to 15-mL RNase free tubes (Fig. 1). Purification of the milk fractions involved an additional centrifugation step in the lipid and skim milk fractions, whilst cells were washed twice in sterile phosphate-buffered saline (PBS, Gibco).

### TOTAL RNA/microRNA EXTRACTION

All RNA/microRNA extractions were done on ice immediately after separation and purification of the three milk fractions (Fig. 1) using eight commercially available kits (Table I). Each fraction of each milk sample was separated into 2–5 identical aliquots depending on the original sample volume and the size of the cell pellet. Each aliquot was used for extraction by a different kit such that the number of aliquots obtained per sample reflected the number of kits used for this sample. Table II shows the number of milk samples tested with

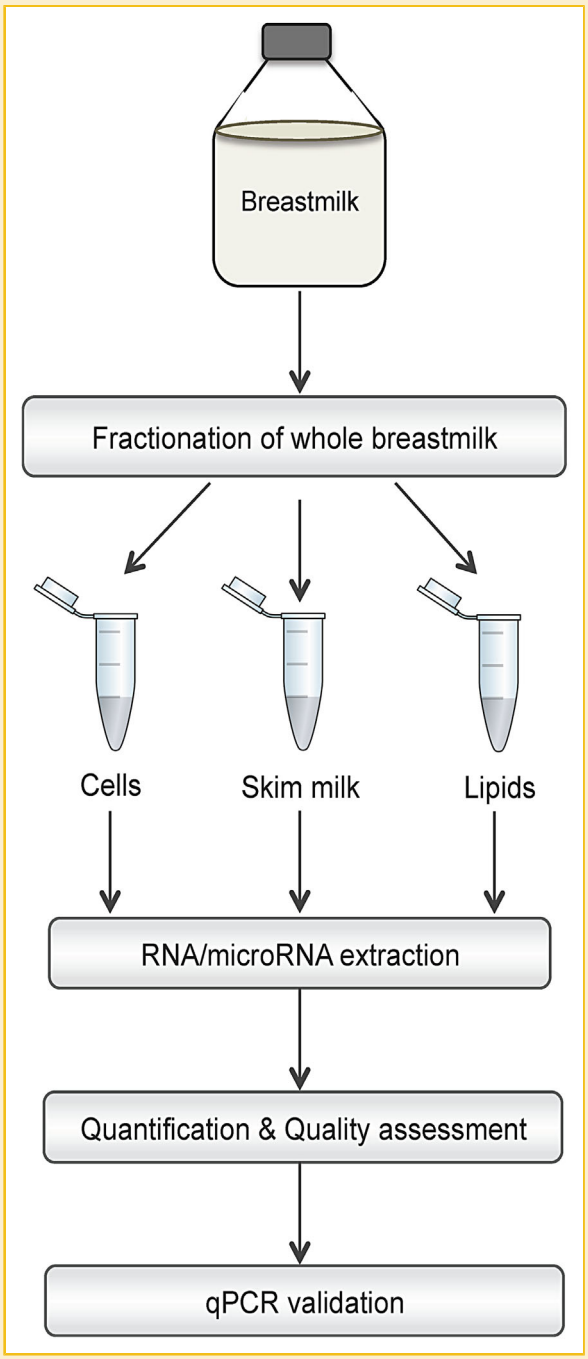


**Fig. 1.** Brief workflow showing the steps to obtain the three main fractions (cells, lipid, and skim milk) of human milk using multiple centrifugation steps depending on the fraction, and quantity, quality and expression level of RNA/microRNA

each extraction kit and for each milk fraction (cells, lipids, and skim milk). Specifically for purified lipids and skim milk, 100–350 μL were used for microRNA extraction, based on the manufacturer's recommendation for each kit.. Briefly, samples were lysed using the lysis reagent provided by the kit, and were homogenized by gentle movement 10 times into and out of a sterile syringe and needle system. Separation of large (DNA, large RNA, and debris) and small (small RNA) phases and precipitation of the former was done with either chloroform or alcohol. After that, isopropanol or column separation was used for RNA precipitation. Then, different washing steps depending on the kit were required to obtain pure small RNA, which was eluted either in elution buffer or suspended in RNase-free water.

### MicroRNA ANALYSIS AND QUANTIFICATION

Concentration and purity (260:280 ratio) of the extracted total RNA was measured using a spectrophotometer (NanoDropTM 1000, Wilmington, DE). The microRNA concentration and microRNA/small RNA ratios were quantified by capillary electrophoresis using the small RNA Chip kit (Agilent, CA) in an Agilent Bioanalyzer 2100 instrument. The amount of small RNA was normalised to 100, and the amount of microRNA was presented as a percentage of this value. Using this kit, we were able to quantify the small RNA in a sample including microRNA, which ranged in size between 6 and 150 nucleotides for small RNAs, and 10–40 nucleotides for microRNAs.

### REVERSE TRANSCRIPTION AND QUANTITATIVE RT-PCR

Two whole human milk samples from two different mothers were fractionated as described above. RNA including microRNA was extracted using recommended kits for each fraction (Table III). RT-PCR was used to validate the presence of two mature microRNAs using the TaqMan miRNA assay (Life Technologies, Foster City, CA). The expression level of hsa-miR-148a-3p and hsa-miR-30a-5p was done in two steps according to the manufacturer's protocol. The reverse transcription was performed using 600 ng of input RNA using the TaqMan miRNA reverse transcription kit and pooled both microRNA primers (5× primers) and endogenous control (RNU48). The RT reaction was processed using BioRad C-1000 thermo cycler (Hercules, CA) as follows: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, then the sample was held at 4°C. The PCR reaction was performed using Fast advanced master mix in triplicates and TaqMan microRNA probes for both examined microRNAs and the endogenous control (20×) using 7500 Fast Real Time-PCR system as follows: 50°C for 2 min; 95°C for 20 s followed by 40 cycles at 95°C for 3 s; and finally 60°C for 30 s. Comparative Ct (RQ) analysis was performed using 7500 software V2.0.6 by normalizing all samples to milk cell A sample.

### STATISTICAL ANALYSIS

Statistical analysis was done using R 2.9.0 for MacOSX (Team Development Core R, 2009) using the base packages, and the libraries nlme, multcomp, and lattice for linear mixed effects models, general linear hypothesis tests, and graphical presentation of data, respectively. Extraction kits were compared in terms of: (a) their efficiency in extracting total RNA, (b) the purity of the extracted RNA (260/280 ratio, whereby values of 1.80–2.19 were considered good; values of 1.51–1.79 were considered moderate; and values $>2.20$ or $<1.50$ were considered poor), (c) their efficiency in extracting microRNA, and (d) the ratio of microRNA to small RNA. This was done separately for each human milk fraction (cells, lipids, and skim milk).

Comparison of the kits was done in two sets reflecting the kits tested in aliquots of the same milk samples (Table I). In the first set

TABLE I. RNA/microRNA Extraction Kits, Suppliers, and the Extraction Based Method That Used for Evaluation and Comparison of Extraction Efficacy in All the Three Breastmilk Fractions in the Two Comparison Sets.

| | Kit | Company | Extraction method |
|---|---|---|---|
| Set 1 | miRNeasy micro Kit | Qiagen | Filter column |
| | mirVana microRNA Isolation Kit | Ambion | Filter column & phenol/guanidine |
| | RNAzol-RT Reagent | Molecular Res. Center | Phenol/guanidine |
| Set 2 | miRNeasy mini Kit | Qiagen | Filter column |
| | TRIzol-LS Reagent | Invitrogen | Phenol/guanidine |
| | miRCURY RNA Isolation-Cell & Plant Kit | Exiqon | Filter column |
| | miRCURY RNA Isolation-Biofluids Kit | Exiqon | Filter column |
| | mirPremier microRNA Isolation Kit | Sigma–Aldrich | Filter column |

(Set 1), three kits were compared: miRNeasy micro Kit (Qiagen, Hilden, Germany), mirVana microRNA Isolation Kit (Ambion, Austin, TX), and RNAzol-RT Reagent (Molecular Research Center, Inc.). In the second set (Set 2), five kits were compared: miRNeasy mini Kit (Qiagen, Hilden, Germany), TRIzol-LS Reagent (Invitrogen, CA), miRCURY RNA Isolation-Cell&Plant Kit, miRCURY RNA Isolation-Biofluids Kit (Exiqon, Vedbaek, Denmark) and mirPremier microRNA Isolation Kit (Sigma–Aldrich, St. Louis, MO).

To determine whether the four measures differed among the kits, linear regression and linear mixed effects models were used, with the measure of interest as the response and the kit (factor with either 3 or 5 levels) as the predictor. Two models were created for each combination of measure, kit set, and milk fraction. Ordinary least squares (OLS) regression, and a linear mixed effects (LME) model with random effects of different baseline levels per sample. Models were compared using analysis of variance. LME models are reported where found to be more appropriate, and regression otherwise. Overall *P*-values for differences among kits were tested using ANOVA, and where this was significantly different Tukey's HSD was used to identify which kits differed. Significance values reported in tables are from the ANOVA, with significances among kits being from Tukey's HSD. Differences were considered to be significant if $P < 0.05$.

## RESULTS

### PURITY OF EXTRACTED RNA

Patterns of RNA purity (Table IV; Suppl. Table 1) were found to be significantly different among the RNA extraction kits examined ($P \leq 0.001$), with the column-based/phenol-free kits being the best performers and the phenol/guanidine-based kits being the worst performers. Moreover, each kit performed differently for different human milk fractions. Notably, the mirPremier microRNA Isolation Kit yielded good 260/280 ratios for the lipid and cellular fractions, but low ratios for the skim milk fraction. In general, all tested kits performed well in the cellular and lipid fractions, with more variation and lower RNA purity seen for the skim milk fraction.

In the milk lipid fraction and comparison Set 1, 260/280 ratios of mirVana and miRNeasy micro kits were significantly higher than those of RNAzol-RT ($P < 0.001$ for both), which had a mean 260/280 ratio that was outside the acceptable range (1.65). Similarly, differences were seen among the TRIzol-LS, miRNeasy mini and miRCURY–Cell&Plant kits, where 260/280 ratios for TRIzol-LS were lower than in the other two kits ($P = 0.016$ and $P = 0.019$, respectively). However, the mean 260/280 ratio for TRIzol-LS was within the acceptable range (1.84), but lower than in the other two kits. Given the 260/280 cut-offs used, RNAzol-RT, and Trizol-LS were outside the acceptable range for lipids, indicating that these are not optimal choices for extracting total RNA or microRNA from milk lipids.

In skim milk and comparison Set 1, 260/280 ratio for mirVana was significantly higher than those of miRNeasy micro ($P < 0.001$) and RNAzol-RT ($P = 0.041$), both of which were outside the acceptable range. In comparison Set 2, although a wide range of average values was obtained among miRNeasy mini, TRIzol-LS, miRCURY–Cell&Plant, miRCURY-Biofluids, and mirRremier, no significant differences were found between the five kits tested ($P = 0.631$). Notably, the mean 260/280 values of TRIzol-LS and mirPremier were outside the acceptable range (1.60 and 1.41, respectively).

In the cellular fraction and comparison Set 1, 260/280 ratios for miRNeasy micro and mirVana were significantly higher than those for RNAzol-RT ($P < 0.001$ and $P < 0.003$, respectively), where RNAzol-RT ratio averaged at 1.61, which is considered to be a low ratio. In comparison Set 2, only the 260/280 ratios for TRIzol-LS were significantly lower than all other kits ($P < 0.001$), and they were also outside the acceptable 260/280 range (1.70).

TABLE II. Number of the Three-breastmilk Fraction Samples Used for RNA/microRNA Extraction in Evaluating Their Performance Using the Eight Kits.

| Kit/Fraction | Cells | Lipids | Skim milk | Total |
|---|---|---|---|---|
| miRNeasy micro Kit | 14 | 27 | 29 | 70 |
| mirVana microRNA Isolation Kit | 11 | 23 | 26 | 60 |
| RNAzol-RT Reagent | 11 | 22 | 25 | 58 |
| miRNeasy mini Kit | 14 | 22 | 22 | 58 |
| TRIzol-LS Reagent | 12 | 21 | 17 | 50 |
| miRCURY RNA Isolation-Cell & Plant Kit | 15 | 19 | 23 | 57 |
| miRCURY RNA Isolation-Biofluids Kit | 15 | 24 | 23 | 62 |
| mirPremier microRNA Isolation Kit | 14 | 24 | 19 | 57 |
| Total | 106 | 182 | 184 | 472 |

TABLE III. The Three Most Effective Kits for Each Breastmilk Fraction in Each of the Four Evaluation Criteria (total RNA Extracted, Quality of Extracted RNA, Total microRNA Extracted, and microRNA/small RNA Ratio). Total RNA and microRNA Are Presented in ng for the Cellular Fraction and in ng/μl for the Lipid and Skim Milk Fractions. All Values Represented Are Means ± standard Deviations.

| | Comparison Set 1 | | | | Comparison Set 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | miRNeasy micro | mirVana | RNAzol-RT | *P*-value | miRNeasy mini | TRIzol-LS | miRCURY Cell & Plant | miRCURY Biofluids | mirPremier | *P*-value |
| Total RNA | | | | | | | | | | |
| Cells (ng) | 6,598 ± 6,275 | 4,816 ± 4,660 | 3,389 ± 3,320 | 0.055* | 11,083 ± 15,106 | 6,354 ± 8,111 | 6,089 ± 2,853 | 5,218 ± 3,050 | 2,153 ± 4,079 | 0.027* |
| Lipids (ng/μL) | 37.5 ± 32.6 | 26.0 ± 26.4 | 29.0 ± 20.0 | 0.600* | 46.9 ± 34.1 | 44.1 ± 26.9 | 49.9 ± 61.1 | 29.2 ± 25.8 | 7.02 ± 4.00 | 0.019* |
| Skim Milk (ng/μL) | 1.92 ± 2.10 | 2.04 ± 2.00 | 6.40 ± 4.30 | <0.001 | 2.70 ± 2.60 | 9.30 ± 18.20 | 4.03 ± 6.18 | 2.22 ± 1.35 | 0.60 ± 0.46 | 0.096 |
| 260/280 ratio | | | | | | | | | | |
| Cells | 2.01 ± 0.17 | 1.98 ± 0.16 | 1.61 ± 0.18 | <0.001 | 2.04 ± 0.03 | 1.72 ± 0.14 | 2.10 ± 0.04 | 2.10 ± 0.09 | 2.01 ± 0.11 | <0.001 |
| Lipids | 2.03 ± 0.07 | 2.03 ± 0.19 | 1.65 ± 0.15 | <0.001 | 2.03 ± 0.05 | 1.84 ± 0.20 | 2.04 ± 0.08 | 1.90 ± 0.20 | 1.90 ± 0.10 | 0.008 |
| Skim Milk | 1.41 ± 0.30 | 1.78 ± 0.27 | 1.54 ± 0.19 | <0.001 | 1.99 ± 0.17 | 1.60 ± 0.21 | 1.93 ± 1.72 | 2.13 ± 2.26 | 1.40 ± 0.70 | 0.631 |
| Total MicroRNA | | | | | | | | | | |
| Cells (ng) | 110.6 ± 95.0 | 65.7 ± 84.0 | 139.1 ± 166.0 | 0.508 | 770.6 ± 2,005 | 1,443 ± 3,448 | 806.9 ± 1,661 | 721.9 ± 1,822 | 126.5 ± 169.5 | 0.722 |
| Lipids (ng/μL) | 3.40 ± 6.30 | 0.80 ± 1.70 | 1.10 ± 1.40 | 0.169 | 6.70 ± 11.1 | 3.20 ± 2.80 | 2.34 ± 1.10 | 12.7 ± 24.4 | 9.40 ± 8.50 | 0.216 |
| Skim Milk (ng/μL) | 0.10 ± 0.09 | 0.10 ± 0.15 | 0.19 ± 0.27 | 0.342 | 0.39 ± 0.29 | 0.19 (0.23) | 2.17 ± 5.66 | 1.00 ± 1.09 | 0.30 ± 0.70 | 0.363 |
| MicroRNA to Small RNA (%) | | | | | | | | | | |
| Cells | 24.4 ± 13.7 | 19.5 ± 8.7 | 33.5% ± 16.0 | 0.152 | 36.1 ± 11.8 | 51.1 ± 24.5 | 19.9 ± 8.1 | 23.8 ± 7.4 | 26.0 ± 13.5 | <0.001 |
| Lipids | 53.2% ± 31.4 | 28.6 ± 22.7 | 46.3% ± 23.9 | 0.041 | 43.7 ± 20.9 | 44.4 ± 20.4 | 34.3 ± 13.9 | 38.9 ± 10.7 | 31.4 ± 17.6 | 0.217 |
| Skim Milk | 20.5 ± 8.5 | 24.0 ± 12.4 | 48.5% ± 20.6 | <0.001 | 35.4 ± 17.1 | 47.7 ± 14.0 | 33.0 ± 6.2 | 47.0 ± 14.5 | 57.8 ± 13.7 | <0.001 |

*Results from linear mixed effects models, indicating that there are significant correlations between measures within a sample.

TABLE IV. Mean and Standard Deviation of Quantity and Quality of RNA and microRNA Extracted From Different Fractions of Breastmilk (cells, Lipids, and Skim Milk) by the Eight Kits in Two Separate Comparison Sets. Total Rna and 260/280 Ratio Were Obtained by Nanodrop, While Total Microrna, and microRNA/small RNA Ratio Were Measured by Bioanalyzer Using Small Rna Kit.

| Milk fraction | microRNA | 260/280 ratio | RNA | microRNA Small RNA Ratio [%] |
|---|---|---|---|---|
| Cells | TRIzol LS (1443.3 ± 3447.6 ng) | miRNeasy mini (100%); (2.04 ± 0.03) | miRNeasy mini (11082.7 ± 15106.3 ng) | TRIzol LS (51.1% ± 24.5) |
| | miRCURY RNA Isolation kit-Cell & Plant (806.9 ± 1660.8 ng) | miRCURY RNA Isolation Cell & Plant (100%); (2.10 ± 0.04) | miRNeasy micro (6598.5 ± 6275.2 ng) | miRNeasy mini (36.1% ± 11.8) |
| | miRNeasy mini (770.6 ± 2005.5 ng) | mirVana microRNA Isolation (85.7% good, 14.3% moderate); (1.98 ± 0.16) | miRCURY RNA Isolation Cell & Plan (6,089.1 ± 2853.2 ng) | RNAzol RT (33.5% ± 16.0) |
| Lipids | miRCURY RNA Isolation Biofluids (12.7 ± 24.4 ng/μL) | miRNeasy mini (100%); (2.03 ± 0.05) | miRCURY RNA Isolation Cell & Plant (49.9 ± 61.1 ng/μL) | miRNeasy micro (53.2% ± 31.4) |
| | mirPremier microRNA Isolation (9.4 ± 8.5 ng/μL) | miRCURY RNA Isolation Cell & Plant (100%); (2.04 ± 0.079) | miRNeasy mini (46.9 ± 34.1 ng/μL) | RNAzol RT (46.3% ± 23.9) |
| | miRNeasy mini (6.65 ± 11.1 ng/μL) | miRNeasy micro (100%); (2.03 ± 0.07) | TRIzol LS (44.1 ± 26.9 ng/μL) | TRIzol LS (44.4% ± 20.4) |
| Skim milk | miRCURY RNA Isolation Cell & Plant (2.17 ± 5.66 ng/μL) | miRNeasy mini (80% good, 13.33 moderate, 13.33 poor); (1.99 ± 0.17) | TRIzol LS (9.26 ± 18.23 ng/μL) | mirPremier microRNA Isolation (57.8% ± 13.7) |
| | miRCURY RNA Isolation Biofluids (1.00 ± 1.09 ng/μL) | mirVana microRNA Isolation (53.33% good, 26.66 moderate, 20% poor); (1.78 ± 0.27) | RNAzol RT (6.4 ± 4.29 ng/μL) | RNAzol RT (48.5% ± 20.6) |
| | miRNeasy mini (0.39 ± 0.29 ng/μL) | miRCURY RNA Isolation Biofluids (33.33% good, 40% moderate, 26.66% poor); (2.13 ± 2.26) | miRCURY RNA Isolation Cell & Plant (4.03 ± 6.18 ng/μL) | TRIzol LS (47.7% ± 14.0) |

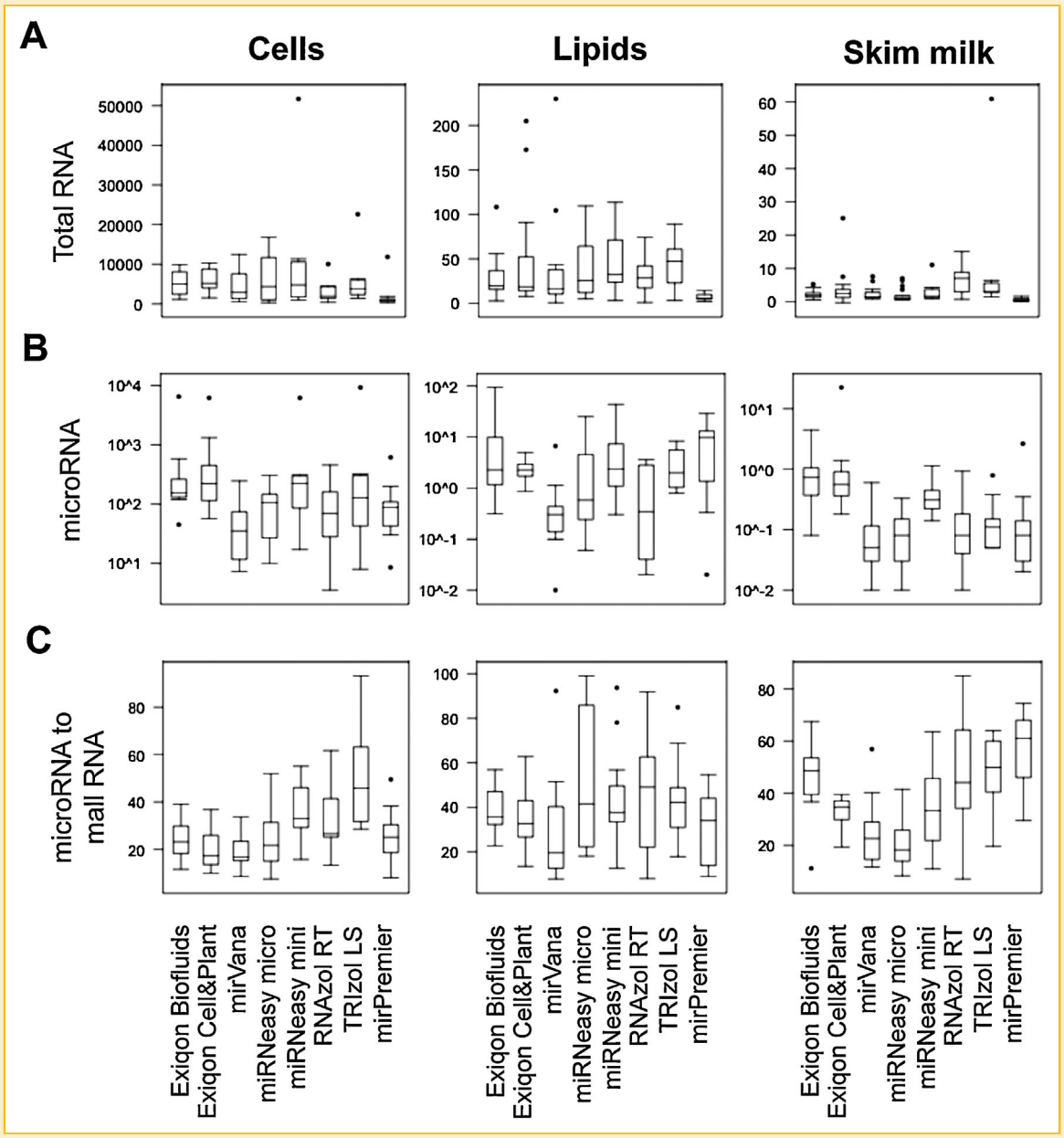


**Fig. 2.** Total RNA (A), microRNA (B) and microRNA to small RNA ratio (%) (C) obtained by the eight kits tested. Different shades of grey represent different individuals (human milk samples). RNA and microRNA (A and B, respectively) are presented in ng for the cellular fraction and in ng/μL for the lipid and skim milk fractions. All values represented are means ± standard deviations.

## TOTAL RNA CONCENTRATION

Total RNA concentration differed both among kits and human milk fractions for the same kit (Table IV, Fig. 2). The lipid fraction contained significantly higher concentration of RNA compered to skim milk ($P < 0.05$) (Fig. 3). In the lipid fraction, the mean ± S.D. of total RNA concentration ranged from 7.02 ± 3.99 ng/μL in the mirPremier kit to 49.90 ± 61.06 ng/μL in the miRCURY–Cell&-Plant kit. In comparison Set 1, no significant difference was found between miRNeasy micro, mirVana, and RNAzol-RT ($P = 0.600$). In comparison Set 2, mirPremier yielded significantly lower total RNA values than TRIzol-LS ($P = 0.05$), miRNeasy micro ($P = 0.007$) and miRCURY–Cell&Plant kits ($P = 0.005$). No significant differences were found among the remaining four kits ($P > 0.05$). TRIzol-LS, miRNeasy mini kit and miRCURY–Cell&Plant kit yielded high amount of total RNA from the human milk lipid fraction.

In the skim milk fraction, the mean ± S.D. of RNA concentration ranged 0.60 ± 0.46 ng/μL (mirPremier) to 9.26 ± 18.23 ng/μL (TRIzol-LS). In comparison Set 1, RNAzol-RT was clearly a better performer than miRNeasy micro and mirVana in terms of amount of extracted RNA ($P < 0.001$). In comparison Set 2, TRIzol-LS was the best performer (mean = 9.26 ng/μL), although this was only

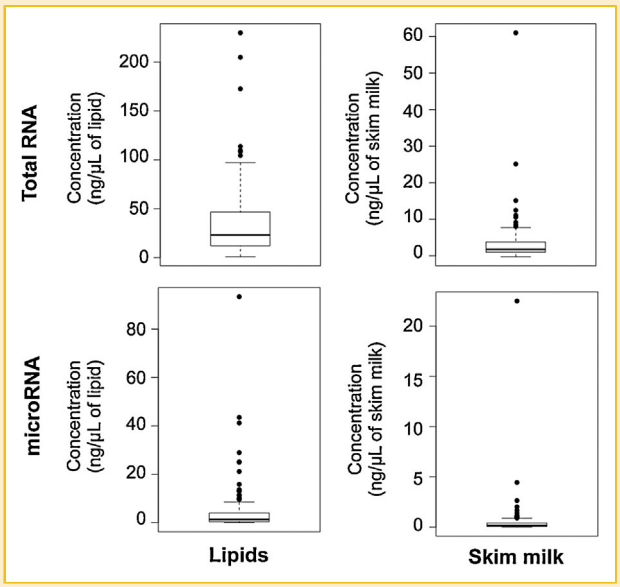


Fig. 3. Comparison of overall concentration of total RNA using NanoDrop 1000, and microRNA using Bioanalyzer 2100, respectively, between the lipid and skim milk fractions obtained for all samples using the eight extraction kits.

significantly different from mirPremier ($P = 0.064$), which had the lowest performance amongst those tested (mean = 0.60 ng/μL).

In human milk cells, the mean ± S.D. of total RNA amount extracted from equal cell aliquots ranged from 1,907 ± 3,321 ng in the mirPremier kit to 11,083 ± 15,106 ng in the miRNeasy mini kit. In comparison Set 1, total RNA extracted with miRNeasy micro kit was significantly higher than that obtained with RNAzol-RT ($P = 0.017$). The highest levels of extracted RNA were seen for the miRNeasy micro kit, followed by mirVana and RNAzol-RT. While the differences between mirVana and the other two kits were not significant, the results were closer to those of the miRNeasy micro kit than to RNAzol-RT. In comparison Set 2, miRNeasy mini yielded significantly higher total RNA amount than mirPremier ($P = 0.003$), with the former kit yielding the highest average amount of RNA among all the other 7 kits (11,083).

## MicroRNA CONCENTRATION

MicroRNA and small RNA were identified on electropherograms in all three human milk fractions, with varying profiles both within and among milk fractions and for different microRNA extraction kits (Fig. 4). Similar to total RNA concentration, microRNA concentration was higher in the lipid fraction compered to skim milk ($P < 0.05$) (Fig. 3). The tested kits performed differently in different human milk fractions ($P = 0.110$ to $P = 0.720$) (Table IV, Fig. 2). In the lipid fraction, the highest mean microRNA concentration was obtained by miRCURY-Biofluids (12.74 ± 24.44 ng/μL), followed by mirPremier (9.43 ± 8.06 ng/μL), while the lowest by mirVana (0.80 ± 1.70 ng/μL). In comparison Set 1, no overall difference was seen among the three kits ($P = 0.169$). Tukey's HSD comparison showed a borderline difference between miRNeasy micro kit and mirVana, with a tendency for higher levels in the former ($P = 0.092$). In comparison Set 2, there was no overall difference among the kits ($P = 0.216$). Further, multiple comparisons of means/Tukey's HSD showed no significant differences, with the smallest $P$-value being 0.210. Thus, all the tested kits performed relatively well in extracting high levels of microRNA from the lipid fraction of human milk.

In skim milk, all kits yielded relatively low quantities of microRNA. Specifically, miRCURY–Cell&Plant kit yielded the highest mean concentration of extracted microRNAs (2.17 ± 5.66 ng/μL), whereas the lowest mean concentration was obtained with mirVana

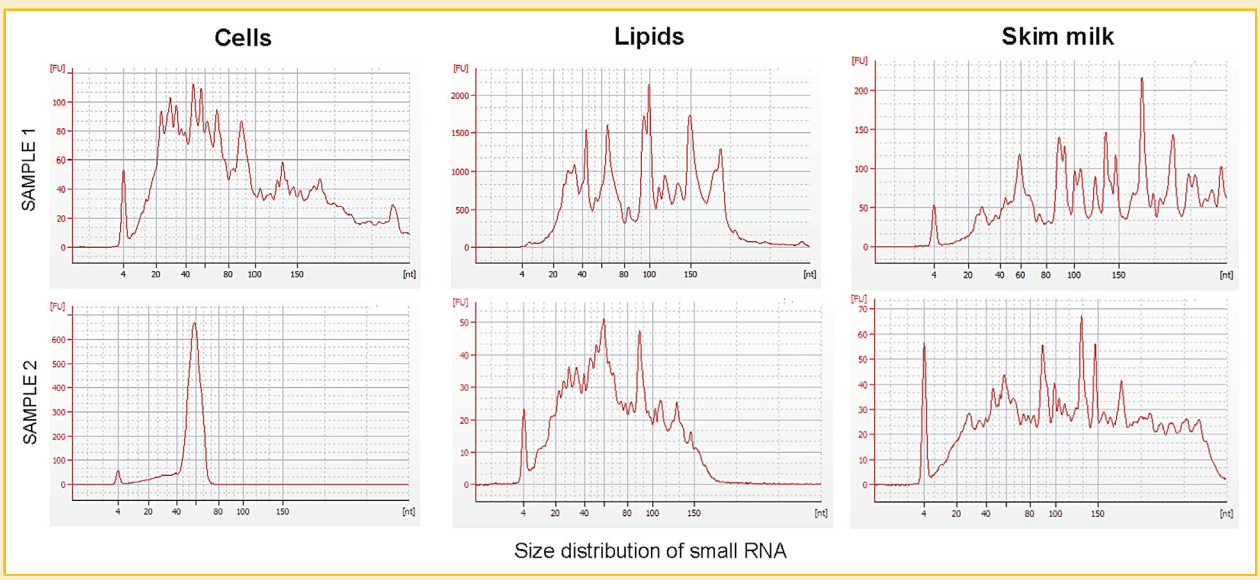


Fig. 4. Examples of electropherograms for different human milk fractions (cells, lipids and skim milk) for two human milk samples obtained using the Agilent 2100 Bioanalyser for small RNA.

($0.10 \pm 0.15$ ng/μL) and miRNeasy micro kits ($0.10 \pm 0.9$ ng/μL). In comparison Set 1, there was no evidence of significant difference among either the three kits overall ($P = 0.342$) or using multiple comparisons of means ($P \geq 0.386$). In comparison Set 2, no evidence of difference was found among the five kits either overall ($P = 0.363$) or using multiple comparisons of means (all $P = 0.449$). Therefore, similar to the lipid fraction, in skim milk all eight tested kits performed similarly in extracting microRNA, although in almost all cases the extracted quantities of microRNA were very low in this fraction compared to the other two human milk fractions.

In human milk cells, TRIzol-LS showed the largest mean amount of microRNA ($1{,}443 \pm 3{,}448$ ng), while mirVana yielded the smallest mean amount of microRNA ($65.67 \pm 84.38$ ng). In comparison Set 1, there was no evidence of difference among the miRNeasy micro kit, mirVana, and RNAzol-RT either overall ($P = 0.308$) or for multiple comparisons of means (all $P > 0.241$). Similarly, in comparison Set 2, no significant difference was found among the five kits in terms of levels of extracted microRNA either overall ($P = 0.722$) or for multiple comparisons of means ($P > 0.614$).

### MicroRNA/SMALL RNA RATIO (%)

To further assess the efficiency of the examined kits to extract microRNA from human milk fractions, we compared the percentage ratio of microRNA to small RNA obtained with each kit (Table 4, Fig. 2). In the lipid fraction, the highest mean microRNA/small RNA ratio was seen with the miRNeasy micro kit (54.4%) and RNAzol-RT (50%). In comparison Set 1, there were significant differences among kits ($P = 0.041$), while in comparison Set 2 there was no overall evidence of significant differences among the five kits compared ($P = 0.217$). Thus, although all the examined kits performed similarly in terms of microRNA/small RNA ratios in the human milk lipid fraction, the highest mean was obtained by the miRNeasy micro kit.

In the skim milk fraction, the highest microRNA/small RNA ratio was obtained by mirPremier (57.8%), followed by RNAzol-RT (48.5%). In comparison Set 1, significant differences were seen among kits ($P < 0.001$). The highest ratios were observed with RNAzol-RT, which were significantly higher than both miRNeasy micro and mirVana ($P < 0.001$), which did not significantly differ from one another ($P = 0.732$). Similarly, comparison Set 2 yielded significant differences among kits ($P < 0.001$). mirPremier kit was higher compared to all other kits (57.8%), and yielded significantly higher microRNA/small RNA ratios than either miRCURY–Cell&Plant or miRNAeasy mini kit ($P < 0.001$). Moreover, ratios obtained by miRCURY-Biofluids and TRIzol-LS were significantly higher than those obtained by miRCURY–Cell&Plant ($P = 0.049$ and $P = 0.063$, respectively), although the significance of the difference between TRIzol-LS and miRCURY–Cell&Plant was much weaker, suggesting that TRIzol-LS yields more variable microRNA/small RNA ratios than miRCURY-Biofluids. Finally, no significant differences were seen between miRCURY-Biofluids and mirPremier ($P = 0.196$). Overall, all examined kits performed similarly in terms of microRNA/small RNA ratios in the skim milk fraction of human milk, with the preference to three kits being mirPremier, RNAzol-RT, and TRIzol-LS.

In human milk cells, TRIzol-LS and miRNeasy mini kit showed the highest mean microRNA/small RNA ratio with 51.1% and 36.1%, respectively. In comparison Set 1, no significant difference was seen among the kits overall ($P = 0.152$). In comparison Set 2, the kits differed significantly ($P < 0.001$). TRIzol-LS yielded higher ratios than miRCURY-Biofluids, miRCURY–Cell&Plant, and mirPremier ($P < 0.001$), while ratios for miRNAeasy mini were significantly higher than those of miRCURY–Cell&Plant ($P = 0.034$).

### VALIDATION OF microRNA EXPRESSION IN DIFFERENT HUMAN MILK FRACTIONS

RT-PCR was used to validate the efficiency of the recommended kits (Table 3) in extracting microRNA using two different samples of each fraction (milk cells, lipids, and skim milk). Using comparative Ct (RQ) analysis, it was found that hsa-miR-148a-3p and hsa-miR-30a-5p were expressed in all three fractions for both milk samples tested (Fig. 5). These two microRNAs showed a similar relative quantification (RQ) value in two different milk lipid samples (A and B samples) (Fig. 5). Despite the low total RNA and microRNA concentration of skim milk compared to the other two human milk fractions, hsa-miR-30a-5p was highly expressed in one of the skim milk samples tested (B); however, the other skim milk sample showed much lower expression for both microRNAs. In the milk cell fraction, both microRNAs were expressed at relatively low levels compared to lipids and skim milk (Fig. 5). In the milk lipid fraction, both microRNAs were expressed more consistently in the two mothers compared to the cellular and skim milk fractions.

## DISCUSSION

Recent advances in human milk compositional studies have revealed the presence of RNA [Lemay et al., 2013] and microRNA molecules [Weber et al., 2010; Munch et al., 2013], similar to those previously found in other biological fluids [Hassiotou et al., 2012]. With breakthrough studies demonstrating an active role of food-derived microRNAs in regulating gene expression in adults [Zhang et al., 2012; Baier et al., 2014], the discovery of these molecules in human

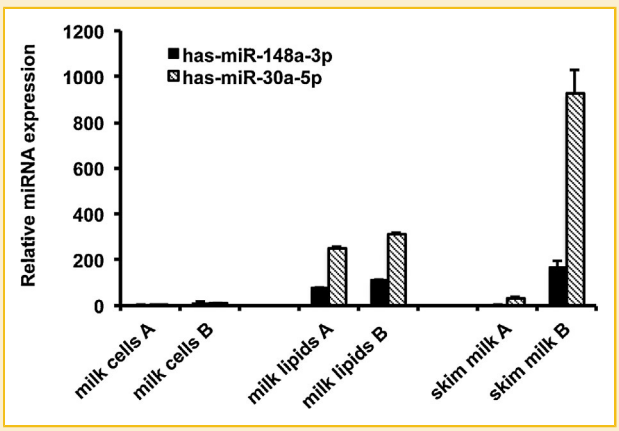


Fig. 5. RT-PCR data for two milk cells, lipids and skim milk samples extracted using three different extraction kits (miRNeasy mini, miRCURY Biofluids, and miRCURY Cell&Plant kits respectively) and analyzed using TaqMan miRNA assay for has-miR-148a-3p (black boxes) and has-miR-30a-5p (grey boxes). RNU48 was used as a housekeeping gene and all samples were normalised to milk cell sample A.

milk highlights their potential significance for the breastfed infant. In addition, these milk molecules may provide novel diagnostic opportunities in relation to disease [Chen et al., 2008; Turchinovich et al., 2011]. Extensive profiling and quantification of microRNA is therefore essential to the understanding and exploration of these molecules and their functions in human milk. The complexity of human milk composition, including lipid, cellular, and skim milk fractions, also suggests that each fraction potentially requires a different handling procedure and extraction kits for optimal RNA and microRNA isolation. In previous studies of human milk microRNA, the expression and type of microRNAs differed between milk lipids and skim milk, with the latter showing lower levels of microRNA expression than the lipid fraction [Kosaka et al., 2010; Weber et al., 2010; Munch et al., 2013]. This suggests that microRNA content may also be different in human milk cells, as we showed in this study. MicroRNAs in the three human milk fractions could be used for different diagnostic and prognostic purposes, especially in monitoring the performance and related pathologies of the lactating mammary gland.

Studies investigating human milk microRNA content have focused mainly on the skim milk fraction and no comparisons have been made with the milk lipid fraction within the same sample. In this study, the human milk lipid and cell fractions clearly contained higher quantities of both total RNA and microRNA ($P < 0.001$) (Fig. 2) compared to skim milk. Specifically, the lipid fraction was on average 10- and 8-fold richer in microRNA and total RNA, respectively, than skim milk (Fig. 3).

With respect to total RNA in milk lipids, all methods performed similarly in our hands (Table 4), except one of the filter column kits (mirPremier), which yielded significantly lower total RNA than the TRIzol-LS ($P = 0.010$), miRNeasy mini ($P = 0.007$), and miRCURY–Cell&Plant ($P = 0.005$) kits. Moreover, RNAzol-RT gave significantly higher total RNA yields than mirVana ($P = 0.002$) and the miRNeasy micro kits ($P = 0.0002$). The above suggest that the phenol/guanidine-based method (RNAzol-RT and TRIzol-LS) yields higher total RNA concentrations in the milk lipid fraction.

Sample RNA purity in milk lipids varied according to the method used. The optimal range considered was between 1.8 and 2.2 (Supp. Table 1). We found that the 260/280 ratios for TRIzol-LS (Table IV) were significantly lower to those of miRNeasy mini ($P = 0.019$) and miRCURY–Cell&Plant ($P = 0.016$). Generally, the phenol/guanidine method (TRIzol-LS) yielded less pure RNA than the other methods examined. However, when phenol/guanidine was combined with filter column (e.g., mirVana), higher microRNA to small RNA ratios were obtained ($P = 0.018$), suggesting it as an appropriate method for extracting high quantities of microRNA from milk lipids.

With respect to microRNA content in the milk lipid fraction, significantly higher microRNA/small RNA ratios were obtained with the miRNeasy micro kit compared to mirVana ($P < 0.035$). No differences were seen between RNAzol-RT and miRNeasy micro kit ($P < 0.190$). TRIzol-LS (phenol/guanidine) was not different to other filter column kits ($P > 0.050$). These findings suggest that both the phenol/guanidine and the filter column methods extract more microRNA than the filter column combined with phenol/guanidine method.

For the cellular portion of the human milk, we present our results as amounts as it was not possible to determine the concentration in this fraction without cell counts of the total sample (whole milk). This limits the comparison to the lipid and skim milk fractions. Nevertheless, RNA/microRNA amounts of the human milk cell fraction were relatively high and comparable to other cells such as mast cells [Eldh et al., 2012]. In the first set of samples, we found that RNAzol-RT yielded less total RNA compared to miRNeasy micro ($P = 0.017$). In the second sample set, mirPremier yielded less total RNA than miRNeasy mini ($P = 0.003$). It is prudent to note that the mirPemier kit yielded the lowest amounts of RNA of all kits. However, no significant difference was seen between kits in the microRNA content. In terms of purity of RNA, the phenol/guanidine method yielded significantly lower values compared to the other methods tested (RNAzol-RT: $P < 0.001$; TRIzol-LS: $P < 0.001$). In terms of microRNA/small RNA ratio, TRIzol-LS yielded significantly more small RNA ($P < 0.001$). In summary, it appears that the filter column-based kits yield similar amounts of microRNA and total RNA with good purity in the milk cell fraction.

In the skim milk fraction, significantly purer RNA was obtained using mirVana than either miRNeasy micro or RNAzol-RT kits ($P < 0.001$). Nevertheless, RNAzol-RT yielded significantly higher total RNA than mirVana ($P = 0.001$). No differences were seen in extracted microRNA levels amongst all kits tested. However, significant differences were observed between kits in the microRNA/small RNA ratios ($P < 0.001$). RNAzol-RT had significantly higher microRNA/small RNA ratio than either miRNeasy micro or mirVana ($P < 0.001$). Further, microRNA/small RNA ratio of mirPremier was higher than of miRCURY–Cell&Plant or miRNAeasy mini kits ($P < 0.001$). Interestingly, although the phenol/guanidine method (RNAzol-RT and TRIzol-LS) was very efficient in extracting high amounts of total RNA and microRNA from skim milk, the purity of the extracted RNA was rather poor.

By using RT-PCR, the validation of microRNA presence in biological samples has been conducted [Chen et al., 2005; Shi and Chiang, 2005; Doleshal et al., 2008]. MicroRNA expression patterns do not correlate with total RNA concentration that is usually measured by Bioanalyzer or NanoDrop [Doleshal et al., 2008; Moret et al., 2013]. We confirmed this in this study by examining hsa-miR-148a-3p and hsa-miR-30a-5p expression in different human milk fractions. These microRNA were detected in all human milk fractions using the most effective extraction kits (Table 3). As expected, both microRNAs were expressed at high levels consistently in two different milk lipid samples; in particular, hsa-miR-30a-5p was expressed at higher levels than hsa-miR-148a-3p in the lipid fraction. In contrast, one skim milk sample had low expression of these microRNAs, whereas the other skim milk sample showed high expression. Moreover, these microRNAs were not expressed at high levels in both milk cell fraction samples compared to the other milk fractions. These findings suggest an enrichment for these microRNAs in the fat globules secreted by lactocytes as well as secretion in the skim milk. Expression levels may change rapidly in cells, whereas the same is not expected for either the fat globule or the skim milk. The above merit further investigation.

Our findings indicate that microRNA in human milk are conserved and protected either within cells or fat globules/other vesicles such as exosomes, and very few can be isolated from the skim milk fraction, which has also been called the plasma phase of milk. Importantly,

most previous studies examined skim milk and not the cellular or the lipid fraction [Kosaka et al., 2010; Weber et al., 2010], and they have therefore excluded the fractions of human milk that appear to be richer in microRNA. Most recently, Munch and colleagues (2013) stated that human milk lipids are richer in microRNAs than skim milk [Munch et al., 2013]. Our findings are in agreement with this and strongly suggest that it is necessary to examine microRNA in all three fractions of human milk and not just in one, to allow complete analysis of this component of human milk, its origin, properties and functions. Also, the microRNA content of the human milk cell fraction has not been profiled as done in human milk lipids, although it potentially conserves more novel microRNAs [Munch et al., 2013]. Finally, our results support the rigorous investigation and standardization of sampling, processing, extraction and storage criteria for the investigation of microRNA in different biofluids. The recommended kits for each human milk fraction based on the quantity and quality of RNA/microRNA were listed in Table 3, and could be applied for highly efficient extraction of RNA/microRNA from exosomes, fat globules, and human fluid cells in addition to human milk fractions.

A potential explanation for the differences between the kits in extraction performance is that the differences in the lysis solution between kits, which is an important step to release intact RNA/microRNA from cells and fat globules. Therefore, the composition and efficiency of the lysis solution must be carefully selected based on the requirements of RNA/microRNA for subsequent studies, such as profiling using qPCR or Microarrays. Importantly, although most of the kits were designed for cellular fractions, we show that they can be used for body fluid samples such as skim milk, with good performance. However, using higher amounts (than those recommended by the manufacturer for fluids) of skim milk for extraction may help to increase the concentration of RNA/microRNA. It is also suggested to use smaller amounts of lysis solution in extracting RNA from skim milk because they are already free in skim milk, and the lysis solution may influence the integrity of RNA transcripts. Another consideration is that microRNAs may be fragmented into smaller pieces during the washing steps, and may not be subsequently conserved in the filter columns. On the other hand, in the phenol/guanidine-based kits, the main issue in our findings was the poor RNA quality, suggesting that RNAs may be influenced during precipitation due to the long term exposure to ethanol and phenol.

This will now generate new avenues for examination of the types, properties and functions of these human milk molecules. Further, the variability amongst and within lactating women and factors that may influence them, such as the stage of lactation or milk removal, can be now robustly and consistently investigated. Opportunities arise for the use of these molecules as diagnostic markers of disease during lactation. Given the recently postulated function of human milk microRNA in providing immunological support to the infant [Kosaka et al., 2010; Zhou et al., 2012]. Although the benefits of oral microRNAs have been recently challenged [Dickinson et al., 2013; Witwer and Hirschi, 2014], our study sets the basis for further examination using sound methodology of the potential significance of microRNAs in the lactating mammary gland and/or in the infant [Heneghan et al., 2009; Gotte, 2010]. Future studies should consider the methodology developed herein to address important questions of immunological as well as developmental benefits conferred to the infant by human milk microRNA, and the potential to use them as diagnostic markers for the human mammary gland.

## CONCLUSIONS

We demonstrated the presence of RNA and specifically microRNA in all three fractions of human milk, including the cells, lipids and skim milk, with the highest levels of both RNA and microRNA obtained in the lipid and cellular fractions. We presented a comparison analysis in a comprehensive dataset of 472 human milk samples, assessing three different extraction methods in eight commercially available kits. These results allow researchers to choose the most appropriate method for measurement of microRNA for their sample composition and fraction of human milk.

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