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RESEARCH ARTICLE

Efficiency and challenges of exosome and microRNA extraction from clinical biofluids in Indonesia: A commercial kit-based approach for qPCR applications

Aprilia Indra Kartika [1,2], Muchamad Dafip [1], Nastiti Wijayanti [3], Didik Setyo Heriyanto [4,5], Sofia Mubarika Haryana [6], Kartika Widayati Taroeno-Hariadi [5,7]

[1]Doctoral Program of Biotechnology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Department of Medical Laboratory Technology, Faculty of Health and Nursing, Universitas Muhammadiyah Semarang, Semarang, Indonesia
[3]Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[4]Department of Anatomical Pathology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[5]Dr. Sardjito General hospital, Yogyakarta, Indonesia
[6]Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[7]Division of Hematology and Medical Oncology, Department of Internal Medicine, Faculty of medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

Correspondence:
Aprilia Indra Kartika
Jl. Kedungmundu No.18, Kedungmundu, Tembalang, Semarang, Central Java – 50273, Indonesia

Email: kartika.widayati@ugm.ac.id

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Abstract

Exosome miRNA has significant potential as biomarker for minimal or non-invasive diagnosis and monitoring of disease progression. However, extraction of miRNA exosomes from biofluids is challenging, and there is a lack of comparative evaluation of commercially available extraction kits for qPCR applications. Therefore, this study aims to obtain information about miRNA exosome extraction using commercial kits and analyze its expression with the qRT-PCR method. The exosome extraction was carried out using 3 types of commercial kits, while the RNA extraction was performed with a spin column. The purity and concentration of exosomes were measured using NTA, while total RNA was measured with nanodrops. Subsequently, miRNA expression was measured using qRT-PCR. The results showed that the concentration of exosomes obtained using kit 1 for plasma isolation was 3.5 x 10^{11} with particle sizes ranging from 30-240 nm. Kit 2 yielded a value of 2.9 x 10^{10} with particle sizes ranging from 30-150 nm, while kit 3 had no measurable value. The average concentration of RNA extracted using the RNA Basic kit from HansaBioMed was 42.53 ng/µL, while the exoRNeasy Midi kit from Qiagen yielded 45.28 ng/µL. Template RNA of 100 ng was converted into cDNA, while the cDNA was diluted at a ratio of 1:20 using RNase free water during the quantification process of miRNA exosome expression with the qRT-PCR method. Based on the results, exosome miRNA quantification process requires the use of the correct method at each stage to obtain the right expression for clinical studies.

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1. INTRODUCTION

Exosomes are extracellular vesicles measuring 30-150 nm that are secreted by various cell types as a form of intercellular communication. Several studies have shown that vesicles consist of phospholipid membranes with receptors on the surface, as well as various biomolecules, including DNA, RNA, miRNA (microRNA), proteins, and lipids. These biomolecules have been reported to describe the physiological or pathological conditions of their originating cells in real-time (1). Exosomes have great potential to be used in non-invasive liquid biopsy methods for disease detection, due to their dispersed nature in various body fluids, such as urine, saliva, serum, and cerebrospinal fluid (2).

Several studies have widely demonstrated the role of exosomes, ranging from delivery agents in pharmaceutical investigations, to the exploration of components as biomarkers for certain diseases (3). Among the exosomal contents, miRNAs have gained significant attention for their stability, abundance, and potential role as diagnostic and prognostic biomarkers, particularly in cancer and other complex diseases (4). However, in Indonesia, miRNA-based studies of exosomes from biofluid clinical samples are still very limited due to technical constraints in the extraction process and availability of infrastructure, particularly rotors for small-volume ultracentrifugation, which is an essential part of the gold-standard exosome extraction method (5).

As alternatives, commercial kits have been widely used to extract exosomes and constituent miRNAs. Although more accessible, some kits in circulation have low capture efficiency and purity, with significant variations in terms of exosome capture efficiency, batch-to-batch consistency, and suitability of the resulting RNA for qPCR-based miRNA analysis (6). Several challenges are also encountered, such as kits not specifically optimized for isolation of miRNA content, lack of detailed protocols regarding input volume, lysis conditions, and elution, leading to variability in results and decreased reproducibility (7-10). Other challenges in the field of exosome studies include the need for specialized tools (such as high-speed cold centrifugation) and supporting materials that are often not provided in kit packages (11). In addition, many commercial kits also do not provide standardized protocols for miRNA extraction from exosomes adapted for sensitive downstream methods, such as qPCR (12).

While commercial kits offer a practical alternative to gold-standard methods, their performance is highly variable and heavily dependent on sample type and intended downstream application. For studies in Indonesia aiming to develop liquid biopsy biomarkers using plasma, there is a critical lack of evidence-based guidance on kit selection. A direct, head-to-head comparison evaluating not just RNA yield but also exosome integrity, and compatibility with sensitive qPCR for miRNA is urgently needed to establish a standardized, accessible, and reliable workflow. Therefore, this study aims to systematically compare 3 commercially available exosome extraction kits based on different isolation principles, namely polymer-based precipitation and membrane affinity, within resource limited settings. The evaluation focused on key practical consideration, including cost, plasma input volume, procedural complexity, and the ability of each kit to generate exosomal miRNA of sufficient quality for downstream qPCR analysis. Performance was assessed using parameters such as RNA concentration, purity, and integrity, as well as overall suitability for clinical study application. The results are expected to support the development of a practical, reproducible, and standardized workflow for exosomal miRNA analysis and to serve as a methodological reference for exosome-based biomarker studies in Indonesia.

2. MATERIALS AND METHODS

This study used a comparative experimental approach to evaluate the performance of 3 commercial kits in extracting RNA from exosomes (Table 1), to identify the most effective and suitable method for clinical applications in Indonesia, particularly for qPCR-based miRNA expression analysis. The evaluation included parameters of RNA concentration, purity, and integrity, as well as the suitability of the results for downstream applications. These kits were selected based on their characteristics of use in the study environment as well as their availability in Indonesia.

The selection of these 3 kits represented 3 different contexts of use (plasma, cell culture, and low-cost approaches), ensuring that the evaluation results provided a comprehensive picture of the performance and suitability of each kit in various exosome study settings. This approach was expected to provide a basis for standardizing exosomal RNA extraction protocols in Indonesia.

2.1. Plasma Specimen Preparation

The study was conducted at Dr. Sardjito General Hospital with permit number DP.04.03/D.XI.2/7470/2024, while samples were obtained from lung cancer patients using procedures that had passed ethical clearance. Plasma samples were obtained from 20 lung cancer patients, and plasma was chosen as the main biofluid in this study because it was a rich source of exosomes and miRNAs that reflected the systemic molecular status of patients non-invasively (13). In addition, plasma was easily obtained through standard clinical procedures and was more representative in biomarker studies in lung cancer than sputum or tissue biopsies, which were more invasive.

Table 1. Justification of exosome extraction kits

Trademarks and specifications	Justification of selection
Kit 1: Total Exosome Isolation Kit from Cell Culture Media (Invitrogen)	is becoming a standard in cell culture-based research, and is widely used in studies of the molecular mechanisms of exosomes. The kit enables efficient isolation of exosomes from culture medium, and is particularly relevant for initial laboratory testing prior to application to clinical samples. The use of this kit helps to ensure that the extracted RNA accurately represents the contents of the exosomes.
Kit 2: Exosome RNA Isolation Kit (HansaBioMed)	offers a cost-effective approach and is an attractive option for laboratories with limited budgets. Although not yet widely used in Indonesia, the kit has been used extensively in various international publications, demonstrating its potential and validity in extracting exosomal RNA. This study is the first to test the effectiveness of the HansaBioMed kit in the local Indonesian context, thus providing a new alternative for domestic researchers.
Kit 3: exoRNeasy Midi Kit (Qiagen)	one of the most commonly used kits in Indonesia, specifically for the extraction of total exosomal RNA from blood plasma specimens. This kit is designed to provide high quality RNA yield from biological fluids, making it relevant to clinical contexts and liquid biopsy methods. The widespread use of this kit in various international studies also supports its validity and reproducibility

Blood was drawn from lung cancer patients using a Vacutainer with EDTA anticoagulant to prevent coagulation and RNA degradation. Subsequently, whole blood was centrifuged at 3,000 rpm for 15 minutes at 4°C to separate plasma from blood cells. This process was performed under cold conditions to maintain RNA stability and prevent enzymatic degradation. After centrifugation, the upper clear plasma layer was carefully removed using an RNase-free pipette to avoid cellular contamination. Plasma was then placed into RNase-free microtubes and stored at -80°C until further use for exosome extraction and miRNA analysis (14).

2.2. Exosome Extraction

The 3 compared kits, namely Invitrogen's Total Exosome Isolation (from cell culture media) (Thermo Fisher Scientific, USA), EXO-Prep for plasma isolation (HansaBioMed Life Sciences: Estonia, Europe), and exoRNeasy Midi Kit (Qiagen: Hilden, Germany), were then used for exosome isolation according to standard procedures. Procedural modifications were made differently depending on the specifications of the samples used and the need to add a step for exosome extraction as confirmation. An initial centrifugation step was added to the Invitrogen kit procedure, due to differences in the sample suggested (cell culture). Thawed plasma samples were centrifuged at 3,000 rpm for 15 minutes, then 1 mL of supernatant was taken, and 500 µL of reagent was added. Modifications to this kit were also carried out at the final stage, namely resuspension of the pellet using 1xPBS (Phosphate-Buffered Saline) with a volume of 100 µL, then the exosome is stored at -20°C.

Extraction of plasma exosomes using EXO-Prep for plasma isolation with the addition of 3 sequential centrifugations. The first centrifugation was done at 300 g for 10 minutes, followed by 1,200 g for 20 minutes, and 10,000 g for 30 minutes. Subsequently, the supernatant volume was taken as 250 µL and added with 62.5 µL EXO-Prep.

Modifications to the exoRNeasy Midi Kit were carried out by adding 1X PBS as an elution solution after the XBP and XWP buffer stage in the manufacturer's procedure. The solution was centrifuged at 8,000x g for 10 minutes to release the exosomes from the filter column membrane. PBS solution was used to match the exosome solvent in the quantification stage of Nanoparticle Tracking Analysis (NTA).

2.3. RNA Extraction

The RNA extraction process from exosomes in this study was carried out using 3 different commercial kits, each of which was applied according to the technical instructions of the manufacturer. The first kit, Quick-RNA Miniprep Plus Kit (Zymo Research), was used to extract total RNA from exosome samples through the stages of lysis, RNA binding, washing, and elution. The second kit, RNA Basic Kit (HansaBioMed), implements 3 main steps, namely exosome lysing, RNA extraction, and final purification, with an approach focused on the efficiency of RNA separation from exosomes. Meanwhile, the third kit, exoRNeasy Midi Kit (Qiagen), integrated exosome isolation and RNA extraction in the same set of reagents. The procedure included binding of exosomes to the column membrane, followed by simultaneous lysis and RNA isolation, thereby providing advantages in time efficiency and minimizing loss of biological material. The comparison of RNA concentration between Kit-2 and Kit-3 was performed using the Wilcoxon signed-rank test due to non-normal data distribution.

2.4. Measurement of Exosome Concentration

NTA machine using ViewSizer 3,000 (Horiba Scientific, PT Dermama Biotechnology Laboratory, Indonesia). The cuvette was cleaned using alcohol and distilled water. Exosome samples were dissolved by 2,000 x using PBS. Alcohol, distilled water, and PBS were filtered using a 0.2 µm filter paper. The cuvette was placed in a magnetic rod positioning box,

which was then inserted into the cuvette. The cuvette insert was placed into the cuvette. Samples were taken as much as 300 µL to rinse the cuvette, and were resuspended to wet the walls of the cuvette. The sample was discarded, then 500 µL was inserted into the cuvette, and the sample was resuspended. Subsequently, the cuvette was closed, making sure there were no bubbles in the cuvette, and then the surface was cleaned using a tissue. The computer and NTA instrument were turned on, and the lid of the NTA instrument as well as the cover of the cuvette holder were opened. The cuvette was inserted according to the direction of the slider and the camera insert vial, then the cuvette holder was closed. Analysis of exosome concentration and particle size using ViewSizer software (15).

2.5. Calculation of Total RNA Purity and Concentration

The maestrogen nanodrop machine was turned on, then the RNA menu was selected, and the sample hole was cleaned using a tissue. A total of 2 µL of blank was dripped into the sample hole, then the sample hole was closed. The blank menu is clicked, and the machine will process. After that, the blank liquid is absorbed with a tissue, 2 µL of the RNA sample is dripped, and then the sample hole is closed. The sample menu was clicked, the machine processed, and the results of RNA concentration and purity were then printed and recorded.

2.6. cDNA Synthesis

The cDNA (Complementary DNA) synthesis process used Qiagen's miRCURY LNA RT Kit. The procedure for cDNA synthesis was in accordance with the insert reagent kit. The recommended concentration of total RNA in the insert kit was 10 ng / 2 µL, while in this study, the total RNA concentration optimization was carried out at 10 ng, 50 ng, and 100 ng. The volume of total RNA and RNase-free water was optional according to the concentration of RNA that was used as a template. Both solutions were added with 5x miRCURY RT Reaction Buffer as much as 2 µL, 10 x miRCURY RT Enzyme Mix as much as 1 µL, and Synthetic RNA spike-ins as much as 0.5 µL, ensuring that the total volume of mixing cDNA was 10 µL. The mixture of solutions was incubated for 60 min at 42°C on an RT-PCR machine, and then incubated for 5 min at 95°C to inactivate the reverse transcriptase enzyme, and immediately cool 4°C. The cDNA was stored at -20°C for further quantification of miRNA exosome expression.

2.7. Quantification of miRNA Exosome Expression

The microRNA exosome quantification process uses Qiagen's miRCURY LNA SYBR Green PCR Kit. cDNA was thawed then diluted in a ratio of 1:20 using RNase-free water (1 µL cDNA was added with 19 µL RNase-free water), then homogenized and spun down. In addition, 2x miRCURY SYBR Green Master Mix was taken as much as 5 µL, and added with ROX reference Dye as much as 0.05 µL, PCR primer mix 1 µL, cDNA 3 µL, and RNase-free water 0.95 µL. 10 µL reaction mixture was spindown then run on the Applied Biosystems 7,500 qRT-PCR machine. The qRT-PCR steps were PCR initial heat activation for 2 minutes at 95°C, 2-step cycling to the denaturation stage for 10 s at 95°C, combined annealing/extension for 60 s at 56°C, 40 cycles, and melting curve analysis process at 60-95°C.

3. RESULTS AND DISCUSSION

3.1. Concentration and Purity of Plasma-Solubilized Exosomes

Exosome extraction results were measured for concentration and purity using NTA, as presented in Table 2. Kit 1 had the highest particle concentration, around 3.5×10^{11}, but its size ranged from 30 to 240 nm (Figure 1), showing that it was larger than exosomes. Kit 1 was not intended for blood plasma specimens, as it was specifically designed for cell culture media samples. Extraction of blood plasma exosomes using kit 1 produced a large amount of sediment, but this sediment did not specifically indicate the size of the exosomes (Figure 2). The pellet formed from kit 1 could be microvesicles (20-5,000 nm) formed through direct division of the plasma membrane. In addition, apoptotic bodies were also released from cells undergoing apoptosis (16).

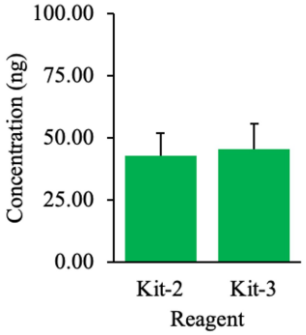

Figure 1. total RNA concentrations of exosomes extracted using kit 2 and kit 3 were not significantly different (p=0.208)

Table 2. Final concentration of exosomes extracted using several commercial extraction kits

Sample name	Concentration minus solvent (particles/mL)
Kit 1	$3,5 \times 10^{11}$
Kit 2	$2,9 \times 10^{10}$
Kit 3	N/A

Note: N/A = Not Applicable

Exosomes extracted using kit 2 had a concentration of 2.9×10^{10}, more than kit 3. In addition, the particle size of exosomes produced by kit 2 showed the specificity of exosomes because it ranged from 30 to 150 nm (Figure 2). Kit 2 was specific for exosome extraction using plasma specimens, resulting in high concentrations and particle sizes in accordance with exosomes.

Kit 3 showed zero concentration, because the particles counted by the NTA machine were equal to the number of negative control particles (buffer). This occurred because kit 3 was not intended solely for extracting exosomes, but it was for extracting total RNA from plasma exosomes. Kit 3 used an affinity membrane to filter exosomes, so there was no specific technique for eluting exosomes bound to the column membrane. The elution technique used in this study was adding 1xPBS and centrifugation, but the NTA results showed that this technique was unsuccessful.

3.2. RNA Plasma Concentration and Purity

RNA concentration and purity were measured using the nanodrop spectrophotometer method (Table 3). RNA extraction using kit 2 and kit 3, the consideration of using kit 2 was the high concentration of exosomes and the appropriate particle size (30-150 nm). The consideration of using kit 3 was because the kit had been frequently used and was known to have good performance from several published references. The use of the exosomal RNA extraction kit 2 and kit 3 both produced pure RNA. The purity ratio value at 260 nm wavelength absorbance divided by 280 nm absorbance ranged from 1.8 to 2 (Table 3).

The results of RNA concentration showed no significant difference between the 2 kits ($p > 0.05$). The mean difference was 2.76 ± 10.5, showing a slightly higher concentration in Kit-3. However, the variation was relatively large. The average concentration of RNA extracted using kit 2 was 42.52 ng/µL (42.52 ± 20.24), while kit 3 was 45.28 ng/µL (45.28 ± 22.59). The total concentration of RNA used for cDNA synthesis was 100 µL, ensuring that the concentration met the requirements for downstream reverse transcriptase PCR. The RNA concentration of samples using the extraction kit 2 and kit 3 showed good results. Based on the procedure manual of the miRCURY LNA RT kit, the required concentration was 5 ng/µL. Kit 2 used a separate procedure between exosome and total RNA extraction, thereby requiring 2 different kits. Kit 2 was a kit that was not commonly used in Indonesia, but was more cost-effective and required a small amount of plasma sample (100 µL). While kit 3 only required 1 type of kit to obtain total RNA from exosomes, this kit was commonly used for transcriptomic study but had a higher price and a large volume of plasma (1 mL). The results of RNA concentration analysis using the 2 kits showed no significant difference ($p>0.05$) (Figure 2). In addition, for cDNA synthesis and qRT-PCR using kit 2, considering that using a lot of samples was more cost-effective and only required 100 µL of plasma specimen.

3.3. RNA Concentration Optimization for cDNA Synthesis and cDNA Dilution for qPCR

The melt peak formed indicated the specificity of the miRNA target (Figure 3). A miRNA target had 1 peak on the melt peak curve. When there were 2 miRNA targets, 2 different peaks were formed. The peak shift on a miRNA target was influenced by cDNA dilution (1:20 and 1:60). The miRNA quantification process required a total RNA concentration with high yield and purity for cDNA synthesis. In addition, cDNA dilution was also needed for miRNA expression quantification. A good concentration for cDNA synthesis based on the procedure manual of the miRCURY LNA RT kit was 10 ng, so in this study, concentration optimization will be carried out at concentrations of 10, 50, and 100 ng. This was carried out because the amount of miRNA in exosomes was very small, to avoid too low expression, the right concentration was required.

RNA template that was synthesized, cDNA must be equalized in the final concentration. This was carried out, ensuring that the measured miRNA expression was appropriate, not influenced by RNA templates with different concentrations. The results of miRNA-103a-5p expression showed that the higher the concentration of RNA to be converted to cDNA, the lower the Ct value (high target miRNA expression). The results of statistical analysis showed that there was a significant difference between RNA concentrations of 10, 50, 100 ng and the expression of target miRNA ($P=0.009$), so it was recommended to use a concentration of 100 ng. The cDNA dilution level contained in the miRCURY LNA SYBR Green PCR Kit procedure manual was 1:60, but because the amount of miRNA in exosomes was not as much as the total miRNA circulated by the body, the dilution optimization is 1:20 and 1:60 (Table 4). The results showed that the lower the dilution, the lower the CT value (high expression of target miRNA) ($P=0.015$). Both RNA concentration and cDNA dilution volume independently had a significant effect on the CT value of miR-103. However, there was no significant interaction between the 2 factors. This suggested that the effects of each factor were independent. The Ct value changed significantly when the RNA concentration or cDNA elution volume was changed, but the change of one did not modify the effect of the other ($p=0.227$) (Figure 4).

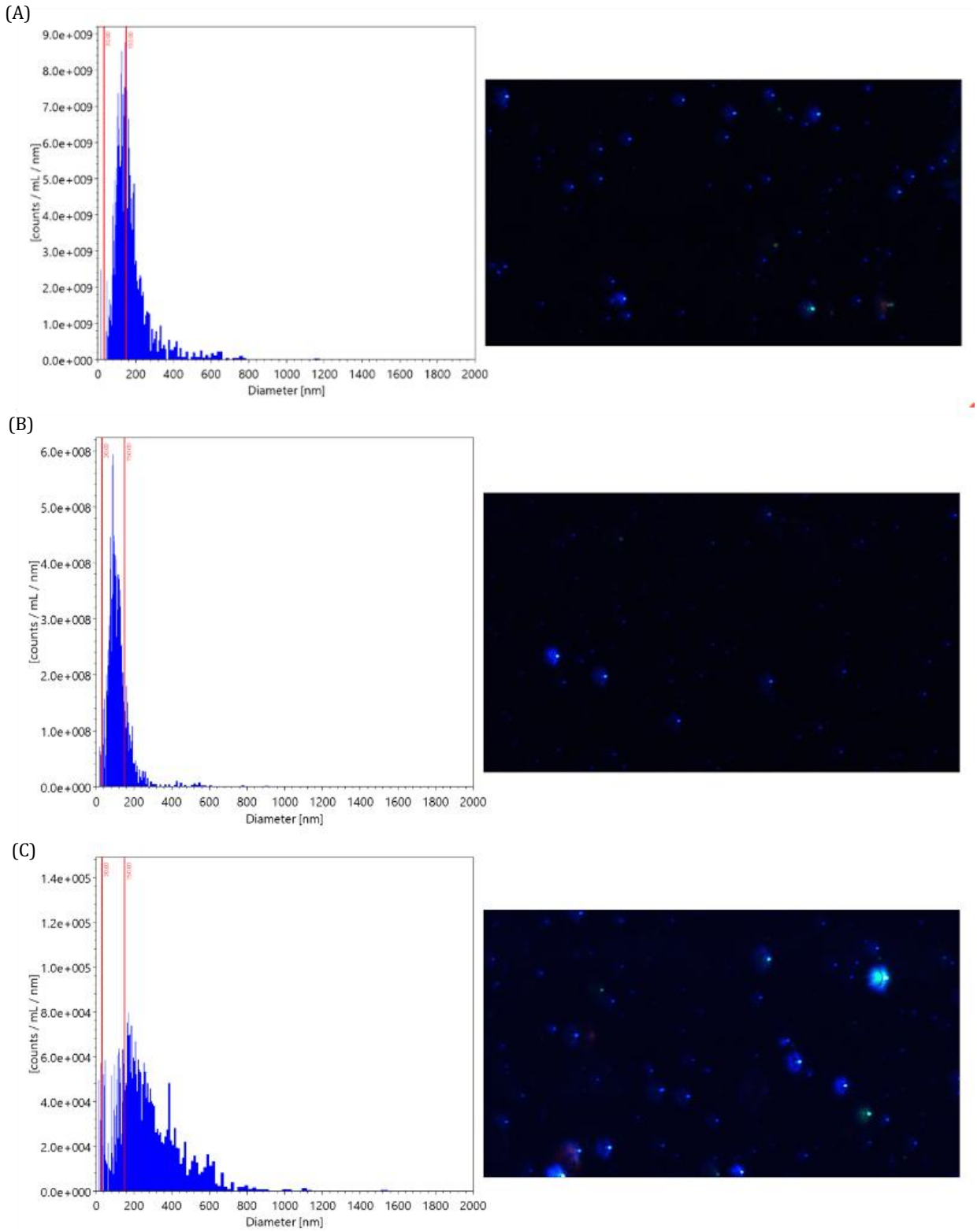

Figure 2. Visualization of exosomes and particle size using NTA machine. (A) exosome extraction performed using Invitrogen's Total Exosome Isolation (from cell culture media), the resulting particle size averaged 110 nm. (B) exosome extraction using HansaBioMed's EXO-Prep for plasma isolation kit, the average particle size was 97 nm, (C) exosome extraction using Qiagen's exoRNeasy Midi Kit, the resulting particles spread between 200 to 400 nm. The exosome diameter is expected to fall within the range defined by the two vertical red lines in the size distribution and concentration graph. Exosome concentration is presented on the Y-axis, while exosome diameter (nm) is shown on the X-axis

Table 3. Total RNA concentration of exosome

Sampel name	Kit 2		Kit 3	
	Purity	Concentration	Purity	Concentration
Sampel 1	1.835	77.59	1.829	79.44
Sampel 2	1.819	33.28	1.927	34.37
Sampel 3	1.927	49.39	1.893	49.30
Sampel 4	1.874	50.14	1.843	71.06
Sampel 5	1.855	46.29	1.907	40.92
Sampel 6	1.928	25.54	1.834	25.91
Sampel 7	1.821	26.12	1.890	29.81
Sampel 8	1.702	66.50	1.897	79.36
Sampel 9	1.892	33.03	1.979	38.95
Sampel 10	1.917	19.90	1.945	20.38
Sampel 11	1.989	43.71	1.876	57.71
Sampel 12	1.922	33.03	1.866	41.02
Sampel 13	1.911	37.37	1.849	36.40
Sampel 14	1.897	67.89	1.831	41.07
Sampel 15	1.956	11.76	1.962	13.62
Sampel 16	1.840	78.46	1.970	85.87
Sampel 17	1.857	27.99	1.825	24.61
Sampel 18	1.838	13.71	1.815	15.02
Sampel 19	1.883	66.50	1.867	77.00
Sampel 20	1.870	42.29	1.880	43.74

Note: Kit 2 = kit EXO-Prep for isolation plasma cat no HBM-EXP-B5 from HansaBioMed; Kit 3 = kit exoRNeasy Midi Kit Qiagen

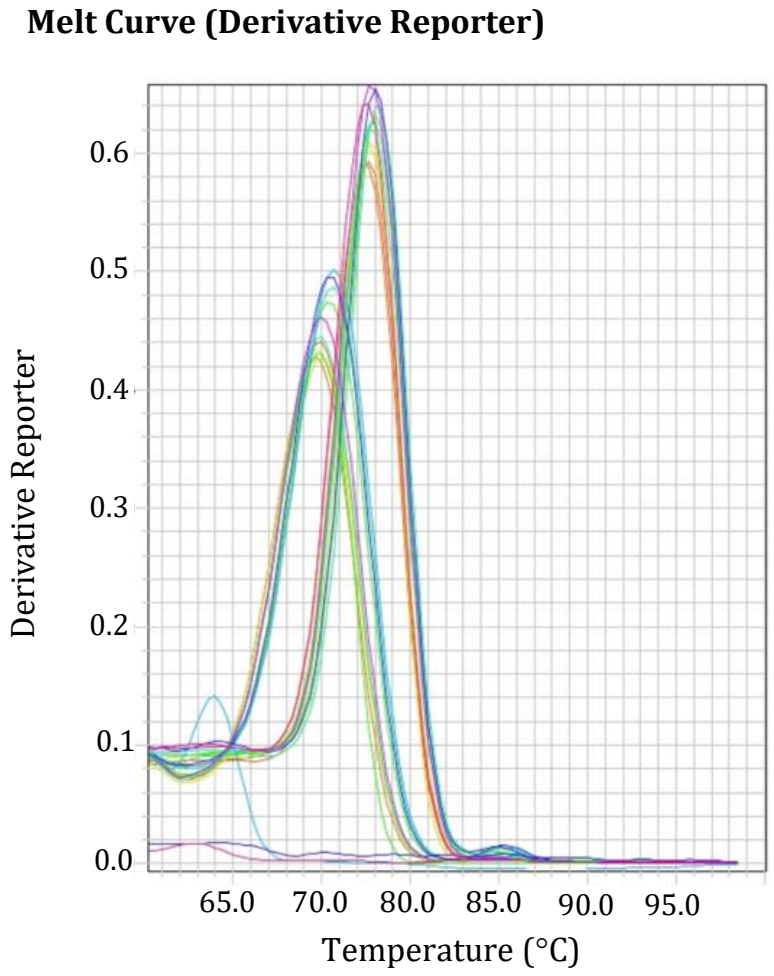

Figure 3. Melt peak shows the anneling temperature of the miRNA primer on the sample target. There are two peaks indicating two miRNA targets (miR-103-5p and spike in). The peaks formed indicate the specificity of the primer binding to its target

Table 4. Expression of miR-103a-5p and Spike in using various RNA concentrations and cDNA dilutions

RNA concentration (ng)	cDNA elution (µL)	Ct miR-103	Ct spike in U	Melt Peak miR-103	Melt Peak Spike in
10	20	32.92	20.55	69.84	72.68
		34.69	20.46	70.01	72.68
	60	33.97	21.49	70.01	72.85
		N/A	21.69	62.64	72.52
50	20	31.44	20.29	70.34	73.02
		31.72	20.63	70.67	73.19
	60	33.08	21.79	69.67	72.51
		33.51	21.82	69.84	72.68
100	20	30.25	20.90	70.67	63.98
		30.99	20.42	70.51	73.02
	60	32.44	21.72	69.84	72.85
		32.51	21.58	69.84	72.85

Note: N/A = Not Applicable

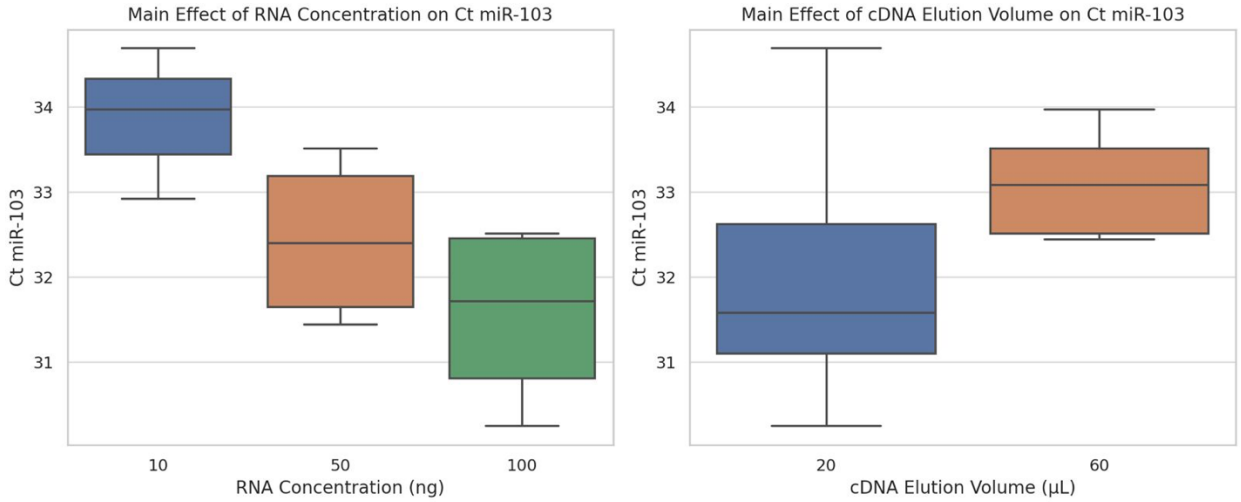

Figure 4. The concentration of RNA to be cDNAized as well as serial dilution of cDNA affects the magnitude of miR-103 expression.

Exosome and miRNA extraction kits had different principles. Kit 1 and kit 2 used a polymer-based precipitation technique, but kit 3 used column affinity. Kit 1 reduced the solubility of exosomes and vesicles, causing precipitation by binding water molecules. The process allowed exosomes to be precipitated using low-speed centrifugation. Kit 1 had a simple procedure and reliable protocol, while also avoiding time-consuming ultracentrifugation. Kit 1 was commonly used for exosome extraction in cell culture media (10,17) for clinical studies (18,19). The results of exosome extraction had also been confirmed using bioimaging, making it suitable for downstream analyses (8). Specimens used for exosome extraction must comply with the provisions of the kit procedure manual. This study used kit 1 to extract exosomes from the plasma of lung cancer patients, but the results were not specific to only precipitated exosomes.

This study compared 2 exosome extraction kits based on polymer-based precipitation, namely Kit 1 and Kit 2, which differed in sample source, processing time, and practical applicability. Kit 1 was designed for exosome isolation from cell culture media, while Kit 2 was optimized for plasma-derived exosomes. A key distinction between the 2 kits was in their processing time. Kit 1 required a substantially longer duration (approximately 13.5 hours), mainly due to the overnight incubation step and extended centrifugation, while Kit 2 enabled a more rapid workflow (approximately 2.5 hours) from whole blood to exosome isolation. This difference had important implications for clinical and translational applications, where time efficiency was critical. In terms of sample requirements, Kit 1 required a relatively large volume of cell culture media (1–10 mL), while Kit 2 required only a minimal volume of plasma (≥100 µL), making it more suitable for clinical samples where volume was often limited. From a practical perspective, both kits offered a significant advantage by eliminating the need for ultracentrifugation, which remained a major limitation in many laboratories due to high cost and specific rotor requirements. This made polymer-based precipitation methods more accessible and feasible, particularly in resource-limited settings. In addition, both kits used PBS as the exosome suspension buffer, which was important for downstream applications, such as quantification using NTA, which required PBS, as well as for molecular extraction processes from exosomes.

Kit 2 and Kit 3 were designed for the extraction of exosomes from various biofluids, including plasma, serum, and cell culture supernatant. Kit 2 and kit 3 also did not require ultracentrifugation for exosome precipitation. Kits 1 and 2 used

PEG (polyethylene glycol), which was a hydrophilic polymer. When added to biofluid specimens, it could bind to water, causing a dehydrated environment. Precipitation methods for exosome extraction in biofluid samples have been widely used, although the gold standard was ultracentrifugation (20). Precipitation using PEG was the most popular agent (10). The precipitation technique made exosomes had a good morphology and function well, when compared to using ultracentrifugation or ultrafiltration. However, the drawback of the precipitation technique using PEG was in the purity of the product exosomes (21). Changes in solubility caused the exosome-vesicle lipid bilayer to precipitate. Kit 2 had the advantages of simplicity, scalability, and the ability to extract exosomes from various biofluid specimens. Kit 2 had not been used in Indonesia, but several published articles used it for clinical studies (22,23).

Kit 3 had 2 extraction processes, namely capturing exosomes using membrane affinity spin columns and extracting RNA from exosomes. Kit 3 was not intended to only capture exosomes in biofluid because there was no reference available on how to elute exosomes trapped in membrane affinity spin columns. This study eluted exosomes on the membrane using 1x PBS and then centrifuged at 8,000 rpm for 10 minutes, but it was not successful, so it did not produce bioimaging (NTA) to determine the specificity of the size and integrity of exosomes. The use of kit 3 for the RNA extraction process was based on the fact that this kit was commonly used in the clinical field (24,25). Kit 3 from Qiagen was also used to measure miRNA expression as a biomarker in various diseases, such as Amyotrophic Lateral Sclerosis (ALS) patients (25), septic and non-septic shock patients (26), non-small cell lung cancer (27), and early pregnancy screening in sows (18). In addition, a study comparing exosome and RNA extraction methods using kit 3 was also widely published (7,28).

Exosome concentration that could be used for miRNA qRT-PCR analysis was > 1 x 10^9 particles (29). The purity of exosomes was observed from the particle size of 30-150 nm or using surface recognition markers consisting of CD (Cluster of Differentiation) 81, CD63, CD9 (1,30), TSG101 (Tumor Susceptibility Gene 101) (31-34). In this study, exosome extraction that had the appropriate particle concentration and size was kit 2, although it did not rule out the possibility that kit 1 and kit 3 were also good. Comparative evaluation revealed distinct performance differences among the tested kits. Kit 2 consistently generated particles within the expected exosome size range and provided sufficient material for subsequent RNA extraction, indicating effective isolation of plasma-derived exosomes. However, kit 1 showed inadequate performance in this context, which was attributable to its design being optimized for exosome isolation from cell culture media rather than plasma specimens, resulting in suboptimal recovery. Meanwhile, Kit 3 was specifically intended for RNA extraction from plasma-derived exosomes; accordingly, while intact vesicles were not detectable during characterization, extractable RNA suitable for downstream analysis was successfully obtained. Taken together, these results suggested that kit 2 offered the most reliable balance of isolation efficiency and exosome specificity for plasma samples, while kit 1 and kit 3 were more appropriately applied within their respective intended sample contexts.

Table 5. Comparison of exosome isolation kits based on technical characteristics and application

Parameter	Kit 1	Kit 2	Kit 3
Method principle	Polymer-based precipitation	Polymer-based precipitation	Membrane affinity (spin column, exoEasy)
Sample source	Cell culture media	Plasma/whloe blood	Serum/plasma
Sample volume	1-10 mL	100 µL plasma	100 µL-1 mL
Processing time	13.5 hours (overnight incubation and centrifugation)	2.5 hours	38 minutes (8 min vesicle binding and 30 min RNA extraction
Centrifugation requirement	10,000 x g (1 hour)	10,000 x g (30 min)	5,000 x g (low-speed centrifugation/spin column)
Ultracentrifugation required	No	No	No
Exosome recovery	Intact exosome	Intact exosome	Not recovered (captured on column)
Exosome quantification	Possible	Possible	Not possible
RNA extraction	Requires additional kit	Requires additional kit	Integrated (exosome+RNA)
Downstream application	Exosome characterization and molecular analysis	Clinical plasma exosome studies	Direct RNA analysis (miRNA, gene expression)
Buffer used	PBS	PBS	Proprietary buffer (column-based)
Advantages	Suitable for large volume samples, intact vesicles	Rapid, low volume, clinically applicable	Fast, integrated workflow, high RNA yield
Limitations	Time-consuming	Moderate yield variability	Cannot recover intact exosomes for characterization

Feasibility and operational efficiency were critical factors from a clinical study perspective, particularly in an Indonesian hospital setting where plasma volume and cost were often limited. Among the evaluated methods, Kit 2 demonstrated a clear practical advantage due to its lower plasma input requirement. While kit 1 and kit 3 required approximately 1 mL of plasma per sample, kit 2 was successfully applied using 250 µL of plasma in this study, despite being originally optimized for volumes as low as 100 µL. This characteristic is clinically relevant, as plasma availability was frequently restricted in patients with advanced disease or when serial sampling was required for monitoring.

Workflow duration further differentiated the kits in a routine laboratory context. Kit 1 required approximately 1.5 hours per sample to obtain exosome preparations, while kit 2 required a longer processing time of around 2.5 hours to achieve exosomal output suitable for downstream analysis. However, kit 3 required approximately 1.5 hours to directly generate RNA. However, unlike kit 3, both kit 1 and kit 2 required an additional RNA extraction step following exosome isolation, which increased total completion time and resource utilization. Despite this, the procedural simplicity and reduced sample volume requirement of kit 2 support its implementation in hospital laboratories with limited technical capacity.

Furthermore, RNA extraction was subsequently performed on exosomes extracted using kit 2, followed by downstream processing with the RNA Basic kits (HansaBioMed). In addition, RNA extraction of exosomes kit 3 was also carried out, considering that this kit was commonly used for biofluid samples, to facilitate a clinical study. The RNA showed purity at an OD 260/280 ratio of approximately 1.8-1.9 (12). The measured RNA concentration also met the requirements for miRNA expression analysis using qRT-PCR. The next step was the synthesis of cDNA from the extracted RNA using RNA Basic kits (HansaBioMed). This kit was selected based on cost-effective considerations and could carry out bioimaging tests on exosomes, even though it must use 2 separate kits for RNA extraction from exosomes. This was confirmed by statistical tests that showed no significant difference in exosome RNA extraction using kit 2 or kit 3.

Assessment of RNA quality and downstream qPCR performance further demonstrated that both kit 2 and kit 3 were capable of generating RNA suitable for miRNA expression analysis. However, the optimal choice between these kits depended on the specific objectives of the study. When visualization, size verification, or characterization of exosomes was required, kit 2 was more appropriate, as it allowed recovery of intact vesicles that could subsequently be subjected to RNA extraction using a separate protocol. Meanwhile, when the primary goal was the acquisition of RNA for qPCR or sequencing and sufficient sample volume was available, kit 3 represented a viable alternative due to its integrated RNA-focused workflow. These results showed that methodology selection must be guided not only by technical performance, but also by intended downstream application and practical constraints of the study setting. The comparison of the 3 kits based on their characteristics and applications was presented in Table 5.

The recommended concentration of RNA for cDNA synthesis was 100 ng, while the recommended elution of cDNA for qRT-PCR was 1:60. The performance of qPCR as a detection and quantification tool for miRNA expression was highly dependent on the quality and purity of the extracted RNA from exosomes (35). Suboptimal extraction could lead to poor sensitivity, low signal-to-noise ratios, and ultimately, unreliable data. Therefore, optimization of each step of the exosomal miRNA workflow, including extraction and qPCR conditions, was essential to improve both the reliability and reproducibility of results.

4. CONCLUSIONS

In conclusion, this study has several limitations that must be acknowledged when interpreting the findings. First, characterization of the isolated vesicles is limited to particle size analysis using NTA. While size distribution provides an important initial indication of exosome presence, additional confirmation using established protein markers such as CD9, CD63, or CD81 is not performed. The absence of immunoblotting validation represents a limitation of the study. However, this approach is beyond the scope of the current work, which primarily focuses on practical kit performance rather than comprehensive vesicle characterization. This study demonstrates that for exosomal miRNA study from plasma in settings where sample volume is limited and exosome visualization is desired, the HansaBioMed EXO-Prep kit (Kit 2) followed by its RNA Basic kit provides an effective and practical solution. While the integrated Qiagen kit (Kit 3) yields high-quality RNA, its inability to elute intact exosomes limits its use for studies requiring vesicle characterization. The critical optimization of using 100 ng RNA input and a 1:20 cDNA dilution further ensures robust qPCR detection of low-abundance exosomal miRNAs. This workflow provides a validated, accessible standard for initiating a liquid biopsy study in Indonesia. Due to the inherent methodological differences among the 3 kits, no single approach fully addresses all analytical needs. Therefore, future study teams are encouraged to adopt complementary strategies by combining different isolation methods to bridge existing methodological gaps. This integrative approach can improve the accuracy of exosome characterization, optimize RNA recovery, and ultimately generate more comprehensive and reliable datasets.

Author contributions: AIK: Contributed to the conceptualization of the study, Developed the methodology, Performed data validation, Drafted the original manuscript. MD: Contributed to the conceptualization of the study and managed project administration. NW: Sontributed to the conceptualization and formal analysis of the study, Oversaw project administration,

and supervised the research activities. DSH: Contributed to the supervision of the study. SMH: Contributed to the supervision of the study. KWTH: Contributed to the supervision of the study.

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