Chapter

Techniques of Using Peripheral Blood Mononuclear Cells as the Cellular System to Investigate How of the Bovine Species (Indian Zebu-Jersey Crossbreds) Responds to *in vitro* Thermal Stress Stimulation (Thermal Assault/Heat Shock)

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Abstract

Animal production is negatively impacted by global warming and is subject to serious consequences for livestock production systems. In order to understand how PBMCs of Indian Zebu-Jersey crossbreds respond to various levels and durations of thermal assault and heat shock, in this chapter we will discuss techniques involving *in vitro* thermal stress stimulation (TSS) to stimulate bovine peripheral blood mononuclear cells (PBMCs) under various thermal assault conditions (TACs), including normal to extreme temperatures and varying durations of thermal exposure (DTEs). The consequences of thermal stress on bovine species can be lessened and managed with an understanding of how PBMCs as a cellular system respond to in *vitro* heat shock and thermal assault. To learn more about how Indian Zebu-Jersey crossbreds respond to *in vitro* thermal conditions, it may also be possible to explore the relationship between the decrease in PBMCs count during *in vitro* TSS and the expression of the heat shock protein genes (HSPs) such as *HSPs* 70 and 90 genes. This will be exploited to discover how Indian Zebu-Jersey crossbreds respond *in vivo* to diverse environmental thermal conditions and will further enable *in vivo* understanding of the

potential for thermotolerance in bovine species for better adaptability, survival, and production performance.

Keywords: heat shock, PBMCs, thermal assault, cellular system, Zebu Cattle, heat stress

1. Introduction

Livestock animals are required to raise their respiratory rate and peripheral blood flow in the tropics due to the harsh weather conditions, which has a detrimental effect on physiological and production performance, including poor milk quality [1]. Environmental thermal conditions have made it difficult for both humans and animals to survive in the face of the existential danger posed by climate change, which has had a variety of negative effects on performance, production, and food security [2]. Cattle as well as other animals can suffer severe effects from thermal stress (TS), including decreased feed intake, low milk production, stunted growth, poor health, decreased activity, and poor performance [3], they also succumb to hyperthermia if thermal assaults are not mitigated [3].

Furthermore, livestock animals are compelled to adapt and survive under assault of thermal conditions or extreme environmental conditions in the tropics, which has a negative impact on their physiology and production performance. The ability of cattle and other animals to maintain homeostasis is negatively impacted by changes in external temperatures and relative humidity, which forces them to actively maintain the internal body temperature (IBT) required for their survival and productivity [4]. Homeothermy is the ability of an animal to regulate its internal body temperature (IBT) in the face of thermal challenges from the environment [1]. TS is attained when an animal's BT is raised over its usual physiological range. The condition results in increased management expenses, lowers food security, and has a negative influence on income production, all of which create economic loss [5].

According to earlier research findings [6–9], variations in thermal assault conditions (TACs) and heat shock have an impact on cellular integrity, proliferation, and viability as well as RNA concentration, making animals more susceptible to opportunistic infections caused by weakened immune systems and a decline in productivity and reproduction [1]. Because RNA is a heat-labile nucleic acid, it is extremely unstable when exposed to harsh environmental conditions, especially heat or thermal assault and as such the degradation of RNA nucleotides and subsequent mutational damage to the structure of nucleic acids that affect nucleic acid synthesis and functions have also been linked to harsh environmental TACs [1]. Previous studies revealed that temperature variations had a significant impact on the production and proliferation of RNA nucleotides [1]. For instance, a moderate 37°C *in vitro* temperature mimics and resembles the BT of mammalian species and increases RNA synthesis and proliferation as well as cell survival [1, 8, 10]. In order for cattle to perform better in terms of production and reproduction abilities, it is necessary to mitigate the effects of harsh environmental conditions [3].

In order to obtain biological information about how cellular systems react to heat shock following exposure to TACs, Onasanya and his team [1] performed *in vitro* TSS of PBMCs on PBMCs of Indian Zebu-Jersey crossbreds maintained under stressful thermal conditions. In this chapter, the methods and techniques employed by the authors will be adequately discussed.

2. Blood sample collection and experimental animals

Seventy (70) Indian Zebu-Jersey crossbred animals were between the age of four and six years. Blood samples (10 mL per animal) were taken aseptically in EDTA bottle (**Figure 1**). After blood collection, the samples were transported in cooled iced-packed box and PBMCs were isolated within two hours of collection [1]. The reason for this is that, immediately the blood is collected the cells will continue to survive on the blood glucose, once the blood glucose is exhausted the cells will begin to die and they won't be able to respond to *In vitro* TSS.

3. Procedures for isolation of peripheral blood mononuclear cells

Ten (10) mL of animal blood samples were homogenized and properly mixed. Then, homogenized blood was added in an equal V/V ratio to 10 mL of previously prepared phosphate-buffered saline (PBS) (HiMedia Laboratories, Mumbai, India). A gentle homogenization and thorough mixing with pipetting up and down followed. A new 50 mL conical tube containing 3 mL of Histopaque®-1077 (Sigma-Aldrich Co. LLC, Darmstadt, Germany) was then carefully filled with the blood-PBS mixture, and the tube was centrifuged at 400 × g for 20 min in a REMI R-4C laboratory centrifuge (Goregaon East, Mumbai - 400 062, India) [1].

Using Histopaque®-1077 (Sigma-Aldrich Co. LLC), fresh whole blood was fractionated, and four separate layers were visible: the top layer was yellowish plasma, the bottom layer was milky PBMCs, and the top layer was histopaque. The top layer

Figure 1.
Showing India Zebu–Jersey crossbred cattle.

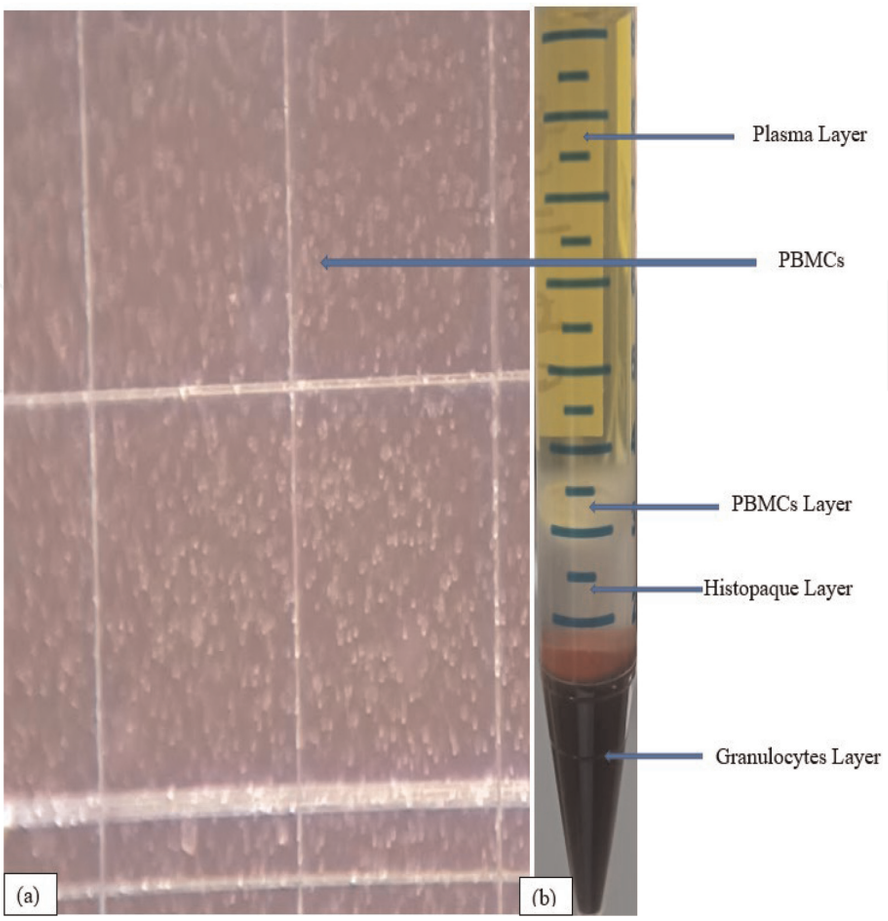

Figure 2.
(a) showing the hemocytometer's ability to detect peripheral blood mononuclear cells under a microscope; (b) shows the fractional separation of a blood sample, which reveals peripheral blood mononuclear cells as well as other fractional layers.

contained erythrocytes and granulocytes (**Figure 2b**). **Figure 2a** show the detection of the PBMCs under microscope.

After centrifugation, PBMCs were collected with the least amount of plasma (and Histopaque®-1077) and transferred into a clean15 mL conical tube. The PBMC suspension was then extensively homogenized by pipetting up and down after adding 10 mL of PBS (PBS was used to wash the PBMCs), followed by 10 min of centrifugation at 100 × g. After that, the supernatant was discarded in order to recover the PBMC pellet. The conical tube was then flicked until the PBMC pellets were fully resuspended in the remaining PBS solution. 10 mL of PBS solution was added to the pellet and thoroughly mixed by up-and-down pipetting. After centrifuging the mixture at 100 × g for 10 min, the supernatant was discarded.

The method (washing with PBS, mixing, and centrifuging at 100 × g for 10 min was performed three times) to recover the PBMCs pellet. The PBMCs were then resuspended in 1 mL of a mixture consisting of 100 mL of fetal bovine serum (FBS) and 900 mL of basic medium (RPMI-1640): 900 mL (HiMedia, Laboratories), and gently mixed by up and down pipetting in the FBS-RPMI mixture. Trypan blue dye exclusion method was used to count and confirm the viability of the isolated PBMCs, and TSS was immediately performed.

4. Procedures for generating various thermal assault conditions

From the previously published study, 70 animals were placed into seven groups with 10 individuals each. In total, 70 Indian Zebu-Jersey crossbred cattle breed's blood samples yielded 70 aliquots of PBMCs, both stressed and unstressed cells. With the exception of the 0°C TAC, the PBMCs were exposed to each of the four TACs for 3 h and 6 h (0, 37, 40, and 45°C) (**Figure 2**). Before the TSS procedure, the number of viable cells were estimated to be between 7.04×10^6-2.56×10^7 cells/mL. About 1 $\times 10^6$ PBMCs/mL were present in each aliquot of 500 μL [1].

All PBMC aliquots were first stabilised for 30 min. at 37°C in a 5% CO_2 incubator with nutritive medium (RPMI 1640; Cole-Parmer Binder C170UL-120V-R CO2 Incubator, Mumbai, India). After 30 min of initial stabilisation of both stressed and unstressed samples in nutrient media at 37°C in 5% CO_2 incubator, the control sample labelled unstressed was immediately harvested.

5. Procedures for thermal stress stimulation of PBMCs

Isolated PBMCs were divided into two groups, one of which underwent TS and the other of which was not. Initially, aliquots of (500 μL) PBMCs were cultured in a nutrient medium (RPMI 1640) at 37°C for 30 min. in a 5% CO_2 incubator for stabilization. Different TACs and DTEs were used to conduct an in vitro TSS of PBMCs. The PBMC aliquots (500 L) were labelled and put through four different TACs in a circulating REMI RSB-12 water bath, as illustrated in **Figure 3**, No TS: Control, 37°C: Normal temperature, 40°C: Moderate heat, and 45°C: Extreme heat) included of four treatment groups, whereas two DTEs were also included (3 and 6 h).

After TSS was completed, the stressed PBMCs were given time to recover at 37°C for 30 min in an incubator with 5% CO_2 before being trypsinized and harvested. Unstressed control samples (500 μL) on the other hand, were stabilized for 30 min before being harvested. They were also not subjected to TAC or DET (0°C or 0 h). Both stressed and unstressed PBMCs will be used for total RNA isolation for heat shock protein gene expression analysis, including *HSP* 70 and 90 genes or other downstream analyses.

6. Evaluation of the PBMC count and viability

Using the Trypan blue dye exclusion method, PBMC number and viability were calculated after PBMC isolation. The trypan exclusion dye method involved the staining of the PBMCs with trypan blue dye such that the viable PBMCs were not stained but dead cells were stained and excluded when observed under a microscope on a haemocytometer (Microyn Improved Neubauer Haemocytometer, Hunt Valley,

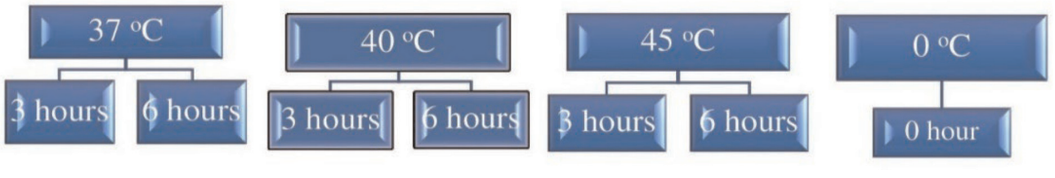

Figure 3.
Illustration of an experimental design with varied thermal assault conditions and durations thermal exposure for stressed and unstressed peripheral blood mononuclear cells of Indian Zebu-Jersey crossbred cattle [1].

Maryland, USA) (CELESTRON Labs CB2000C Compound Microscope, Celestron, LLC., California, USA) (**Figure 2a**). Before TSS, it was estimated that there were 7.04 $\times$ 10^6 to 2.56 $\times$ 10^7 viable cells per millilitre. Total RNA was extracted from the isolated PBMCs. Using a Thermo-Scientific-Nano Drop 2000 spectrophotometer, total RNA was quantitated (Shimadzu co-operation, Kyoto, Japan).

7. Estimation of viable peripheral blood mononuclear cells and quantitation of viable peripheral blood mononuclear cells

As earlier published by Onasanya and his co-workers [1], the PBMCs were estimated, and viability was checked using various parameters shown in Eqs. (1)–(4).

$$\%\text{Viability of PBMCs} = \text{Total viable PBMCs/Total number of PBMCs(Viable + Dead)} \times 100 \quad (1)$$

$$\text{Average number of viable PBMCs per square} = \text{Total number of viable PBMCs in 4 Squares/4} \quad (2)$$

$$\text{Dilution factor} = \text{Total volume (Volume of PBMCs + Volume of trypan blue dye/Volume of PBMCs} \quad (3)$$

$$\text{Concentration of Viable PBMCs/Square} = \text{Average number of viable PBMCs} \times \text{Dilution factor} \times 10^4 \quad (4)$$

For this study, two dilution factors were used.

In their previously published data (**Table 1**) on the TSS of PBMCs, Onasanya and his coworkers [1] found that heat shock/thermal assault at 45°C for 6 h-DTE had the greatest impact on PBMCs of Indian Zebu-Jersey crossbreds than at any other thermal conditions examined.

8. Processing and preservation of Isolated PBMCs for the extraction of total RNAs and mRNA expression analyses

PBMCs to meant for total RNA isolation, especially for gene expression analyses, should be aliquoted into convenient volumes, such as 250 μL of equal PBMCs per

TAC/DTE	Concentration of PBMCs (Cells/mL)	Concentration of total RNA (ng/μL)
37°C/3 h	$1.64 \times 10^6 \pm 0.12^b$	$9.89 \times 10^3 \pm 0.15^a$
37°C/6 h	$9.19 \times 10^5 \pm 0.11^c$	$6.91 \times 0^3 \pm 0.13^c$
40°C/3 h	$7.80 \times 10^5 \pm 0.22^d$	$1.88 \times 10^3 \pm 0.22^d$
40°C/6 h	$4.00 \times 10^5 \pm 0.33^f$	$7.23 \times 10^2 \pm 0.44^e$
45°C/3 h	$6.04 \times 10^5 \pm 0.45^e$	$7.57 \times 10^2 \pm 0.32^e$
45°C/6 h	$3.59 \times 10^5 \pm 0.34^g$	$6.99 \times 10^2 \pm 0.51^f$
0°C/0 h	$2.56 \times 10^7 \pm 0.22^a$	$8.95 \times 10^3 \pm 0.15^b$

$^{a-g}$Means within the same column having different superscripts are significantly different $^{***}P < 0.001$; TACs: Thermal Assault Conditions; DTEs: Durations of Thermal Exposures.

Table 1.
Mean values for PBMCs and RNAs after TSS at different TACs and DTEs in Indian-Jersey Crossbreds [1].

treatment group. The purpose of this is meant to eliminate error of orthogonality that could arise from variation in the numbers of PBMCs among treatment groups, so that variation in RNA concentration will not be due to differences in PBMC numbers among the treatment groups. Thereafter, the PBMC was centrifuged at 8000 rpm for 5 min, gently pipette out the supernatant without disturbing the pellet, add 250 μL of RNAlater® and store at 4°C for 24 h to stabilize the RNA and internal environment of the cells. After the cellular environment of the PBMC has stabilised for 24 hours, centrifuge the PBMCs at 10,000 rpm for 10 min to remove the RNAlater® gently without disturbing the PBMCs pellet and store the recovered PBMCs at −80°C for downstream analyses.

9. Computation of equal number for PBMCs across the treatment groups

How to guarantee that each treatment group has equal number of PBMCs is shown in **Table 2**. For instance, the maximum PBMC count in the four treatment groups is 3.08×10^7. Note that, the treatment group with the highest PBMC count will be used as the benchmark for the computation to guarantee that PBMC counts are equal across treatment groups. Eqs. (5–7) demonstrate the various equations to make the estimations.

$$\text{Numbers of PBMCs in 250 } \mu\text{L} = \frac{\text{PBMCs in 250 } \mu\text{L}}{250} \tag{5}$$

$$\text{Numbers PBMCs per } 3.08 \times 10^7 = \frac{3.08 \times 10^7}{\text{PBMCs}/\mu\text{L}} \tag{6}$$

Volume (μL) to remove from PBMCs of each treatment to generate equal number PBMCs across the treatment groups

$$= 250 - \text{PBMCs per } 3.08 \times 10^7 \tag{7}$$

10. Procedures for isolation of total RNA from PBMCs pellet

1. Spin the PBMC pellet at 10 000 rpm for 10 to recover the PBMCs pellet (for freshly isolated PBMCs)

2. For the already processed PBMCs pellet, add the PBMCs pellet + 1 mL of Trizol in 1.5 μL eppendorf tube, mix gently by up and down pipetting for 5 times,

TAC (°C/Min)	PBMCs/250 μL	PBMCs/μL	PBMCs per 3.08×10^7	Vol of PBMCs to remove
37/15	3.68×10^7	147, 200	209	250–209 = 41
45/15	3.25×10^7	130, 000	237	250–237 = 13
37/30	3.55×10^7	142, 000	217	250–217 = 33
45/30	3.08×10^7	123, 200	250	250–250 = 0

Table 2.
Computation of equal PBMC count across the treatment groups.

subsequently cover the tube and mix thoroughly for 5 times in the right – left direction.

3. Incubate at room temperature (15–25 °C) for 10 min

4. Add 200 μL of chloroform into the above mixture, mix gently for 5 times in the right– left direction for 5 times

5. Centrifuge the above at 10 000 rpm for 20 min at 4°C

6. After spinning, transfer the supernatant into a new 1.5 μL eppendorf tube and add ethanol (100%) in equal V/V ratio. The supernatant is the clear transparent/ upper layer while the Trizol is the bottom layer found beneath the supernatant layer

7. Mix the above mixture thoroughly for 5 times in the right – left direction and fetch 500 μL of supernatant-ethanol mixture

8. Transfer the above mixture into spin column placed in the collection tube

9. Incubate at room temperature (15–25°C) for 5 min

10. Centrifuge the above at 10 000 rpm for 2 min at 4°C

11. Discard the flow-through and re-use the collection tube

12. Then, transfer the spin column back into the same collection tube

13. Add the remaining supernatant-ethanol mixture obtained in (7) above into the spin column and collection tube at (12) above

14. Incubate at room temperature (15–25°C) for 5 min

15. Centrifuge the above at 10 000 rpm for 2 min at 4°C

16. Discard the flow-through and re-use the collection tube

17. Place the spin column back into the same collection tube

18. Add 700 μL RWI wash buffer into the above spin column and collection tube

19. Centrifuge the above at 10 000 rpm for 2 min at 4°C

20. Discard the flow-through and re-use the collection tube

21. Place the spin column back into the same collection tube

22. Add 500 μL RPE wash buffer into the above spin column and collection tube

23. Centrifuge the above at 10 000 rpm for 2 min at 4°C

24. Discard the flow-through and re-use the collection tube

25. Place the spin column back into the same collection tube

26. Centrifuge the empty column obtained above at step 25 and centrifuge at 10 000 rpm for 5 min at 4°C

27. Discard the flow-through and the collection tube

28. Transfer the empty spin column into a new 1.5 μL eppendorf tube

29. Add 15 μL nuclease free water (65°C sterilized in dry bath or water bath for 10 min) into the empty spin column

30. Centrifuge at 10 000 rpm for 5 min at 4°C

31. Repeat step 29 by adding 10 μL sterilized nuclease free water into the empty spin column placed same 1.5 μL eppendorf tube obtained at step 28

32. Incubate the above at room temperature (15–25°C) for 5 min

33. Centrifuge at 10 000 rpm for 5 min at 4°C

34. Discard the collection tube and keep the eppendorf tube

35. Total RNA is isolated into 1.5 μL eppendorf tube

36. Quantitate the concentration of RNA and estimate the purity (1.7–2.0 optical density is consider good for gene expression heat shock protein genes)

37. Store at −80°C for gene express analyses of heat shock protein genes

11. How to preform TapeStation quantity control check for total RNA integrity for preparation of mRNA library and mRNA expression

A 4150 TapeStation System (Catalog: G2992AA, Agilent) that is intended for analysing Eukaryote and Prokaryote RNA can be used to perform the RNA quality assessment [10]. Total RNA molecules with lengths between 50 and 6000 nt are used to compute the RNA integrity (RINe) values, which are used to assess the quality of the total RNA. 3μL of RNA ScreenTape were combined with 1 μL of total RNA sample. Sample buffer was heated at 72°C for 3 min to denature it, after which the sample was immediately put on ice for 2 min before being loaded onto the Agilent 4150 TapeStation equipment. The software assigns total RNA integrity number (RINe) that indicates the integrity of the total RNA [10]. RINe values were graded from 1 to 10, with values between 1 and 5 indicating fully degraded total RNA, 5–7 indicating moderately degraded total RNA, and values above 8 indicating high-quality total RNA. Total RNA whose RINe number falls within the range of 6 and above are of good quality hence they are recommended for preparation mRNA library and gene expression.

12. Procedures for total RNA quantitation

RNA concentration was determined on Qubit® 3.0 Fluorometer using the Qubit™ RNA BR Assay Kit (Catalog: Q10211, ThermoFisher Scientific), which contains RNA reagents consisting of buffers, dye that binds specifically to RNA with linear fluorescence detection in the range of 20 ng/ul to 1000 ng/ul and two RNA standards [11]. The dye and the buffer were diluted at 1:200 ratio and 1 μl of the RNA sample was mixed with the dye mix and incubated at RT for 2 min and the readings were taken in the Qubit.3 Fluorometer. Prior to the sample's measurement, the instrument was calibrated using the two standards provided in the kit [11] (**Table 3**).

13. Conclusion

The cellular systems of livestock animals are exposed to heat shock under prolonged and extreme TACs such high tropical temperatures, which prevents proper cell performance and may even cause cell death or prompt apoptosis. Consequently, severe TAC-DTE combinations have an adverse effect on cell count and survival by causing prompt apoptosis. Therefore, PBMCs can be employed as a cellular model or biological indicator to learn more about how animals' response to thermal assault conditions both *in vitro* and/or *in vivo*. In order to better understand how livestock animals, react to *in vitro,* it will be established in the future whether there is a relationship between the decreased PBMC count following *in vitro* TSS and the expression of the heat shock protein genes. This will enable better understanding of the thermotolerance ability of bovine species and other livestock animals under real-life scenarios/conditions for improved adaptability, survivability, and production performance, the biological data obtained from such study will be used to understand the *in vivo* response of livestock animals to different environmental TACs.

Finally, researches, academics, and livestock farmers will all profit greatly from the *in vitro* thermal stimulation and associated methodologies /procedures as presented in this chapter regarding the function PBMCs can play as a biological indicator in the monitoring and control of heat stress challenges in farm animals.

Sample details		TapeStation QC		QUBIT quantification		Test results	
S/N	Sample ID	RIN	28S/18S Ratio	Conc. (ng/ul)	Volume (ul)	Total RNA mass (ng)	QC status
1	S1	7	1.3	9.74	40	389.6	Pass
2	S2	6.3	1.2	13.8	40	552	Border-Line
3	S3	7.5	1.9	10.7	40	428	Pass
4	S4	7.9	1.6	15.8	40	632	Pass
5	S5	7.2	1.1	17.3	40	692	Pass
6	S6	7.5	0.7	6.08	40	243.2	Pass

Table 3.
TapeStation quality control check for total RNA quantitation and total RNA integrity values.

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Conflict of interest

The authors declare no conflict of interest

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