**Title:**

Circulating microRNAs in Cancer.

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**Running head:** Circulating microRNAs

**Summary**:

It is believed that microRNAs have potential as circulating biomarkers of disease, however successful clinical implementation remains a challenge. This chapter highlights broad variations in approaches to microRNA analysis where whole blood, serum and plasma have each been employed as viable sources. Further discrepancies in approaches are seen in endogenous controls and extraction methods utilised. This has resulted in contradictory publications, even when the same microRNA is targeted in the same disease setting.

Analysis of blood samples highlighted the impact of both collection method and storage, on the microRNA profile. Analysis of a panel of microRNAs across whole blood, serum and plasma originating from the same individual emphasised the impact of starting material on microRNA profile. This is a highly topical field of research with immense potential for translation into the clinical setting. Standardisation of sample harvesting, processing and analysis will be key to this translation. Methods of sample harvesting, preservation and analysis are outlined, with important mitigating factors highlighted.

**Keywords**:

Circulating microRNAs, Breast Cancer, Whole blood, Serum, Plasma.

## 1. Introduction

### *1.1 MicroRNAs:*

Once microRNAs were shown to be detectable in the circulation of patients with cancer, a surge of interest regarding these molecules implementation as biomarkers for the disease quickly ensued. Further research discovered that microRNAs could be protein bound or encapsulated in vesicles in the circulation [1].

In the breast cancer field alone, this breakthrough has resulted in the emergence of a significant number of studies analysing breast cancer patient blood samples for the possibility of identifying clinically relevant microRNAs. Despite tremendous potential, microRNAs have not yet been implemented in the clinical setting as a biomarker of disease. There is not a standardised approach to investigating these molecules, resulting in many different methods being employed. All three starting materials (whole blood, plasma or serum) have been analysed following differing methods of extraction (Trizol or column-based) with data generated being normalised to a large variety of endogenous controls. This variance in approaches to circulating microRNAs has resulted in opposing published results. Contradictory results can be seen in this field even when the same target microRNA is being investigated. For example, in one study circulating levels of miR-10b were found at significantly higher levels in the serum of breast cancer patients when compared to healthy controls, while another study reported no significant difference in the whole blood of patients versus healthy individuals [2,3]. This pattern was mirrored in other studies where miR-106a and miR-155 were found to be elevated in the serum of patients with breast cancer when compared to healthy individuals [4,5]. However, when these microRNAs were analysed in plasma samples by other research groups, no significant change was observed in breast cancer patients compared to healthy controls [5,4]. miR-145 was also analysed in the plasma and serum of breast cancer patients and compared to healthy controls by two separate groups

[2,6]. Analysing miR-145 in the serum suggested a significant increase in the patient cohort, while it was found to be decreased in the plasma of patients when compared to healthy controls [6,2].

However, separate studies using different source materials can achieve similar results. For example, two separate groups carried out analysis of miR-21 in serum and plasma respectively, and both concluded that it was up-regulated in patients when compared to healthy controls [6,4].

Variation is not limited to starting material, but is also witnessed in methods of extracting microRNA with some implementing TRIzol based methods, while others opt for column-based approaches on the same source material [7,8]. Storage and handling of samples vary across the studies also, with some utilising storage of whole blood at -80 °C in PAXgene™ tubes while others report storage in EDTA tubes at 4 °C [9,3]. These contrasting approaches to analysis could impact results seen, thus inhibiting publication of consistent findings and preventing the progression of the field into the clinical setting.

1.2 *Sample Source:*

Circulating microRNAs have been analysed in serum, plasma and whole blood of breast cancer patients and healthy controls. MicroRNAs are found to be relatively stable in these starting materials as well as other fluids such as saliva and urine. This is due to being either protein bound or encapsulated in exosomes, this makes each source material a viable option for analysis [1]. It is imperative for these samples to be stored appropriately. Whichever starting material is routinely collected in the host lab is likely to be the greatest influencing factor for researchers when choosing a starting material. A review of the literature revealed publications using each of these sources, with serum employed in the majority of studies (Table 2).

1.3 *Whole Blood:*

8 papers have published results analysing circulating microRNAs in the whole blood of patients with breast cancer. This would be an ideal source of identifying and analysing circulating microRNAs as it could be tested after taking a simple pin prick sample from an individual. While some studies reported use of whole blood collected in standard EDTA tubes, more recently, PAXgene™ tubes have been employed for analysis of whole blood. PAXgene™ tubes contain a blend of proprietary reagents that lyse all cells, allowing immediate stabilisation of RNA. This stability is maintained for 3 days when stored at room temperature, and up to 8 years when stored at -80 °C [10]. RNA extracted from whole blood will result in greater amounts of RNA available for downstream analysis when compared to the lower, but adequate, quantity of RNA extracted from serum and plasma.

However, whole blood contains many cellular constituents, which may impact upon levels of microRNAs being detected. The presence of red blood cells can impact particular microRNAs. For example, miR-16 and miR-451 have been found to be at much higher levels on erythrocytes [11]. As a result of the many cellular elements existing in whole blood, a published study took white cell counts, haemoglobin and haematocrit levels into account in order to reduce the likelihood of sample-to-sample variability [3].

An issue when using whole blood as a source of microRNAs is storage of the sample. Samples can be collected in EDTA and PAXgene™ tubes and can then be frozen for long term storage at -80 °C. However, some studies also reported the long term storage of whole blood in EDTA tubes at 4 °C [3]. Stabilising the RNA in the collected sample is crucial, as it is now understood that certain microRNAs have very short half-lives, some as short as an hour [12]. This highlights the necessity to standardise methods of collecting samples in order to reduce potential variability associated with particular microRNA instability.

*1.4 Plasma:*

As whole blood contains many factors such as erythrocytes that are capable of effecting levels of particular microRNAs, cell free sources have been employed to analyse circulating microRNAs. There have been 13 published papers that analysed this source in the breast cancer setting. As with the two other sources of circulating microRNAs, many publications did not provide a rationale as to why plasma was chosen. Plasma is obtained through a centrifugation process and contains certain clotting factors such as fibrinogen, requiring the addition of anti-coagulants such as Heparin. This addition has been shown to inhibit the downstream process of PCR analysis while citrate and EDTA have been deemed acceptable [13]. Plasma is advantageous when used for retrospective studies as it is routinely stored at -80 °C where it has been reported to remain stable and suitable for subsequent analysis. One study has shown that freeze thawing of plasma samples stored at -80 °C does not affect microRNAs present at high levels [14]. There is a risk of contaminating plasma samples with cells when aspirating the sample as this can subsequently result in detection of cellular based microRNAs as well as increasing levels of certain circulating microRNAs in the extracted RNA [13].

There are certain pre-analytic variabilities associated with plasma. The time between sample collection and processing can significantly impact sample quality so it is important to standardise this time for each sample [13]. Differences can also be seen in how samples are centrifuged. Some studies report that samples were spun at 1,300 *x g* for 20 min at 10 °C, and others at 600 *x g* for 15 min at room temperature [15,6].

*1.5 Serum:*

The most studied source of circulating microRNAs is serum and this is represented by 26 published studies in breast cancer alone. Serum is also a cell free source, but unlike plasma,

the sample must first undergo the coagulation process prior to centrifugation [13]. Similar to plasma, serum can be stored at -80 °C for long periods of time. A study was carried out where plasma and serum samples from the same group of individuals were compared for certain microRNAs [16]. This study found miR-15b, -16 and -24 to be detected at higher levels in plasma when compared to serum of matched individuals. This study stated that results from serum and plasma samples are not interchangeable when looking at microRNA levels. It states the need for a rigorous protocol for centrifugation to be set in place in order to standardise this method. It was also discovered that haemolysis can lead to the detection of artificially high levels of miR-15b and miR-16 [16].

**2. Materials:**

1. EDTA tubes, serum-separating tubes and PAXgene™ tubes
2. TRIzol, Bromoanisole (BAN), Isopropanol and 75% Ethanol, store at room temperature (RT)
3. PreAnalytix kit (Qiagen/BD; all product mentioned below is provided with the kit)
    a. RNase free water
    b. Buffer BM1
    c. Buffer BM2
    d. Buffer BM3 (add 100% ethanol as indicated)
    e. Buffer BM4 (add 100% ethanol as indicated)
    f. BR5
    g. Proteinase K
    h. DNase 1 stock solution (dissolve in RNase-free water) store at 4 °C
    i. Buffer RDD

j. PAXgene™ shredder spin column

k. PAXgene™ RNA spin column

4. miRCURY™ kit (Exiqon; all product mentioned below is provided with the kit):
    a. Collection tubes
    b. microRNA mini spin column BF
    c. Lysis solution BF
    d. Protein precipitation solution BF
    e. Wash solution 1 BF
    f. Wash solution 2 BF (add 100% as outlined)
    g. RNase free water
5. Nuclease Free Water,store at 4 °C
6. Isopropanol
7. NanoDrop-1000 (ND-1000) Spectrophotometer
8. Deoxynucleotide mix, 10X RT Buffer (100mM), RNase Inhibitor (20U/µL), Multiscribe (50U/µL), Stem loop primer and a Probe (Applied Biosystems) store at -20 °C
9. GeneAmp PCR system 9700 Applied Biosystems
10. TaqMan Fast mix (Applied Biosystems) store at 4 °C
11. MicroAmp® Fast Optical 96-well Reaction Plate with Barcode (0.1 mL) and a MicroAmp optical adhesive film (Applied Biosystems)
12. 7900HT Fast Real Time PCR system (Life Technologies)
13. Shaker-Incubator PHMT (Grant-Bio)
14. 75% Ethanol: 125 mL $dH_20$ and 375 mL 100% Ethanol (make up to 500 mL and store at RT

15. 100% Ethanol

## 3. Methods:

### 3.1 SERUM AND PLASMA SEPARATION:

1. Serum: collect whole blood in serum separating tubes and let stand at RT for 30 min for sample to coagulate prior to centrifugation at 3,000 *x g* for 5 min, remove supernatant and subsequently store at -80 °C
2. Plasma: collect whole blood directly into EDTA tubes in order to prevent coagulation of the sample. Samples are then centrifuged at 3,000 *x g* for 5 min, remove supernatant and store at -80 °C

### 3.2 EXTRACTION OF RNA:

3.2.1 From EDTA collected whole blood using TrizolBD [17] method:

1. Collect whole blood directly into an EDTA tube to prevent any coagulation of the sample and store sample at 4 °C as quickly as possible until extraction.
2. Add 3 mL of TRIzol to a 5 mL tube.
3. Add 200 µL of BAN to the TRIzol and the sample is mixed.
4. Add 1 mL of whole blood to the mixture and sample is thoroughly mixed until entire sample is homogenous.
5. Stand samples at RT for 5 min prior to centrifugation @ 18,000 *x g* for 15 min at 4 °C where samples will undergo phase separation leaving a clear aqueous upper phase which contains the required RNA.

6. Remove 1 mL of the aqueous phase without interfering with the middle interphase layer. Discard remaining sample.
7. Add 1 mL of isopropanol to the aqueous phase and stand at RT for 5 min.
8. Spin sample at 18,000 *x g* for 5 min at 18 °C.
9. Remove supernatant completely from the pellet.
10. Add 1 mL of 75% ethanol to pellet and mix by vortex.
11. Centrifuge sample at 18,000 *x g* for 5 min at 18 °C
12. Remove ethanol without disturbing the pellet and repeat addition of 75% ethanol and centrifugation step.
13. After removing 75% ethanol for a second time allow pellet to air dry at RT for up to 5 min, or until the pellet has dried sufficiently.
14. Add 30 µL of NFW and vortex the sample before leaving it stand at RT for 5 min.

3.2.2 From PAXgene™ collected whole blood:

1. Collect whole blood directly into PAXgene™ tube and store at RT for 2h prior to long term storage at -80 °C (*see* **Note 1**).
2. Thaw sample at RT for at least 1 hr prior to extraction.
3. Centrifuge sample at 4,500 *x g* for 10 min.
4. Remove and discard supernatant from pellet
5. Add 4 ml RNase-free water, seal tube using a new Hemogard closure and vortex sample to suspend pellet.
6. Centrifuge at 4,500 *x g* for 10 min.

7. Remove and discard supernatant, add 350 µL of buffer BM1 and vortex until pellet is dissolved.
8. Transfer sample into 1.5 ml microcentrifuge tube.
9. Add 300 µL buffer BM2 to sample and then 40 µL proteinase K and vortex for 5 sec.
10. Incubate sample for 10 min at 55 °C and 900 R.P.M. on shaker-incubator.
11. Pipet sample into PAXgene™ Shredder spin column and centrifuge at 20,000 *x g* for 3 min.
12. Transfer supernatant to fresh microcentrifuge tube without disrupting pellet.
13. Add 700 µL of isopropanol to supernatant and vortex sample to mix.
14. Pipet 700 µL of sample into PAXgene™ RNA spin column and centrifuge at 20,000 *x g* for 1 min.
15. Discard flow-through and repeat Step 14 until entire sample has passed through PAXgene™ RNA spin column.
16. Add 350 µL of buffer BM3 and centrifuge sample at 20,000 *x g* for 15 sec.
17. Add 10 µL DNase 1 stock solution to 70 µL buffer RDD in a separate microcentrifuge tube.
18. Add 80 µL of mixture directly onto PAXgene™ RNA spin column membrane and incubate at RT for 15 min.
19. Add 350 µL of buffer BM3 to column and centrifuge at 20,000 *x g* for 15 sec.
20. Discard flow-through, add 500 µL of buffer BM4 to column and centrifuge at 20,000 *x g* for 2 min.
21. Repeat Step 20.
22. Discard flow-through and centrifuge column at 20,000 *x g* for 1 min.

23. Place column into fresh collection tube and pipet 40 µL of buffer BR5 directly onto column membrane and centrifuge at 20,000 *x g* for 1 min.
24. Pipet another 40 µL of buffer BR5 onto column membrane and centrifuge at 20,000 *x g* for 1 min.
25. Incubate eluate at 65 °C for 5 min in shaker-incubator.
26. Following incubation place sample directly on ice or store at -80 °C for future use.

3.2.3 From serum and plasma:

1. Allow samples to thaw on ice.
2. Once thawed, centrifuge samples at 3,000 *x g* for 5 min
3. Transfer 200 µL of sample to microcentrifuge tube and add 60 µL of Lysis solution BF.
4. Vortex for 5 sec and incubate for 5 min at RT.
5. Add 20 µL of protein precipitation solution BF and vortex for 5 sec.
6. Incubate at RT for 1 min and then centrifuge at 11,000 *x g* for 3 min.
7. Transfer supernatant to fresh tube without disturbing pellet.
8. Add 270 µL isopropanol to sample and vortex for 5 sec.
9. Put microRNA Mini Spin Column BF into a collection tube and add sample to column.
10. Incubate at RT for 2 min and then centrifuge at 11,000 *x g* for 30 sec.
11. Discard flow-through and repeat Step 9 and 10 if there is sample remaining.
12. Add 100 µL wash solution 1 BF to column and centrifuge at 11,000 *x g* for 30 sec.
13. Discard flow-through, add 700 µL wash solution 2 BF to column and centrifuge at 11,000 *x g* for 30 sec.

14. Discard flow-through, add 250 µL wash solution 2 BF to column and centrifuge at 11,000 *x g* for 2 min.
15. Place column in fresh collection tube and add 50 µL RNase free water directly onto the column membrane.
16. Incubate for 1 min at RT and centrifuge at 11,000 *x g* for 1 min.
17. Store samples at -80 °C for future use.

### 3.3 DETERMINING RNA QUALITY AND QUANTITY:

1. Place 1.1 µL of NFW on pedestal of ND-1000 spectrophotometer to act as a blank (*see* **Note 2**).
2. Adjust wavelength to 260 nm for analysis of RNA. Add 1.1 µL of sample onto pedestal and acquire reading to determine quantity and quality of RNA extracted.

### 3.4 cDNA SYNTHESIS OF MICRORNA:

1. Appropriate amount of RNA required for cDNA synthesis (25-100 ng) is calculated based on yield following analysis on ND-1000 spectrophotometer.
2. NFW is added to RNA to achieve a final volume of 5µl.
3. A premix of 10 µL is made up for each sample of extracted RNA and each microRNA being reverse transcribed as follows (Table 2):
4. Add components of the premix as outlined in Table 2.
5. Add 10 µL of premix to 5 µL of RNA. Samples are thoroughly mixed and centrifuged in a microcentrifuge for < 1 min.

6. Place samples into a GeneAmp PCR system 9700 and is set to one cycle of 30 mins @ 16 °C, 30 mins @ 42 °C and 5 mins @ 4 °C. Samples are then maintained @ 4 °C until required.
7. Store samples at -20 °C until further use.

### 3.5 RQ-PCR ANALYSIS OF MICRORNA:

1. Make premix up to 9.3 µL for each sample and target microRNA being analysed, consisting of 5 µL Fast Master mix, 3.8 µL of NFW and 0.5 µL of microRNA Probe.
2. Add 0.7 µL of cDNA to each well of the 96-well plate.
3. Add the corresponding 9.3 µL of premix to the appropriate cDNA sample where every sample is run in triplicate (*see* **Note 3**).
4. After all samples and premix are added seal the plate with MicroAmp optical adhesive film and centrifuged at 1,000 *x g* for 1 min.
5. Plates are then run on a Thermocycler where cycles of 20 sec @ 95 °C, 1 sec @ 95 °C and 20 sec @ 60 °C are repeated.
6. Data is collected and analysed.

### 3.6 Comparing microRNA Profile in Whole Blood, Serum and Plasma from Same Individual:

Whole blood, plasma and serum samples, originating from the same patient were collected and subsequently had the microRNA profile analysed (Figure 1). Whole blood was collected and stored in PAXgene™ tubes while, plasma samples were collected in EDTA tubes and serum samples were collected in serum separating tubes. Plasma and serum samples were

centrifuged at 3,000 *x g* for 5 min. miR-16 was used for this comparison due to its frequent use as an endogenous control.

miR-16 was detectable in all samples analysed (Figure 1). Different trends in miR-16 levels can be seen across the three sources with similar patterns noticed between certain individuals when analysing plasma and serum. However, the miR-16 values were clearly more variable in plasma (Ct range: 17-27) and serum (Ct range: 18-26) than in PAXgene™ stabilised blood (Ct range: 14-16) from the same individuals.

Two more microRNAs were also analysed across these same matching samples, miR-138 and miR-504. Firstly, miR-504 was detected in all whole blood samples. In contrast, it was detected in 46% of serum samples and 75% of plasma samples (Figure 2). This suggests that when analysing particular microRNAs, source material may be crucial for accurate analysis.

Analysis of miR-138 in the same cohort of samples yielded similar results with the microRNA detectable in every whole blood sample analysed, while it was detected in only 57% of serum samples and 68% of plasma samples (data not shown).

**3.7 Impact of Haemolysis:**

A very important issue to consider when using serum and plasma as a source for microRNA analysis is haemolysis. Haemolysis is the rupturing of erythrocytes and can be measured by quantifying levels of free haemoglobin in the sample (*see* **Note 4**). This is achieved using a spectrophotometer to analyse levels of oxy-haemoglobin, which is detected when peaks at wavelength $\lambda$= 414 are observed indicating that a sample is haemolysed [11]. Another method of detecting haemolysis is to analyse particular microRNAs that are known to be enriched in erythrocytes, such as miR- 451 and miR-144 [11]. These steps are necessary when determining the quality of the source material as haemolysis does have an effect on the portrait of microRNAs seen in these samples.

Haemolysis is an issue that was ignored at first but as the field has developed it became far too great a problem not to be addressed. It has been shown to effect levels of certain microRNAs, such as miR-16, miR-15b and miR-24 [11]. As miR-16 has been employed as endogenous control for studies looking at circulating microRNAs in both serum and plasma, it makes the issue of haemolysis a major factor in data analysis and something that cannot be overlooked [18,19].

### 3.8 Impact of Variations in extraction methods:

There is a large variation in extraction methods when looking across all publications regarding circulating microRNAs in breast cancer, with nine different methods employed, eight of which are column-based (Table 3).

As part of the current study, analysis of particular microRNAs in whole blood was carried out on samples stored in EDTA tubes followed by TRIzol BD extraction, as well as microRNAs analysed in whole blood samples collected in PAXgene™ tubes followed by extraction using the PreAnalytix kit. Levels of miR-16 were analysed and detected using RQ-PCR analysis (Figure 3).

miR-16 was detected at high levels (low Ct value) as it is robustly present in whole blood. Expression was found to be more stable in the PAXgene™ processed samples, with miR-16 ranging from 14-16 Ct. In contrast, whole blood collected in EDTA tubes with subsequent TRIzol extraction was found to be much less stable, with miR-16 ranging from 14-34 Ct. In samples collected, stored and extracted in this manner, miR-16 would not be deemed a suitable endogenous control.

### 3.9 Impact of Endogenous Controls:

Following a similar pattern, there is also inconsistency in relation to which endogenous controls are employed for the analysis of circulating microRNAs in breast cancer, with twelve different endogenous controls used for analysis of serum microRNAs alone (Table 4). An endogenous control would ideally be stably expressed across all samples used for analysis so that sample-to-sample variability can be accounted for, as well as variations in template loading and varying efficiencies of reaction.

These endogenous controls range from microRNAs to ribosomal RNAs, to spiked in controls. Entire studies are carried out to establish appropriate endogenous controls for analysis of blood [20]. Many published studies determine suitability of endogenous control across a range of samples to ensure that it is stably expressed prior to employment [21].

miR-16 is seen as an appropriate endogenous control for each source material and one that has featured in several publications [18,22,7,19,23,24,9,25]. There has also been work stating that miR-16 along with other microRNAs are impacted by blood cells and haemolysis [26]. As shown in Figure 3, miR-16 can fluctuate depending on storage and extraction method. In our hands it was found to be robust and stable in PAXgene™ collected whole blood. One study chose to normalise the data to the mean and median of all microRNAs measured in a sample [27]. As endogenous controls heavily influence data analysis it is crucial that there is not a large degree of variability seen in this sector of the field. Standardisation of starting material, methods of harvesting, storage and extraction will impact endogenous controls.

**3.10 Conclusion:**

This chapter highlights a number of issues that have a significant impact on the outcome of circulating microRNA studies. This in turn is preventing the realisation of the full potential of microRNAs as biomarkers of disease. Standardisation of approaches to analysing microRNAs in the circulation could help establishment of these molecules as clinically relevant biomarkers.

Whole blood, plasma and serum have all been shown to be appropriate for analysing circulating microRNAs, and each with varying methods of sample collection and storage. This shows that there are many ways of analysing microRNAs and that overall, no one method is necessarily superior to another. The issue is reproducibility, and that the different starting materials are not suitable for comparison. Consistency is required to support reliable comparison of data generated by different groups. Until the factors influencing microRNA presence, stability and detection in the circulation are clearly defined it is imperative that there is an attempt at standardisation.

Understanding and addressing the mitigating factors that can negatively impact upon a study, such as haemolysis, is critical in achieving standardisation across this field and resulting in more consistent and reproducible findings. If achieved, this may allow microRNAs to fulfil their potential as circulating biomarkers of many diseases.

### 3.11 Outcome:

If using whole blood, authors recommend collection in PAXgene™ tubes where the collection and storage guidelines are strictly adhered to. For plasma and serum collection it is crucial that samples are handled carefully and guidelines for temperature, time and centrifugation are followed rigidly. It is necessary to check and compensate for haemolysis in both plasma and serum samples. It is important to note that each starting material is a viable source, however, certain microRNAs are not present at sufficiently high levels to be detected in particular fractions. Therefore it is necessary to carry out preliminary analysis prior to commencing a large study analysing a particular microRNA in the circulation.

## 4. **Notes**

1. When collecting whole blood into PAXgene™ tubes it is important for samples to be incubated for 2 hours at RT in order to allow the reagents in the tube to lyse the whole blood sample.
2. When analysing samples on the ND-1000 it is important to pay particular attention to both the 260/230 nm and 260/280 nm ratios. If ratios are not in the required range of 1.8-2.1 for samples analysed, it could impact subsequent RQ-PCR analysis.
3. For RQ-PCR analysis keep mixtures containing the probe in opaque tubes in order to prevent any photo-bleaching of the fluorophore which may impact analysis.
4. When harvesting serum and plasma, care in handling is critical in order to prevent haemolysis of the sample. When carrying out analysis on a patient cohort it is important to take haemolysis into consideration. As haemolysis is not always readily detectable through visual analysis, it is important to analyse samples by means mentioned previously in this chapter.

**Conflict of Interest:**

The authors declare no conflict of interest.

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**References:**

1. Chen X, Liang H, Zhang J, Zen K, Zhang CY (2012) Secreted microRNAs: a new form of intercellular communication. Trends in cell biology 22 (3):125-132. doi:10.1016/j.tcb.2011.12.001
2. Mar-Aguilar F, Mendoza-Ramirez JA, Malagon-Santiago I, Espino-Silva PK, Santuario-Facio SK, Ruiz-Flores P, Rodriguez-Padilla C, Resendez-Perez D (2013) Serum circulating microRNA profiling for identification of potential breast cancer biomarkers. Dis Markers 34 (3):163-169. doi:Doi 10.3233/Dma-120957
3. Heneghan HM, Miller N, Kelly R, Newell J, Kerin MJ (2010) Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. The oncologist 15 (7):673-682. doi:10.1634/theoncologist.2010-0103
4. Wang F, Zheng Z, Guo J, Ding X (2010) Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. Gynecologic oncology 119 (3):586-593. doi:10.1016/j.ygyno.2010.07.021
5. Cookson VJ, Bentley MA, Hogan BV, Horgan K, Hayward BE, Hazelwood LD, Hughes TA (2012) Circulating microRNA profiles reflect the presence of breast tumours but not the profiles of microRNAs within the tumours. Cell Oncol (Dordr) 35 (4):301-308. doi:10.1007/s13402-012-0089-1
6. Ng EKO, Li RFN, Shin VY, Jin HC, Leung CPH, Ma ESK, Pang R, Chua D, Chu KM, Law WL, Law SYK, Poon RTP, Kwong A (2013) Circulating microRNAs as Specific Biomarkers for Breast Cancer Detection. PloS one 8 (1). doi:ARTN e53141

DOI 10.1371/journal.pone.0053141
7. Wu XW, Somlo G, Yu Y, Palomares MR, Li AX, Zhou WY, Chow A, Yen Y, Rossi JJ, Gao H, Wang JH, Yuan YC, Frankel P, Li S, Ashing-Giwa KT, Sun GH, Wang YF, Smith R, Robinson K, Ren XB, Wang SE (2012) De novo sequencing of circulating miRNAs identifies novel markers predicting clinical outcome of locally advanced breast cancer. Journal of translational medicine 10. doi:Artn 42

Doi 10.1186/1479-5876-10-42
8. Schwarzenbach H, Milde-Langosch K, Steinbach B, Muller V, Pantel K (2012) Diagnostic potential of PTEN-targeting miR-214 in the blood of breast cancer patients. Breast Cancer Res Tr 134 (3):933-941. doi:DOI 10.1007/s10549-012-1988-6
9. Schrauder MG, Strick R, Schulz-Wendtland R, Strissel PL, Kahmann L, Loehberg CR, Lux MP, Jud SM, Hartmann A, Hein A, Bayer CM, Bani MR, Richter S, Adamietz BR, Wenkel E, Rauh C, Beckmann MW, Fasching PA (2012) Circulating micro-RNAs as potential blood-based markers for early stage breast cancer detection. PloS one 7 (1):e29770. doi:10.1371/journal.pone.0029770
10. Shaffer J (2012) miRNA profiling from blood — challenges and recommendations. Qiagen
11. Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, van Zandwijk N, Reid G (2011) Haemolysis during sample preparation alters microRNA content of plasma. PloS one 6 (9):e24145. doi:10.1371/journal.pone.0024145
12. Sethi P, Lukiw WJ (2009) Micro-RNA abundance and stability in human brain: specific alterations in Alzheimer's disease temporal lobe neocortex. Neuroscience letters 459 (2):100-104. doi:10.1016/j.neulet.2009.04.052
13. Kroh EM, Parkin RK, Mitchell PS, Tewari M (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods 50 (4):298-301. doi:10.1016/j.ymeth.2010.01.032
14. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proceedings of the National Academy of Sciences of the United States of America 105 (30):10513-10518. doi:DOI 10.1073/pnas.0804549105
15. Leidner RS, Li L, Thompson CL (2013) Dampening Enthusiasm for Circulating MicroRNA in Breast Cancer. PloS one 8 (3). doi:ARTN e57841

DOI 10.1371/journal.pone.0057841

16. McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A (2011) Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem 57 (6):833-840. doi:10.1373/clinchem.2010.157198
17. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ (2010) Circulating microRNAs as Novel Minimally Invasive Biomarkers for Breast Cancer. Ann Surg 251 (3):499-505. doi:Doi 10.1097/Sla.0b013e3181cc939f
18. Si HY, Sun XM, Chen YJ, Cao Y, Chen SM, Wang HC, Hu CJ (2013) Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. J Cancer Res Clin 139 (2):223-229. doi:DOI 10.1007/s00432-012-1315-y
19. Zeng RC, Zhang W, Yan XQ, Ye ZQ, Chen ED, Huang DP, Zhang XH, Huang GL (2013) Down-regulation of miRNA-30a in human plasma is a novel marker for breast cancer. Med Oncol 30 (1). doi:Artn 477

Doi 10.1007/S12032-013-0477-Z
20. McDermott AM, Kerin MJ, Miller N (2013) Identification and Validation of miRNAs as Endogenous Controls for RQ-PCR in Blood Specimens for Breast Cancer Studies. PloS one 8 (12). doi:ARTN e83718

DOI 10.1371/journal.pone.0083718
21. Anfossi S, Giordano A, Gao H, Cohen EN, Tin S, Wu Q, Garza RJ, Debeb BG, Alvarez RH, Valero V, Hortobagyi GN, Calin GA, Ueno NT, Woodward WA, Reuben JM (2014) High Serum miR-19a Levels Are Associated with Inflammatory Breast Cancer and Are Predictive of Favorable Clinical Outcome in Patients with Metastatic HER2(+) Inflammatory Breast Cancer. PloS one 9 (1):e83113. doi:10.1371/journal.pone.0083113
22. Wang HJ, Tan G, Dong L, Cheng L, Li KJ, Wang ZY, Luo HF (2012) Circulating MiR-125b as a Marker Predicting Chemoresistance in Breast Cancer. PloS one 7 (4). doi:ARTN e34210

DOI 10.1371/journal.pone.0034210
23. Chen W, Cai F, Zhang B, Barekati Z, Zhong XY (2013) The level of circulating miRNA-10b and miRNA-373 in detecting lymph node metastasis of breast cancer: potential biomarkers. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 34 (1):455-462. doi:10.1007/s13277-012-0570-5
24. Kumar S, Keerthana R, Pazhanimuthu A, Perumal P (2013) Overexpression of circulating miRNA-21 and miRNA-146a in plasma samples of breast cancer patients. Indian journal of biochemistry & biophysics 50 (3):210-214
25. McDermott AM, Kerin MJ, Miller N (2013) Identification and Validation of miRNAs as Endogenous Controls for RQ-PCR in Blood Specimens for Breast Cancer Studies. PloS one 8 (12):e83718. doi:10.1371/journal.pone.0083718
26. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M (2012) Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res (Phila) 5 (3):492-497. doi:10.1158/1940-6207.CAPR-11-0370
27. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, Dirix LY, van Dam PA, Van Galen A, de Weerd V, Kraan J, van der Spoel P, Ramirez-Moreno R, van Deurzen CH, Smid M, Yu JX, Jiang J, Wang Y, Gratama JW, Sleijfer S, Foekens JA, Martens JW (2011) mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clinical cancer research : an official journal of the American Association for Cancer Research 17 (11):3600-3618. doi:10.1158/1078-0432.CCR-11-0255
28. Khan S, Brougham CL, Ryan J, Sahrudin A, O'Neill G, Wall D, Curran C, Newell J, Kerin MJ, Dwyer RM (2013) miR-379 Regulates Cyclin B1 Expression and Is Decreased in Breast Cancer. PloS one 8 (7). doi:ARTN e68753

DOI 10.1371/journal.pone.0068753

29. Waters PS, Dwyer RM, Brougham C, Glynn CL, Wall D, Hyland P, Duignan M, McLoughlin M, Newell J, Kerin MJ (2014) Impact of Tumour Epithelial Subtype on Circulating microRNAs in Breast Cancer Patients. PloS one 9 (3). doi:ARTN e90605

DOI 10.1371/journal.pone.0090605
30. Waters PS, McDermott AM, Wall D, Heneghan HM, Miller N, Newell J, Kerin MJ, Dwyer RM (2012) Relationship between Circulating and Tissue microRNAs in a Murine Model of Breast Cancer. PloS one 7 (11). doi:ARTN e50459

DOI 10.1371/journal.pone.0050459
31. Wang PY, Gong HT, Li BF, Lv CL, Wang HT, Zhou HH, Li XX, Xie SY, Jiang BF (2013) Higher expression of circulating miR-182 as a novel biomarker for breast cancer. Oncol Lett 6 (6):1681-1686. doi:Doi 10.3892/Ol.2013.1593
32. Eichelser C, Flesch-Janys D, Chang-Claude J, Pantel K, Schwarzenbach H (2013) Deregulated Serum Concentrations of Circulating Cell-Free MicroRNAs miR-17, miR-34a, miR-155, and miR-373 in Human Breast Cancer Development and Progression. Clin Chem 59 (10):1489-1496. doi:DOI 10.1373/clinchem.2013.205161
33. Sun Y, Wang MJ, Lin GG, Sun SP, Li XX, Qi J, Li JM (2012) Serum MicroRNA-155 as a Potential Biomarker to Track Disease in Breast Cancer. PloS one 7 (10). doi:ARTN e47003

DOI 10.1371/journal.pone.0047003
34. van Schooneveld E, Wouters MCA, Van der Auwera I, Peeters DJ, Wildiers H, Van Dam PA, Vergote I, Vermeulen PB, Dirix LY, Van Laere SJ (2012) Expression profiling of cancerous and normal breast tissues identifies microRNAs that are differentially expressed in serum from patients with (metastatic) breast cancer and healthy volunteers. Breast Cancer Research 14 (1). doi:Artn R34

Doi 10.1186/Bcr3127
35. Asaga S, Kuo C, Nguyen T, Terpenning M, Giuliano AE, Hoon DSB (2011) Direct Serum Assay for MicroRNA-21 Concentrations in Early and Advanced Breast Cancer. Clin Chem 57 (1):84-91. doi:DOI 10.1373/clinchem.2010.151845
36. Zhu W, Qin W, Atasoy U, Sauter ER (2009) Circulating microRNAs in breast cancer and healthy subjects. BMC Res Notes 2:89. doi:10.1186/1756-0500-2-89
37. Li J, Zhang Y, Zhang W, Jia S, Tian R, Kang Y, Ma Y, Li D (2013) Genetic heterogeneity of breast cancer metastasis may be related to miR-21 regulation of TIMP-3 in translation. Int J Surg Oncol 2013:875078. doi:10.1155/2013/875078
38. Godfrey AC, Xu Z, Weinberg CR, Getts RC, Wade PA, Deroo LA, Sandler DP, Taylor JA (2013) Serum microRNA expression as an early marker for breast cancer risk in prospectively collected samples from the Sister Study cohort. Breast Cancer Res 15 (3):R42. doi:10.1186/bcr3428
39. Tang D, Zhang Q, Zhao S, Wang J, Lu K, Song Y, Zhao L, Kang X, Xu S, Tian L (2013) The expression and clinical significance of microRNA-1258 and heparanase in human breast cancer. Clinical biochemistry 46 (10-11):926-932. doi:10.1016/j.clinbiochem.2013.01.027
40. Zhao FL, Hu GD, Wang XF, Zhang XH, Zhang YK, Yu ZS (2012) Serum overexpression of microRNA-10b in patients with bone metastatic primary breast cancer. The Journal of international medical research 40 (3):859-866
41. Guo LJ, Zhang QY (2012) Decreased serum miR-181a is a potential new tool for breast cancer screening. International journal of molecular medicine 30 (3):680-686. doi:10.3892/ijmm.2012.1021
42. Wang X, Wu X, Yan L, Shao J (2012) [Serum miR-103 as a potential diagnostic biomarker for breast cancer]. Nan fang yi ke da xue xue bao = Journal of Southern Medical University 32 (5):631-634
43. Wu Q, Wang C, Lu Z, Guo L, Ge Q (2012) Analysis of serum genome-wide microRNAs for breast cancer detection. Clinica chimica acta; international journal of clinical chemistry 413 (13-14):1058-1065. doi:10.1016/j.cca.2012.02.016

44. Hu Z, Dong J, Wang LE, Ma H, Liu J, Zhao Y, Tang J, Chen X, Dai J, Wei Q, Zhang C, Shen H (2012) Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. Carcinogenesis 33 (4):828-834. doi:10.1093/carcin/bgs030
45. Appaiah HN, Goswami CP, Mina LA, Badve S, Sledge GW, Jr., Liu Y, Nakshatri H (2011) Persistent upregulation of U6:SNORD44 small RNA ratio in the serum of breast cancer patients. Breast cancer research : BCR 13 (5):R86. doi:10.1186/bcr2943
46. Wu Q, Lu Z, Li H, Lu J, Guo L, Ge Q (2011) Next-generation sequencing of microRNAs for breast cancer detection. Journal of biomedicine & biotechnology 2011:597145. doi:10.1155/2011/597145
47. Gotte M (2010) MicroRNAs in breast cancer pathogenesis. Minerva ginecologica 62 (6):559-571
48. Roth C, Rack B, Muller V, Janni W, Pantel K, Schwarzenbach H (2010) Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. Breast cancer research : BCR 12 (6):R90. doi:10.1186/bcr2766
49. Guo L, Zhao Y, Yang S, Cai M, Wu Q, Chen F (2013) Genome-wide screen for aberrantly expressed miRNAs reveals miRNA profile signature in breast cancer. Molecular biology reports 40 (3):2175-2186. doi:10.1007/s11033-012-2277-5
50. Jung EJ, Santarpia L, Kim J, Esteva FJ, Moretti E, Buzdar AU, Di Leo A, Le XF, Bast RC, Jr., Park ST, Pusztai L, Calin GA (2012) Plasma microRNA 210 levels correlate with sensitivity to trastuzumab and tumor presence in breast cancer patients. Cancer 118 (10):2603-2614. doi:10.1002/cncr.26565
51. Cuk K, Zucknick M, Madhavan D, Schott S, Golatta M, Heil J, Marme F, Turchinovich A, Sinn P, Sohn C, Junkermann H, Schneeweiss A, Burwinkel B (2013) Plasma microRNA panel for minimally invasive detection of breast cancer. PloS one 8 (10):e76729. doi:10.1371/journal.pone.0076729
52. Liu JJ, Mao QX, Liu Y, Hao XM, Zhang S, Zhang J (2013) Analysis of miR-205 and miR-155 expression in the blood of breast cancer patients. Chinese J Cancer Res 25 (1):46-54. doi:DOI 10.3978/j.issn.1000-9604.2012.11.04
53. Madhavan D, Zucknick M, Wallwiener M, Cuk K, Modugno C, Scharpff M, Schott S, Heil J, Turchinovich A, Yang RX, Benner A, Riethdorf S, Trumpp A, Sohn C, Pantel K, Schneeweiss A, Burwinkel B (2012) Circulating miRNAs as Surrogate Markers for Circulating Tumor Cells and Prognostic Markers in Metastatic Breast Cancer. Clinical Cancer Research 18 (21):5972-5982. doi:Doi 10.1158/1078-0432.Ccr-12-1407
54. Zhao RH, Wu JN, Jia WJ, Gong C, Yu FY, Ren ZF, Chen K, He JR, Su FX (2011) Plasma miR-221 as a Predictive Biomarker for Chemoresistance in Breast Cancer Patients who Previously Received Neoadjuvant Chemotherapy. Onkologie 34 (12):675-680. doi:Doi 10.1159/000334552
55. Zhao H, Shen J, Medico L, Wang D, Ambrosone CB, Liu S (2010) A Pilot Study of Circulating miRNAs as Potential Biomarkers of Early Stage Breast Cancer. PloS one 5 (10). doi:ARTN e13735

DOI 10.1371/journal.pone.0013735
56. Tjensvoll K, Svendsen KN, Reuben JM, Oltedal S, Gilje B, Smaaland R, Nordgard O (2012) miRNA expression profiling for identification of potential breast cancer biomarkers. Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals 17 (5):463-470. doi:10.3109/1354750X.2012.686061

Figures:

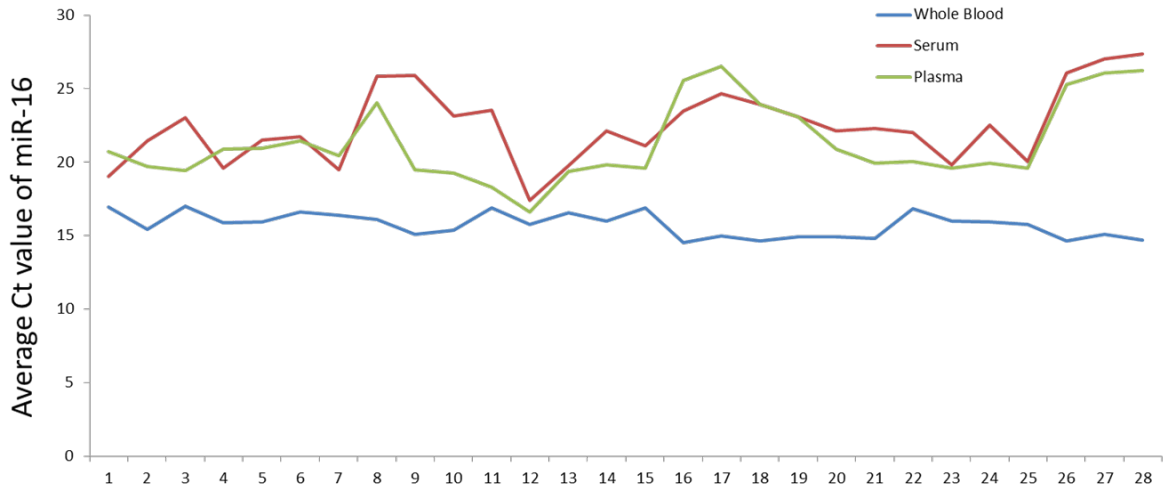


Figure 1: Comparing levels of miR-16 across whole blood, plasma and serum from the same individuals.

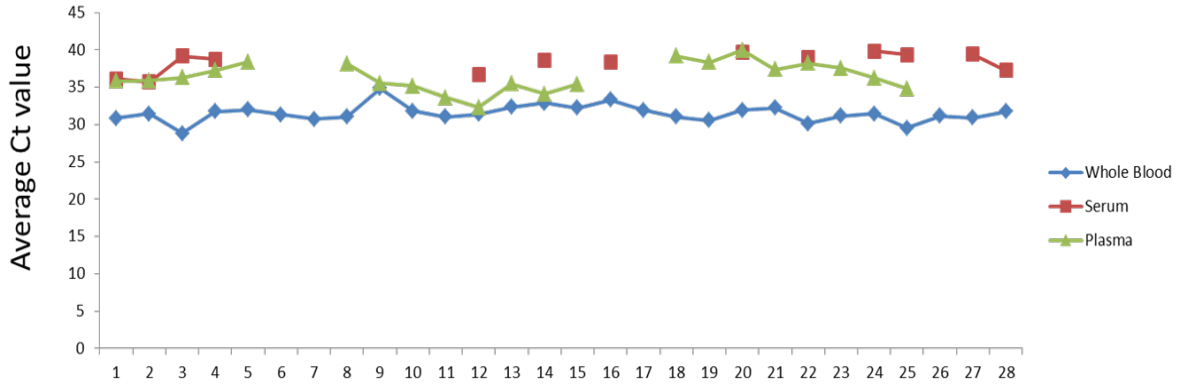


Figure 2: Comparing levels of miR-504 across whole blood, plasma and serum from the same individuals.

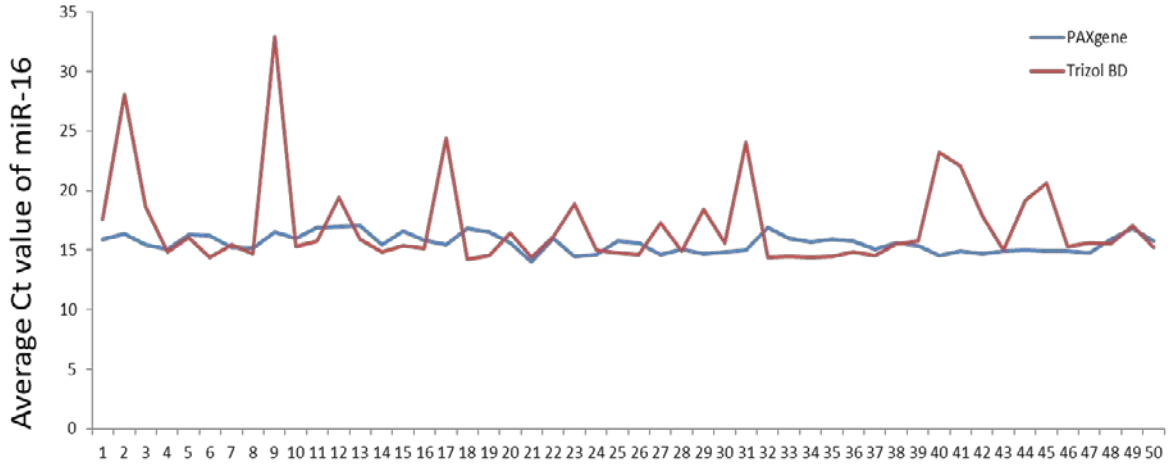


Figure 3: Comparison of miR-16 levels in whole blood harvested into PAXgene™ tubes followed by PreAnalytix extraction and EDTA tubes followed by TRIzol BD method respectively.

**Tables:**

| **Component** | **Volume** |
|---|---|
| dNTP mix (100mM) | 0.17 µL |
| 10X RT Buffer | 1.65 µL |
| NFW | 4.57 µL |
| RNase Inhibitor (20U/µL) | 0.21 µL |
| Multiscribe (50U/µL) | 1.1 µL |
| Stem Loop Primer | 3.1 µL |

**Table 1:** Components of cDNA synthesis premix

| **Source of extracted microRNAs** | **Studies Published to Date** | **References** |
|---|---|---|
| Whole Blood | 8 | [27,9,28,3,17,25,29,30] |
| Serum | 26 | [31,32,2,33,18,22,7,34,8,35,36,4,37-48,21,49] |
| Plasma | 13 | [50,51,15,19,52,6,23,24,53,5,54-56] |

**Table 2:** Overview of starting material used in studies analysing circulating microRNAs in breast cancer.

| Method | Whole Blood | Serum | Plasma |
|---|---|---|---|
| mirVana miRNA isolation kit | | **X** | **X** |
| mirVana PARIS kit | | **X** | |
| miRNeasy mini kit | **X** | **X** | **X** |
| Trizol LS method | | **X** | **X** |
| Trizol BD method | **X** | **X** | **X** |
| BioChain miRNA isolation kit | | **X** | |
| MagMax viral RNA isolation kit | | **X** | |
| Norgens RNA purification kit | | **X** | **X** |
| Allprep DNA/RNA micro kit | **X** | | |

**Table 3:** Different extraction methods used in analysis of circulating microRNAs in breast cancer. (X indicating at least 1 published use of technique)

| **Endogenous Control** | **Whole Blood** | **Serum** | **Plasma** |
|---|---|---|---|
| 5s rRNA | | **X** | |
| 18S rRNA | | **X** | |
| cel-miR-39 | | **X** | **X** |
| miR-16 | **X** | **X** | **X** |
| GAPDH | | **X** | |
| miR-1825 | | **X** | |
| U6 snRNA | | **X** | |
| RNU6B | | **X** | **X** |
| miR-191 | | **X** | |
| miR-484 | | **X** | |
| SNORD44 | | **X** | |
| miR-192 | | **X** | |
| U6 | | | **X** |
| miR-92 | | | **X** |

**Table 4:** Variation in endogenous controls used in literature of circulating microRNAs in breast cancer.