International Journal on Advanced Science Engineering Information Technology

Vol.11 (2021) No. 3
ISSN: 2088-5334

Carrier RNA (cRNA) Enhances dsDNA Recovery Extracted from Small Volume Spent Embryo Culture Medium

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***Abstract*— Embryo spent culture medium has been intensively investigated, considering its promising feature for non-invasive bioanalytical techniques in in-vitro fertilization (IVF). Despite, isolating DNA from such samples is quite challenging due to its small volume. Carrier RNA is reported to exhibit DNA retrieval effects and commonly employed in various limited biological samples, but there are no reports regarding its benefit on embryo media. Therefore, we aim to evaluate the competence of cRNA on isolated DNA from embryo medium and analyzed its optimal volume as there are also no records respecting its ideal volume to obtain decent outcomes. Results showed that cRNA significantly increases DNA amounts in the cRNA treated group ($p<0.001$), but the D-4/D-5 medium yielded similar ($p=0.684$). Pearson test demonstrated no correlation between cRNA volume vs. total retrieved DNA ($r=0.760$, $p=0.80$), and Whole Genome Amplification (WGA) was shown to increase DNA in the treated group ($p=0.022$), but not in the untreated group ($p=0.128$). Additionally, electrophoresis successfully resulting in a thick and thin band of TH01 locus signifies the cRNA competence. In conclusion, our study suggests that cRNA addition is essential in embryo medium extraction as it increases initial DNA that crucial for downstream application. However, the optimal volume could not be determined in the current study since the initial amount of DNA in the medium is unknown. Obtained findings are expected to be a new input for subsequent research on DNA extraction.**

***Keywords*— Assisted reproductive technology; carrier RNA; in-vitro fertilization; spent embryo medium.**

Manuscript received 28 Oct. 2020; revised 31 Jan. 2021; accepted 24 Feb. 2021. Date of publication 30 Jun. 2021.

I. INTRODUCTION

In-Vitro Fertilization (IVF) is one kind of Assisted Reproductive Technology (ART) that has been continuously investigated. However, despite the increasing trend of the procedure, it has still been facing challenges in terms of a low success rate, which only 10-40% globally [1] and 20-50% locally in Indonesia [2]. Apart from maternal factors, another reason for this sub-optimal outcome is the high number of embryo transfer failures [3], [4], [5]. Failure transfer is related to the insufficient embryo quality to develop reaches pregnancy [6]. Several reasons, including chromosomal abnormality [6], [7], and cellular energy disruptions, is thought to be the primary cause of this phenomenon [8]. Hence, methods such as Preimplantation Genetic Testing (PGT) and Gardner's morphology grading are continuously developed for embryo selection to ease these challenges. However, PGT requires an embryo biopsy on either the 8-cells or trophectoderm, which are costly and invasive [9]. It was reported to increases the risk of embryo trauma, undetected mosaicism, and segmental aneuploidy, leading to false diagnosis [10]. Consequently, non-invasive methods are now more attractive for embryo selection.

One of the emerging non-invasive approaches that further investigated is the detection and selection of biomarkers present in the embryo culture medium [11]. It was reported

that the spent medium contains three significant materials, including translated proteins of particular gene expression (proteomics), the end-products of biological processes (metabolomics), and small non-coding RNAs. In particular, the initial discovery of embryonic DNA in medium culture leads to various reports on other specific biomarkers' characterization, such as mitochondrial and genomic DNA [12], [13]. Measurement of these substances eventually can be utilized for various downstream applications comprising broad purposes in embryo selection. As reported, Stigliani *et al.* found that genomic DNA (gDNA) and mitochondrial DNA (mtDNA) are released into the medium, in which embryos with high mtDNA/gDNA ratio are known to have better development potential, embodied a novel non-invasive biomarker for embryo selection [14]. Additionally, Cimadomo *et al.* reported that microRNA (miRNA) is secreted into embryo medium and proven to associate with blastocyst development prediction [15]. Therefore, investigating embryo secretomes in a medium is a promising non-invasive measure to alleviate the low IVF's success rate.

However, the mentioned methods are sometimes could not be performed despite the advantages due to low input extracted DNA resulting from small sample volumes [16]. Besides, DNA amounts in the medium are affected by culture days, which vary over time [13]. Consequently, the standard extraction method sometimes does not yield sufficient DNA for further analysis, whereas DNA extraction is the initial-crucial step in this improved embryo assessment workflow, given its result is determining the advance of subsequent applications.

The silica-based solid-phase extraction is a general method to purify DNA. It utilizes a chaotropic agent, which serves as a denaturant and cofactor, to promotes nucleic acid adsorption [17]. As regards, carrier molecules are often added during the extraction process to increase DNA yields, such as linear polyacrylamide, glycogen, and RNA. These carriers recovered nucleic acid by preventing it from being irretrievably bonding [18].

Carrier RNA (cRNA) is reported as the most suitable for DNA recovery since the carrier molecule is required to be >200nt to bind to the membrane [18]. Since the amount of DNA bound to the membrane is thought to be the same as those in the lysate, cRNA addition increases the specific binding of DNA-membrane, which subsequently increases the overall yield of DNA. Additionally, cRNA reduces DNA lost during extraction by competes with DNA in non-specific binding to the membrane and tube wall [19]. To date, various studies have been successfully reporting expected results of cRNA on numerous samples.

However, its competence in DNA extracted from the embryo culture medium has not been investigated. Additionally, there are also no reports regarding its optimal volume needed for optimal recovery. Therefore, we evaluated the effect of cRNA addition on DNA yield derived from small volumes embryo culture medium and analyzed its optimal volume. Also, since the DNA release is known to increase over time, we examined the DNA yield derived from 4- and 5- day culture to determine the appropriate time to perform the extraction.

II. Materials and Methods

A. Sample Collection

Six enrolled participants who signed written informed consent were recruited from IVF Center, Yasmin Clinic, Cipto Mangunkusumo General Hospital, Jakarta. IVF procedure comprises controlled ovarian stimulation (COS), recombinant human chorionic gonadotropin (hCG) administration, oocyte retrieval, and intracytoplasmic sperm injection (ICSI) were sequentially conducted as described elsewhere. After 19+1 h post-ICSI, fertilization assessment was performed, then two pronuclei embryos were individually cultured in IVF cleavage medium (Cook Medical, Bloomington, IN, USA) up to day-3 culture. Embryos were then transferred to blastocyst medium (Cook Medical, Bloomington, IN, USA) until uterine transfer. Following embryo removal for transfer, the spent medium sample was collected into sterile polymerase chain reaction (PCR) tubes (SPL Life Sciences, Korea) and stored at 80ºC until DNA extraction.

B. DNA Purification and Spectrophotometry

Samples were purified using QIAamp Circulating Nucleic Acid (Qiagen, Halden, Germany) based on manufacturer protocol. Before extraction, samples were thawed adjusted into room temperatures. In order to evaluate the effect of day culture on the yield of DNA, samples were extracted with cRNA according to manufacturer protocol classified into two groups: 1) 4-day medium (n=7), and 2) 5-day medium (n=7). Furthermore, to evaluate the effect of cRNA, samples were divided into two groups: 1) cRNA-free (-) group (n=7), and 2) cRNA-treated (+) group (n=7). Meanwhile, another separate group (n=6) was extracted with cRNA ranging from 2.8-5.6 ul to evaluate the optimal cRNA volumes. Each treatment's DNA concentration was subsequently measured using UV Spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA) at 260 nm wavelength.

C. Whole Genome Amplification (WGA) and Purification

To further assess the competence of cRNA, all DNA isolates of both untreated and treated group was subsequently processed by whole genome amplification using GenomePlex® Single Cell Whole Genome Amplification Kit according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, USA) comprises lysis & fragmentation, OmniPlex library generation, and PCR amplification stages. This performed procedure was intended to confirm does WGA itself could enhance DNA yield without cRNA addition. For library preparation, samples were placed in a thermal cycler and incubated: 16°C for 20 minutes, 24°C for 20 minutes, 37°C for 20 minutes, and 75°C for 5 minutes prior to 4°C storage. The amplification thermocycling step was conducted 25 cycles according to the following thermal profiles:

- Initial step: 95°C for 3 minutes
- Denaturation: 94°C for 30 seconds
- Annealing: 65°C for 5 minutes
- Storage: 4°C until further analysis was performed

The WGA product was then qualitatively analyzed by loading 4 µl of the final reaction onto a 1.5% agarose gel. Successful amplification resulting DNA ranging from 100-

1000 bp in size, with the mean size about 400 bp. According to the manufacturer's protocol to determine the DNA yield of post WGA, purification was performed using The GenElute PCR Clean-Up Kit according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, USA). DNA quantification was subsequently carried out using UV Spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA) at 260 nm wavelength.

D. *Electrophoresis*

In order to assess the quality and integrity of the obtained DNA, the WGA products were further proceeded by PCR to amplify a specific microsatellite as described previously [19]. THO1 microsatellite locus was amplified using forward and reverse primers as follows:

F: 5'-GTGGGCTGAAAAGCTCCCGATTAT-3'
R: 5'-GTGATTCCCATTGGCC TGTTCCTC-3'

All samples, including negative control, subsequently amplified using a thermal cycler for 28 cycles for 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, sequentially. Afterward, the PCR product was confirmed by loaded into an agarose gel.

E. *Statistical Analysis*

Data were expressed as mean ± standard deviation analyzed by SPSS ver. 24 (SPSS Inc., Armonk, New York, USA). Student's t-test and Pearson correlation were performed to determine the mean differences and correlation between cRNA volumes and DNA concentration, respectively. P-value <0.05 is considered statistically significant. All performed methods were summarized in Figure 1A-C.

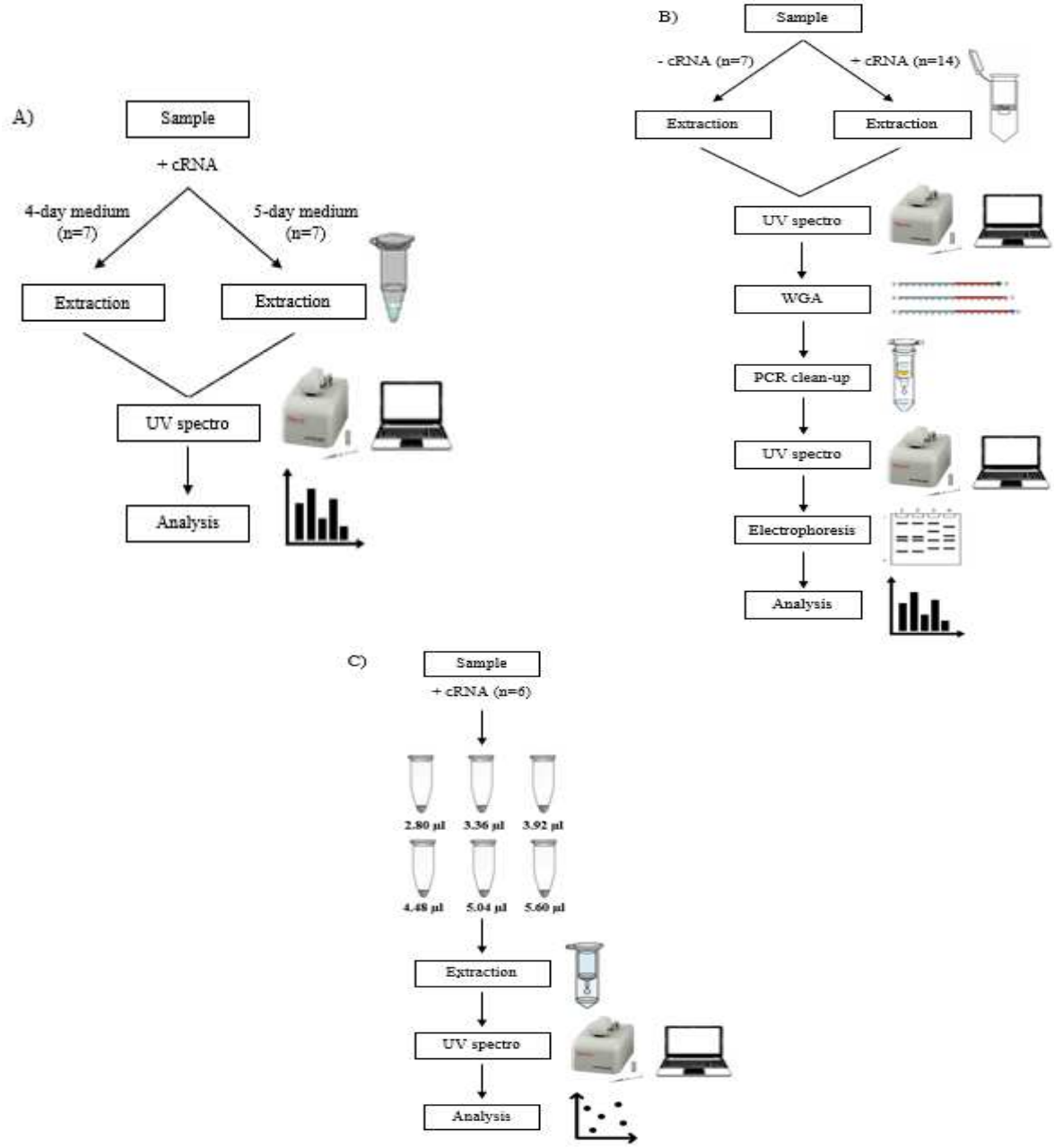

Fig. 1 Schematic representation of methods used in this study to evaluate: A) days culture on DNA yield, B) cRNA addition and WGA procedure on DNA yield, and C) optimal cRNA volumes.

III. Results and Discussion

Our study is the first to clarify the beneficial manner of cRNA to DNA yield derived from small volumes of embryo spent culture medium. The result of the DNA concentration of both control (-) and cRNA-treated groups (+) derived from Spectrophotometry is shown in Figure 2. As shown in the figure, the mean count of the cRNA-treated group is significantly higher than control (14.52 ± 5.08 vs. 2.24 ± 1.99, $p<0.001$), signifying the beneficial effect of cRNA on DNA yield. This finding is in line with Shaw *et al.* who reported that carrier RNA addition to chaotropic salt resulted in a notable increase of recovered DNA from 5 ng to 25 ng extracted from monoliths [19]. Besides, Pearlman *et al.* found that cRNA removal from extraction kits significantly reduced DNA retrieval efficiency extracted from urine and sputum samples [20]. Although the exact action of cRNA in recovering DNA is unknown, Kishore *et al*. revealed two possible hypotheses regarding how cRNA increased DNA yield. It assumed that this molecule might block any binding sites in the side of tubes, centrifugal filter devices, as well as on the surface of the silica membrane, which consequently retains DNA. Moreover, in the thought of DNA adsorption directed by entropy, cRNA might enhance DNA adsorption by competing for remaining solvent that does not bind to chaotropic salt [21]. Thus, we assumed that the cRNA effect on this study has occurred through similar action.

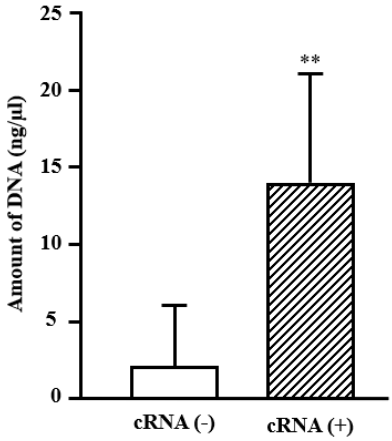

Fig. 2 Mean count of DNA yield of 1) control group and 2) cRNA-treated group analyzed by Student T-test. A double asterisk (**) indicates significant differences ($p<0.01$), and the error bars represent standard deviation.

Regarding the effect of culture day on DNA, statistical analysis showed that DNA concentration in D-4 media was not different from D-5 media (13.79 ± 3.94 vs. 14.56 ± 2.91, $p=0.684$) as presented in Figure 2. Yang *et al.* reported that total DNA in the embryo culture medium was detected from day 3 to 6, given released DNA was proportional to fertilization and cleavage. Based on their experiments, it was concluded that DNA accumulation increased with the culture and cleavage times [13]. Yang *et al.* also revealed that the DNA level increases during culture, suggesting the proper time sampling to obtain expected adequate DNA. However, our results produce the opposite, following Galluzi *et al.* reported that no significant difference observed between day-3 and day 5/6 medium. It was assumed the insignificant result of DNA yield between media with different culture days was presumably due to the low volume of culture media used and the lack of DNA purification procedures [22]. Considering employed volumes in the current study ranged from 15-25 µl, we assumed that limited sample volumes were also the main driver of our inconclusive result. However, our finding has successfully designated that a 5-day medium is more reliable for subsequent procedures requiring a large enough initial DNA since it contains more DNA than the 3-day medium. Further analysis must be conducted to elucidate another mechanism responsible for this phenomenon apart from small sample volumes.

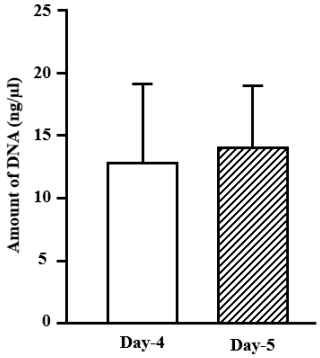

Fig. 3 Mean count of DNA yield extracted from 1) D-4 spent medium, and 2) D-5 spent medium analyzed by Student T-test ($p>0.05$). The error bars represent standard deviation.

We extracted samples with varying cRNA volumes to find out the optimal volume required for optimal results. However, the Pearson correlation test shows no correlation exists between cRNA volumes added to the obtained DNA ($r=0.760$) (Figure 3), which opposed Shaw *et al*. reported that cRNA increase leads to optimal recovery. In summary, it was concluded that the ideal ratio for optimal recovery is 10:1 and 50:1 each for RNA to DNA, respectively [19]. They denote that cRNA does not exert any effect on DNA recovery for samples outside these ratios. Besides, Reddy *et al.* revealed that prolonged storage time affected DNA quality by decreasing recovered DNA [23].

In the present study, the initial DNA secreted in each sample is unfortunately unknown. Consequently, we were unable to determine the cRNA to DNA ratio to achieve optimal recovery, as in Figure 4.

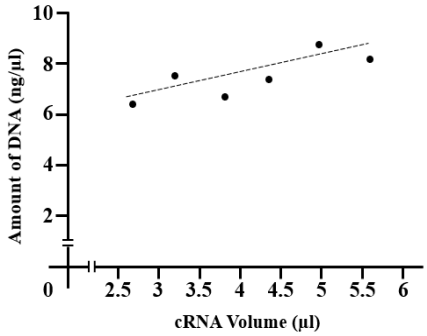

Fig. 4 Correlation between cRNA volume added to the extraction system and retrieved DNA yield. Data were analyzed using the Pearson correlation test ($r=0.760$, $p=0.80$)

Moreover, employed samples were already stored in a -80ºC freezer for a while with several thawing processes

during extraction. Therefore, we assumed that both reasons were leading to an insignificant correlation result. Besides, we employed a small sample number that, although technically valid, might alter the statistic power caused by a lack of recognizing the possibly relevant deviation due to large standard error.

To further assess cRNA competence, we performed whole-genome amplification to both treated and untreated groups to confirm whether this procedure alone could enhance DNA yield without cRNA addition so that the function can be interchanged. The result showed that amplification was significantly improved DNA yield in the treated group, as presented in Figure 5A, in which the mean count before and after WGA was 14.52 ± 5.08 vs. 29.73 ± 8.85, p=0.022. However, WGA was shown to did not affect DNA amount in untreated groups as the mean yield before and after was 2.24 ± 1.99 vs. 5.03 ± 4.12, p=0.128 (Figure 5B).

Finding on the treated group agrees with Rodriguez *et al.* (2018), which revealed that WGA significantly increases DNA from spent culture media as this technique enabled to amplified an entire genome-worth of DNA starting from single cells [24], [25]. Considering that low initial DNA is a limiting factor in an advanced IVF procedure, such as PGT, WGA is an essential preemptive measure for solving. Meanwhile, inconclusive data on the untreated group signifies that cRNA addition is essential in embryo medium extraction as WGA alone proved to could not improve DNA yield significantly.

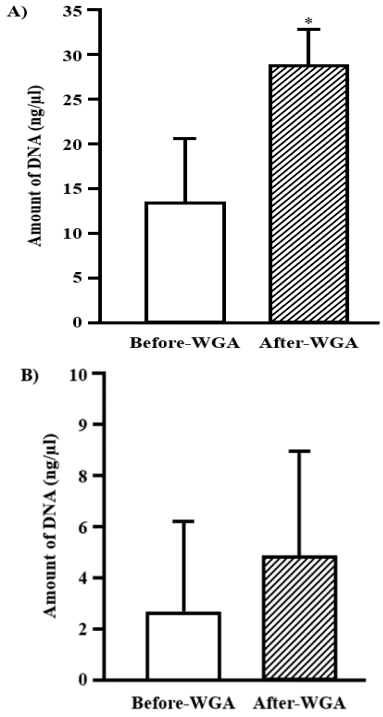

Fig. 5 Meant count of WGA product from A) treated group, and B) untreated group analyzed by Student T-test. A single asterisk (*) indicates a significant difference ($p<0.05$), and the error bars represent standard deviation.

PCR analysis was performed to confirm the integrity of extracted dsDNA in both treated and untreated groups as well as to prove that cRNA is reliable and does not yield a detrimental effect on downstream methods. As seen in Figures 5A-B, our results were in accordance with Shaw *et al.*'s study, in which THO1 was successfully amplified, indicates that obtained DNA is stable for subsequent procedures. Moreover, results were also signified that cRNA addition to the extraction system does not hinder any supposed reaction [19].

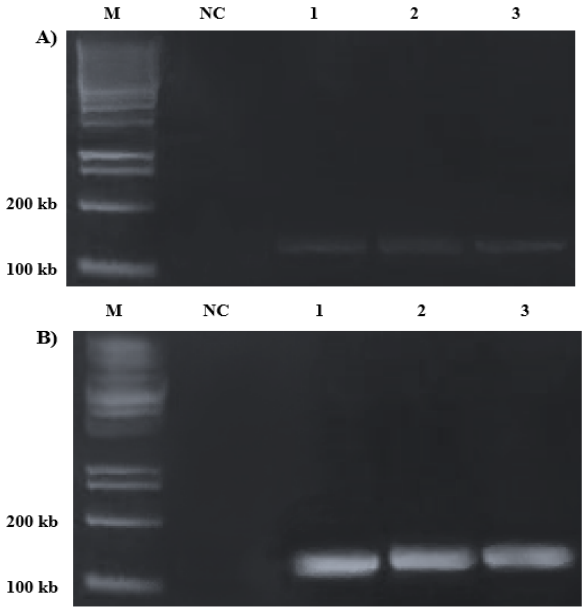

Fig. 6 Agarose gel showing PCR products amplified from A) untreated group without cRNA, B) treated group with cRNA. M: Marker, NC: negative control containing no DNA, 1: sample 1, 2: sample 2, 3: sample 3.

In addition to quality and integrity information, PCR results on agarose gel also indirectly represented the amount of amplified DNA in the samples. As seen in Figure 5A, the untreated group's PCR product results in a thin and faint band compared to the treated group with a thicker and darker band. A thicker and darker band suggests more DNA present resulting from the PCR process as the size difference observed in the agarose band is arouse from the cycling amplification, as demonstrated by Wahyuningsih *et al.* It was reported that the thickness and clarity of the band observed in agarose gel are matched with the estimated PCR product. Additionally, it was represented that a thicker agarose band is proportional to a faster fluorescence increase, indicates the higher of initial DNA molecules in the sample [26]. Therefore, it can be concluded that the cRNA-treated group has a higher DNA amount compared to the untreated group. Obtained results confirmed that cRNA has a retrieval effect on DNA extracted from the spent embryo culture medium, reliable for subsequent analysis.

IV. Conclusion

In conclusion, our study demonstrates that cRNA addition is essential in embryo medium extraction as it enhances initial DNA that is crucial for subsequent application, as confirmed by WGA and electrophoresis. Moreover, the day-5 medium is known to contained more expected DNA compared to the day-4 medium.

Acknowledgment

This study is funded by the Directorate of Research and Community Service, University of Indonesia, and supported

by an international publication grant: International Research Collaboration Grant (NKB-1944/UN2.R3.1/HKP.05.00/2019) of the year 2019.

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