Validation of extraction methods for total RNA and miRNA from bovine blood prior to quantitative gene expression analyses

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28 **Supplementary Data**

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31 **Methods**

32 *Extraction of total RNA from whole blood after blood collection (WB) and from*

33 *leukocytes after lysis of erythrocytes (LY)*

34 Total RNA was extracted from samples homogenized in TriFast with minor
35 modifications according to the manufacturer's instructions (Peqlab Biotechnologie).
36 Therefore samples were kept for 30 min at room temperature until complete thawing.
37 For phase separation 200 µl chloroform (-20℃) for 1 mL TriFast (originally used) was
38 added following vortexing for 10 sec, and incubation at room temperature for 10 min.
39 Centrifugation was done at 12000 rpm for 15 min at 4 ℃. RNA containing phase was
40 transferred to a fresh tube and RNA precipitation was performed adding 500ul
41 isopropanol (-20℃) for 500µl of RNA aqueous phase. Samples were mixed,
42 incubated at room temperature for 10 min and centrifuged for 10 min at 4 ℃ at
43 12000 rpm. Supernatant was carefully removed and wash of the RNA pellet with
44 500µl of 75% ethanol by vortexing and subsequent centrifugation for 5 min at 4 ℃ at
45 12000 rpm was performed twice. To remove excess of alcohol, samples were placed
46 in a heat block at 50 ℃. Solubilisation of RNA pellet was done using Rnase-free
47 water pipetting it up and down following 10 min of heating at 50 ℃ and storage at 80
48 ℃.

49 *Extraction of mRNA and miRNA from leukocytes stabilized by LeukoLOCK (LL)*

50 Locked cells in filter cartidges were flushed with 2.5 ml of mirVana miRNA Lysis
51 Solution and collected in 15 ml conical tubes. To this solution 1/10 miRNA
52 Homogenate Additive was added and vortexed. Filter cartidges were flushed with 1.5
53 ml acid phenol and collected in the same tube. Solution was vigorously vortexed for

54 30 sec, kept 5 min at room temperature and centrifuged for 10 min at 3200 rpm.
55 Supernatant was carefully removed and transferred to a fresh 15 ml tube, mixed with
56 1.25 volumes of ethanol. Solution was multiple loaded over filter cartidge, centrifuged
57 at room temperature for 15 sec at 10000 rpm and flow-through was collected in a
58 fresh 15 ml tube and saved at 4 °C for miRNA recovery. To the filter 700 µl of miRNA
59 Wash Solution was applied and centrifuged at room temperature for 10 sec at 10000
60 rpm. Flow-through was discarded and filter was washed twice with 500µl Wash
61 Solution following centrifugation at room temperature for 1 minute at 10000 rpm.
62 RNA was recovered applying 100 µL of pre-heated (95°C) Elution Solution to the
63 filter, centrifuged for 30 sec at 12000 rpm and storage at -80 °C.
64 To the Saved flow-through containing the miRNA portion 2/3 volume of 100% ethanol
65 was added and mixed thoroughly. Solution was multiple loaded over a filter cartridge,
66 centrifuged at room temperature for 15 sec at 10000 rpm and flow-through was
67 discarded. To the filter 700 µl of miRNA Wash Solution was applied and centrifuged
68 at room temperature for 10 sec at 10000 rpm. Flow-through was discarded and filter
69 was washed twice with 500µl Wash Solution following centrifugation at room
70 temperature for 1 minute at 10000 rpm. miRNA was recovered applying 100 µL of
71 pre-heated (95°C) Elution Solution to the filter, centrifuged for 30 sec at 12000 rpm
72 and storage at -80 °C.
73 *Extraction of mRNA and miRNA from leukocytes after blood fractionation (PI)*
74 Total RNA was extracted from samples homogenized in Qiazol reagent (Qiagen).
75 Therefore samples were kept for 30 min at room temperature until complete thawing.
76 For phase separation, 140 µl chloroform was added following vortexing for 15 sec
77 and incubation at room temperature for 3 min. Centrifugation was done at 12000 rpm
78 for 15 min at 4 °C. RNA containing phase was carefully transferred to a fresh tube
79 and RNA precipitation was performed adding 1.0 volume of 70% ethanol. Solution

80 was multiple loaded over RNeasy Mini Spin filter, centrifuged at room temperature for
81 15 sec at 10000 rpm and flow-through was collected in a fresh 15 ml tube and saved
82 at 4 °C for miRNA recovery. To the RNeasy Mini Spin filter 700 µl Buffer Wash
83 Solution was applied and centrifuged at room temperature for 15 sec at 10000 rpm.
84 Flow-through was discarded and filter was washed twice with 500µl Buffer Solution
85 following centrifugation at room temperature for 15 sec at 10000 rpm. RNA was
86 recovered applying 30 µL of RNA free water to the filter, centrifuged for 1 minute at
87 10000 rpm and storage at -80 °C.
88 To the Saved flow-through containing the miRNA portion 2/3 volume of 100% ethanol
89 was added and mixed thoroughly. Solution was multiple loaded over a MinELute filter
90 cartridge, centrifuged at room temperature for 15 sec at 10000 rpm and flow-through
91 was discarded. Filter was washed with 500µl Buffer Solution following centrifugation
92 at room temperature for 15 sec at 10000 rpm. Following wash step was done
93 applying 500 µl of 80% ethanol to the filter following centrifugation at room
94 temperature for 2 min at 10000 rpm. miRNA was recovered applying 14 µL of RNA
95 free water to the filter, centrifuged for 1 minute at 10000 rpm and storage at -80 °C.
96 *Extraction of mRNA and miRNA from whole blood collected in PAXgene tubes (PAX*
97 Samples were thawed in a wire rack at ambient temperature for 2 hours. After
98 PAXgene tube centrifugation for 10 min at 3000 rpm the supernatant was discarded.
99 To the pellet 5 ml RNA free water was added, vortexed, centrifuged for 10 min at
100 3000 rpm and supernatant was discarded. For dissolving the pellet 360 µl of Buffer
101 BR1 was used and solution was transferred into a 1.5 ml tube. To this solution 300 µl
102 Buffer BR2 and 40 µl Proteinase K was added, vortexed and incubated for 10 min at
103 55 °C using a shaker incubator at 1400 rpm. Samples were centrifuged at 13000 rpm
104 and supernatant was transferred to a fresh tube. Precipitation was done adding 350
105 µl of 100% ethanol, mixing and centrifuged briefly. Solution was multiple loaded over

106 a PAXgene spin column, centrifuged at room temperature for 1 min at 10000 rpm
107 and flow-through was collected in a fresh 15 ml tube and saved at 4 ℃ for miRNA
108 recovery. To the PAXgene spin column filter 700 µl Buffer BR3 was applied and
109 centrifuged at room temperature for 1 min at 10000 rpm. Flow-through was discarded
110 and filter was washed twice with 500µl Buffer BR4 following centrifugation at room
111 temperature for 3 min at 13000 rpm. RNA was recovered applying 40 µL of Buffer
112 BR5 directly onto the filter, centrifuged for 1 minute at 10000 rpm and storage at -80
113 ℃.
114 To the Saved flow-through containing the miRNA portion 1.4 volume of 98% ethanol
115 was added and mixed thoroughly. Solution was multiple loaded over a RNeasy mini
116 spin column, centrifuged at room temperature for 1 min at 13000 rpm and flow-
117 through was discarded. Filter was washed twice with 500µl Buffer Solution following
118 centrifugation at room temperature for 15 sec at 13000 rpm. miRNA was recovered
119 applying 30 µL of RNA free water to the filter, centrifuged for 1 minute at 13000 rpm
120 and storage at -80 ℃.