Plasma Exosomal microRNA Purification from Hitachi's ExoComplete™ 96-Well Plate Kit using Norgen's Plasma/Serum RNA Purification Mini Kit

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INTRODUCTION

Exosomes are small vesicles of about 40 –100 nm in diameter originating from within multi-vesicular bodies, which are secreted into the extra-cellular space. The contents of these exosomes reflect the origin and the physiological status of the source cells. Exosomes are found in different bodily fluids such as blood, blood derivatives, urine, amniotic fluid, and malignant ascitic fluid. Evidence has been accumulating recently that these vesicles act as cellular messengers, conveying information to distant cells and tissues within the body. Recent work has demonstrated the presence of distinct subsets of mRNA and microRNAs within exosomes which depend upon the tumour cell type from which they are secreted. For this reason exosomal RNAs may serve as biomarkers for various diseases including cancer. The number of exosomes varies significantly in different bodily fluids and they are usually present in low amounts. Consequently, effective detection of exosomes in plasma requires both the isolation and the detection method to be sensitive and consistent.

Although a significant amount of research has been done on exosome concentration for RNA isolation from plasma, the isolation of plasma exosomal RNA still a challenge. Exosome precipitation reagents followed by RNA isolation and/ or direct phenol:chloroform silica-based isolation of plasma exosomal RNA have been widely used for this purpose. In addition to being expensive, lengthy and hazardous, these methods could result in significant carryover contamination that may affect sensitive downstream applications such as RT-qPCR or microarrays.

The ExoComplete system from Hitachi isolates exosomal mRNA from biological samples such as plasma in a 96-well filter plate format. The process is streamlined from sample to mRNA isolation by the direct lysis of the captured extracellular vesicles on 96-well filter plate using a buffer that effectively inactivate ribonucleases to ensure isolation of intact mRNA. The exosomal mRNA will be then isolated through hybridization with single-strand oligos (dT) immobilized in the wells of the mRNA capture plate. For the synthesis of cDNA, an antisense primer of mRNA targets of interest can be added to the lysis buffer that will hybridize to the target mRNAs and maximize the efficiency and specificity of the reverse transcription reaction.

The ExoComplete™ 96-Well Plate Kit from Hitachi only targets plasma exosomal mRNA without purifying the other important RNA subset that is also found within the exosomes, which is miRNA. Therefore Norgen Biotek has developed a method for the isolation of plasma exosomal miRNA from the lysate after the hybridization step of the mRNA on Hitachi's mRNA capture plate using a modified procedure from Norgen's Plasma/Serum RNA Purification Mini Kit (Cat# 55000). This modified procedure was able to efficiently purify the plasma exosomal miRNAs that were not purified using Hitachi's ExoComplete™ 96-Well Plate Kit.

MATERIALS AND METHODS

Sample Collection

Blood samples were collected on K2EDTA tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ). Plasma was separated from each aliquot by centrifuging for 20 minutes at room temperature at 1600 × g.

Exosome Purification and RNA Isolation

Exosomes were purified in triplicate and total exosomal RNA was isolated from 300 µL plasma using Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Cat# 58300) (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions.

Exosomes were purified in triplicate and exosomal mRNA was isolated from 300 µL plasma using Hitachi's ExoComplete™ 96-Well Plate Kit (Cat# 880002) (Hitachi Chemical Diagnostics, Inc., Mountain View, CA, USA) according to the manufacturer's instructions.

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To isolate miRNA from exosomes captured on Hitachi's Exosome Isolation Plate, Hitachi's Lysis Buffer was spun down for 5 minutes after it was applied to the Exosome Isolation plate, then Norgen's Plasma/Serum RNA Purification Mini Kit (Cat# 55000) (Norgen Biotek Corp., Thorold, ON, Canada) was used to isolate the miRNA from the lysate flowthrough. According to Hitachi's protocol, the Exosome Isolation Plate should be washed after the exosome cell lysis step with Wash Buffer A and Wash Buffer B, respectively. Wash Buffer A and Wash Buffer B were also kept for miRNA isolation using Norgen's Plasma/Serum RNA Purification Mini Kit (Cat# 55000) (Norgen Biotek Corp., Thorold, ON, Canada). All isolations were done in triplicate.

RT-PCR Amplification

The purified RNA from both Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit and Hitachi's ExoComplete™ 96-Well Plate Kit was then used as the template in a real-time PCR (qPCR) reaction to assess the recovery of both mRNA and miRNA in comparison to the miRNA that could be isolated from Hitachi's lysate, Wash Buffer A and Wash Buffer B. Three different miRNA targets that are highly abundant in plasma exosomes were used for the evaluation, namely miR-26a, miR-92a and miR 99a. The HPRT-1 mRNA was also used to compare the mRNA purified from both Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit and Hitachi's ExoComplete™ 96-Well Plate Kit. Briefly, 2 µL of isolated RNA was added to 20 µL of rt-PCR reaction mixture containing 5X first strand Buffer, 10mM dNTPs, McLAB reverse transcriptase and a final concentration of 0.25 µM from one of the stem-loop miRNA primer or the reverse primer for the HPRT-1 mRNA. The cDNA synthesis was performed at 50°C for 30 minutes followed by 50°C for 10 minutes. The synthesized cDNA from each target was then used as a template for real time PCR amplification using Norgen's 2X PCR Master Mix (Cat# 28007) which was spiked with SYBR® Green dye. The PCR samples were amplified under the real-time program; 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. The reaction was run using the CFX Real-time System (Bio-Rad).

RESULTS AND DISCUSSION

Exosomal RNA was isolated in triplicate from 300 µL plasma using Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit, Hitachi's ExoComplete™ 96-Well Plate Kit and from Hitachi's lysate flowthrough, Wash Buffer A and Wash Buffer B. The HPRT-1 mRNA was amplified from all isolated exosomal RNA and average Ct values are shown in **Figure 1**. It was found that Hitachi's ExoComplete™ 96-Well Plate Kit (Hitachi's Control) and Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen's Control) equivalently purified the HPRT-1 mRNA from plasma. RNA isolated from either Hitachi's lysate flowthrough, Wash Buffer A or Wash Buffer B showed that all the mRNA has been efficiently bound and captured using Hitachi's Exosome Lysis buffer, and no mRNA was detected in the lysate flowthrough, Wash Buffer A and Wash Buffer B.

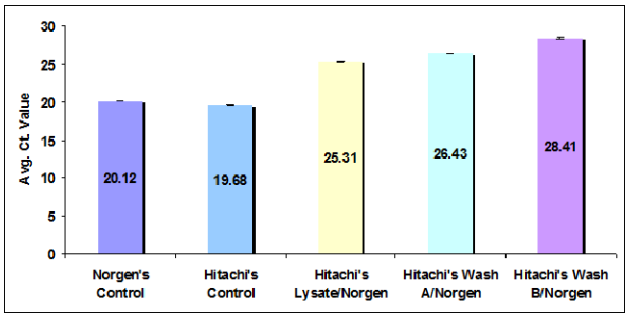

Figure 1. HPRT-1 mRNA rt-qPCR amplification from RNA purified from exosomes captured on Hitachi's ExoComplete™ 96-Well Plate Kit (Hitachi's Control), Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen's Control), or from Hitachi's Lysate flowthrough, Wash Buffer A or Wash Buffer B using Norgen's Plasma/Serum RNA Purification Mini Kit.

Next was to investigate how the exosomal miRNA can be rescued from either Hitachi's lysate after lysing the exosomes for mRNA capture, or from the different Hitachi's washes. Norgen's Plasma/Serum RNA Purification Mini Kit (Cat# 55000) was then used to isolate miRNA from Hitachi's lysate flowthrough, Wash Buffer B and Wash Buffer A. The miRNA isolated from the lysate or from the wash buffers was compared to the exosomal miRNA isolated using Norgen's Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit. Three highly abundant miRNA were used for that purpose which are miR-26a, miR-92a and miR-99a. All Ct. Values for the amplification of miR-26a, miR-92a and miR-99a from all fractions are shown in **Figure 2, Figure 3 and Figure 4,** respectively**.**

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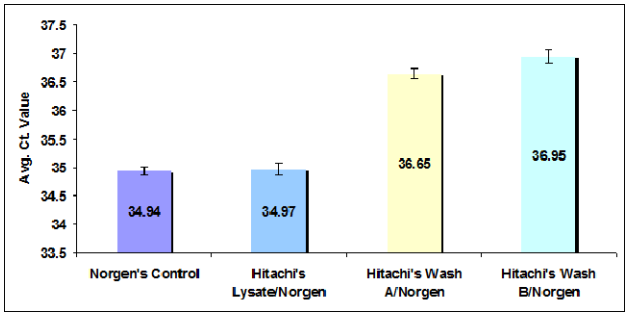

Figure 2. miR-26a rt-qPCR amplification from RNA purified from exosomes captured on Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen's Control), or from Hitachi's Lysate, Wash Buffer A or Wash Buffer B using Norgen's Plasma/Serum RNA Purification Mini Kit.

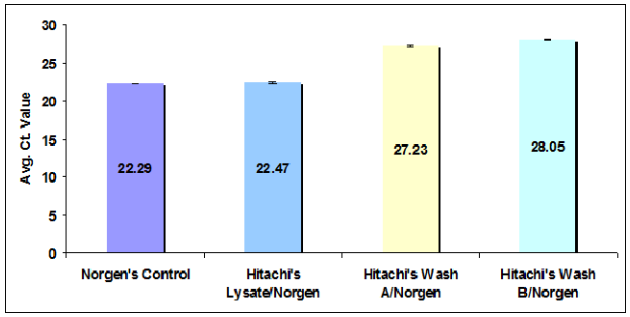

Figure 3. miR-92a rt-qPCR amplification from RNA purified from Exosomes captured on Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen's Control), or from Hitachi's Lysate, Wash Buffer A or Wash Buffer B using Norgen's Plasma/Serum RNA Purification Mini Kit.

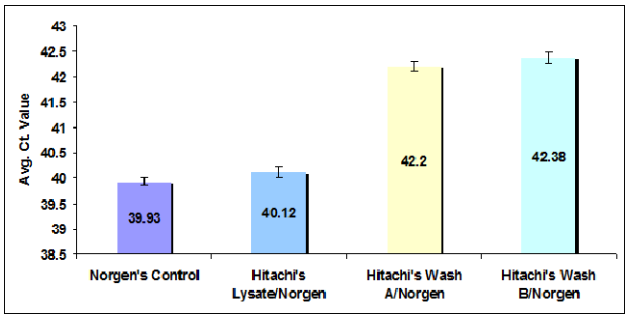

Figure 4. miR-92a rt-qPCR amplification from RNA purified from Exosomes captured on Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen's Control), or from Hitachi's Lysate, Wash Buffer A or Wash

Buffer B using Norgen's Plasma/Serum RNA Purification Mini Kit.

It has been found as shown in Figures 2, 3 and 4 that miRNA can be rescued from lysate used for the lysing of the exosomes captured on Hitaach's Exosome Capture plate. The three miRNAs used for the evaluations showed similar Ct values to the miRNA detected from exosomes purified using Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit control kit.

CONCLUSIONS

From the data presented in this report, the following can be concluded:

1. Norgen's Plasma/Serum Exosome Purification and RNA Isolation Mini Kit and Hitachi's ExoComplete™ 96-Well Plate Kit can equivalently isolate mRNA from plasma exosomes.
2. Norgen's Plasma/Serum RNA Purification Mini Kit can be integrated in Hitachi's ExoComplete™ 96-Well Plate Kit workflow for the purification of plasma exosomal miRNA as represented in **Figure 5**.

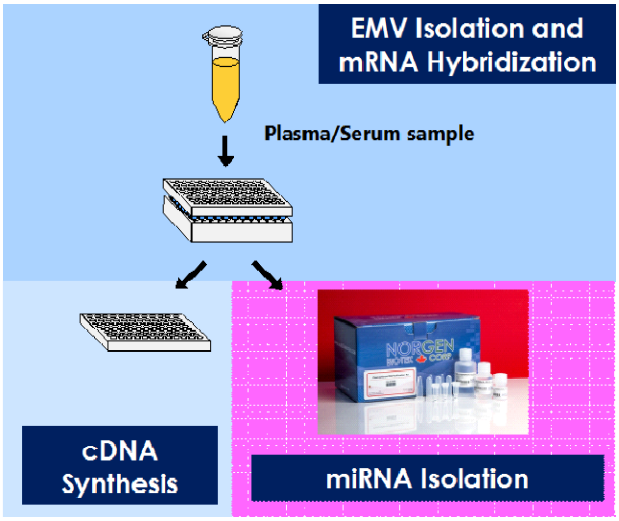

Figure 5. Hitachi/Norgen Workflow for the isolation of exosomal mRNA and miRNA.

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