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Improved Protocol for High-Quality Co-Extraction of DNA and RNA from Rumen Digesta

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ABSTRACT. We report an improved method for total nucleic acids extraction from rumen content samples. The method employs bead beating, and phenol–chloroform extraction followed by saline–alcohol precipitation. Total nucleic acids and RNA yield and purity were assessed by spectrophotometric measurements; RNA integrity was estimated using Agilent RNA 6000 Nano Kit on an *Agilent* 2100 Bioanalyzer. The method provided total nucleic acids and RNA extracts of good quantity and quality. The extraction is not time consuming and it is valuable for ecological studies of rumen microbial community structure and gene expression.

Similar to other environments, the rumen contains numerous microbial species that have not yet been cultured. In order to overcome culture limits, nucleic acid-based approaches have been applied to this ecosystem (Kobayashi *et al.* 2006). These approaches use DNA extracted from the rumen contents for downstream applications, such as cloning, PCR amplification and sequencing of target genes. Analyses based on genomic DNA do not take into account viability and metabolic state of microbes, while analyses based on RNA are directly correlated with growth activity (Wagner *et al.* 1994). RNA is unstable, which is probably the reason why only few have studied gene expression in the rumen (Bera-Maillet *et al*. 2009; Guo *et al*. 2008; Kang *et al*. 2009). Moreover, none of these studies compared gene abundance in DNA and RNA. Simultaneous DNA and RNA analysis should allow a better comprehension of the structure and dynamics of the microbial populations in the complex rumen ecosystem.

Here we describe a protocol for total nucleic acid extraction from rumen content that allows simultaneously obtaining of high quality DNA and RNA.

MATERIALS AND METHODS

Sampling and extraction protocol. Rumen content samples were collected from wethers fitted with rumen cannulas. Samples were homogenized using a Polytron grinding mill at speed 5, for three times 1 min with 1-min stands in order to avoid sample heating; 0.5 g was transferred to a 2-mL Eppendorf tube in duplicate and mixed with 1 mL of RNA*Later*® Tissu Collection: RNA Stabilization Solution (*Applied Biosystems*, USA). Tubes were kept at 4 °C overnight to allow the solution to penetrate through the cells as suggested by the manufacturer and stored at –80 °C for at least two weeks.

Prior to nucleic acid extraction, all solutions and glassware were rendered RNase-free by diethyl dicarbonate treatment, and only certified RNase- and DNase-free plasticware was used.

The detailed protocol for total nucleic acids extraction is described in Table I. Nucleic acids were extracted from 0.5 g (wet mass) of rumen digesta. RNA*Later* solution was removed from the sample by phosphate-buffered saline (PBS) washing prior to extraction. Cell lysis was achieved by mechanical and chemical disruption using bead beating and hexadecyltrimethylammonium bromide (CTAB) buffer (Griffiths *et al*. 2000). Precipitation of total nucleic acids was performed with ethanol (100 %) and sodium acetate overnight at –20 °C.

DNA digestion. The total nucleic acid sample was divided into two equal fractions named "DNA sample" and "RNA sample". DNA in the "RNA sample" was digested by rDNase, RNase-free (from the Total RNA Isolation Kit, *Macherey Nagel*) as described in Table I.

Comparative studies. We tested three different protocols for total nucleic acids (Griffiths *et al*. 2000), DNA (Persoh *et al.* 2008) or RNA (Trizol method; Kang *et al.* 2009) extraction.

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Other DNA digestion protocols tested were RNase-Free DNase Set (*Qiagen*, Germany) and RQ1 RNase-Free DNase (*Promega*, USA). The comparative assessment of total nucleic acids yield and purity and RNA quality is discussed.

Table I. Improved protocol for co-extraction of DNA and RNA

I. Elimination of RNALater Solution
1. Centrifuge thawed samples at 7000 ***g*** for 10 min at 4 °C *2*. Remove the clear part of the supernatant and note its volume (V_1); add V_1 of PBS 1× and centrifuge (7000 ***g***, 10 min, 4 °C); repeat once.
II. Cell lysis
3. Transfer 0.5 g of sample into 2-mL screw-cap tube loaded with 0.8 g silica beads (0.5 mm diameter). *4*. Add 0.5 mL of CTAB buffer and 0.5 mL of phenol–chloroform–3-methyl-1-butanol (25 : 24 : 1) mixture. *5*. Homogenize on a Precellys two cycles of 30 s at speed 6200 with a 30 s interval between cycles; incubate on ice for 5 min. *6*. Centrifuge at room temperature (13 000 ***g***, 15 min); transfer the supernatant to a new 2-mL Eppendorf® tube; note the volume (V_2). *7*. Add V_2 of chloroform–3-methyl-1-butanol (24 : 1) mixture. *8*. Centrifuge (13 000 ***g***, 15 min, 4 °C). *9*. Repeat steps *7* to *8*.
III. Nucleic acids precipitation
10. Transfer the supernatant to a new 2-mL Eppendorf® tube; note the volume (V_3). *11*. Add 1/10 V_3 of 3 mol/L sodium acetate and 2 V_3 ethanol (100 %); leave at –20 °C overnight. *12*. Centrifuge (16 000 ***g***, 45 min, 4 °C). *13*. Remove the supernatant and wash the nucleic acid pellet with 70 % ethanol with DEPC treated water; air dry for 5 min; repeat washing and air drying at least once. *14*. Dissolve the nucleic acid pellet in 100 µL Tris–EDTA buffer. *15*. Divide the resuspended total nucleic acids in two equal parts: "DNA sample" and "RNA sample".
IV. DNA digestion (NucleoSpin RNA I kit)
15. Load the silica membrane with 3 µg total nucleic acids from the RNA sample. *16*. Spin (11 000 ***g***, 30 s, 4 °C). *17*. Add 350 µL membrane desalting buffer (MDB) and spin (11 000 ***g***, 30 s, 4 °C). *18*. Prepare DNase reaction mixture: for each sample 10 µL rDNase to 90 µL reaction buffer for rDNase; apply 95 µL DNase reaction mixture directly in the center of the silica membrane of the column. Incubate at room temperature for 1 h. *19*. Add 200 µL RA2 buffer to the column and spin (11 000 ***g***, 30 s, 4 °C); place the column in a new collection tube. *20*. Add 600 µL RA3 buffer to the column and spin (11 000 ***g***, 30 s, 4 °C); place the column in a new collection tube. *21*. Add 250 µL RA3 buffer to the column and spin (11 000 ***g***, 30 s, 4 °C); place the column in a new collection tube. *22*. Elute RNA in 30 µL RNase-free water and spin (11 000 ***g***, 1 min, 4 °C).

Polymerase chain reaction. PCR primers mcrA-f and mcrA-r (Luton *et al.* 2002) were used to amplify the *mcr*A gene of methanogenic *Archaea*. PCR amplifications were performed using MyCycler thermal Cycler (*BioRad*, USA) in 50 µL volume according to Luton *et al.* (2002).

Quantity and quality assessment. The yield and the purity of the extracted total nucleic acids and RNA alone were assessed by absorbance measurement using a NanoQuant Plate on an Infinity spectrophotometer (*Tecan*, Switzerland).

RNA quality was assessed using Agilent RNA 6000 Nano Kit on an Agilent 2100 bioanalyzer (*Agilent Technologies*, USA).

RESULTS

Extraction protocol. Four extraction methods were compared using samples conserved in RNA*later* solution and stored at –80 °C. Table II shows the mean extraction yields and absorbance ratios. Our improved protocol recovered approximately 224 µg of total nucleic acids per g of rumen contents (wet mass), which represented 89, 50 and 61 % higher yield than the output obtained by the methods of Persôh *et al.* (2008), Griffiths *et al.* (2000) and Kang *et al.* (2009), respectively. The 260/280 and 230/280 absorbance ratios

obtained with our protocol were both close to 2, while the 230/260 ratio obtained with the other previously published protocols was <1.

Table II. Total nucleic acids yield (NA; μg/g wet mass) and purity (absorbance ratio)[a]

Extraction method	Absorbance ratios		NA
	260/280	230/280	
Persôh *et al.* (2008)	1.54	0.96	23.85
Griffiths *et al.* (2000)	1.45	0.89	111.83
Kang *et al.* (2009)	1.81	0.47	86.99
Improved protocol	1.99	1.87	223.99

[a]Means of duplicates.

DNA digestion and RNA quality. Total nucleic acids extract was divided into two equal parts, and the "DNA sample" was directly used for PCR or qPCR amplification (Fig. 1). The co-extracted DNA in "RNA sample" was removed by digestion. The protocol of Persôh *et al*. (2008) recovered only low quantities of total nucleic acids and therefore was not used in further studies.

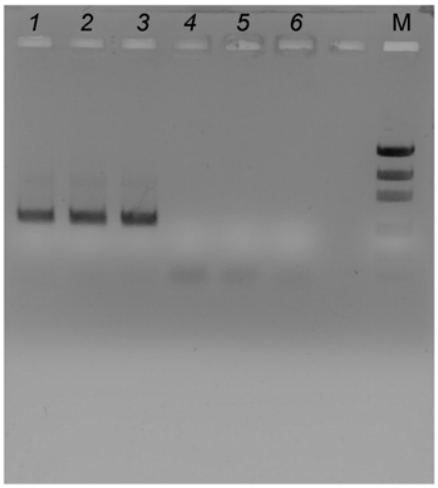

Fig. 1. Agarose gel electrophoresis of PCR products using as template extracted DNA (lanes *1*–*3*) and RNA (*4*–*6*); M – low DNA mass ladder (*Invitrogen*).

Three commercial protocols for DNA digestion were tested: RNase-Free DNase Set (*Qiagen*, Germany), RQ1 RNase-Free DNase (*Promega*, USA), and rDNase from the kit Total RNA Isolation, NucleoSpin RNA II (*Macherey Nagel*, Germany). Yield and purity of the samples were similar independently of the method used to digest DNA (Table III). Complete DNA digestion was confirmed by the absence of PCR amplicon in the "RNA samples", while a single PCR product of the expected size (464–491 bp) was generated when "DNA samples" were used as template for the amplification (Fig. 1).

The quality of the extracted RNA was analyzed with the *Agilent* RNA 6000 Nano Kit. Fig. 2 presents the electropherograms obtained for RNA extracted with the improved protocol with each DNase treatment. Two major peaks displayed in each electropherogram correspond to the 16S and 23S ribosomal RNA. The RNA Integrity Number (RIN) represents the general total RNA integrity (Schroeder *et al.* 2006). The indicators of RNA integrity are the RIN (maximum value 10), the 23S-to-16S ratio (expected value 2) and the absence of degradation (low baseline). Relatively high amount of low-molar-mass material is present after digestion with *Promega* DNase protocol. Marginally degraded RNA, detected as a noise between the 16S and the 23S peaks, and a smaller 23S than 16S peak are observed with both *Promega* and *Qiagen* digestion methods. On-column digestion (rDNase *Macherey Nagel*) apparently improved RNA quality with higher RIN values, improved 23S-to-16S ratios and no background degradation was noted. The RNA quality indicators for the RNA extracted with the methods

Table III. Total RNA yield (μg/g wet mass) and purity (absorbance ratio)[a] using three (*Qiagen*, *Promega*, *Macherey Nagel*) DNase treatments

Extraction method	DNase I (*Qiagen*)		RQ1 DNase (*Promega*)		rDNase (*Macherey Nagel*)	
	260/280	RNA yield	260/280	RNA yield	260/280	RNA yield
Griffiths *et al.* (2000)	1.98	20.0	1.98	20.5	2	16.0
Kang *et al.* (2009)	1.95	37.8	1.85	30.1	2	29.1
Improved protocol	1.99	38.0	1.99	36.4	2	34.9

[a]Means of 2 DNA digestions.

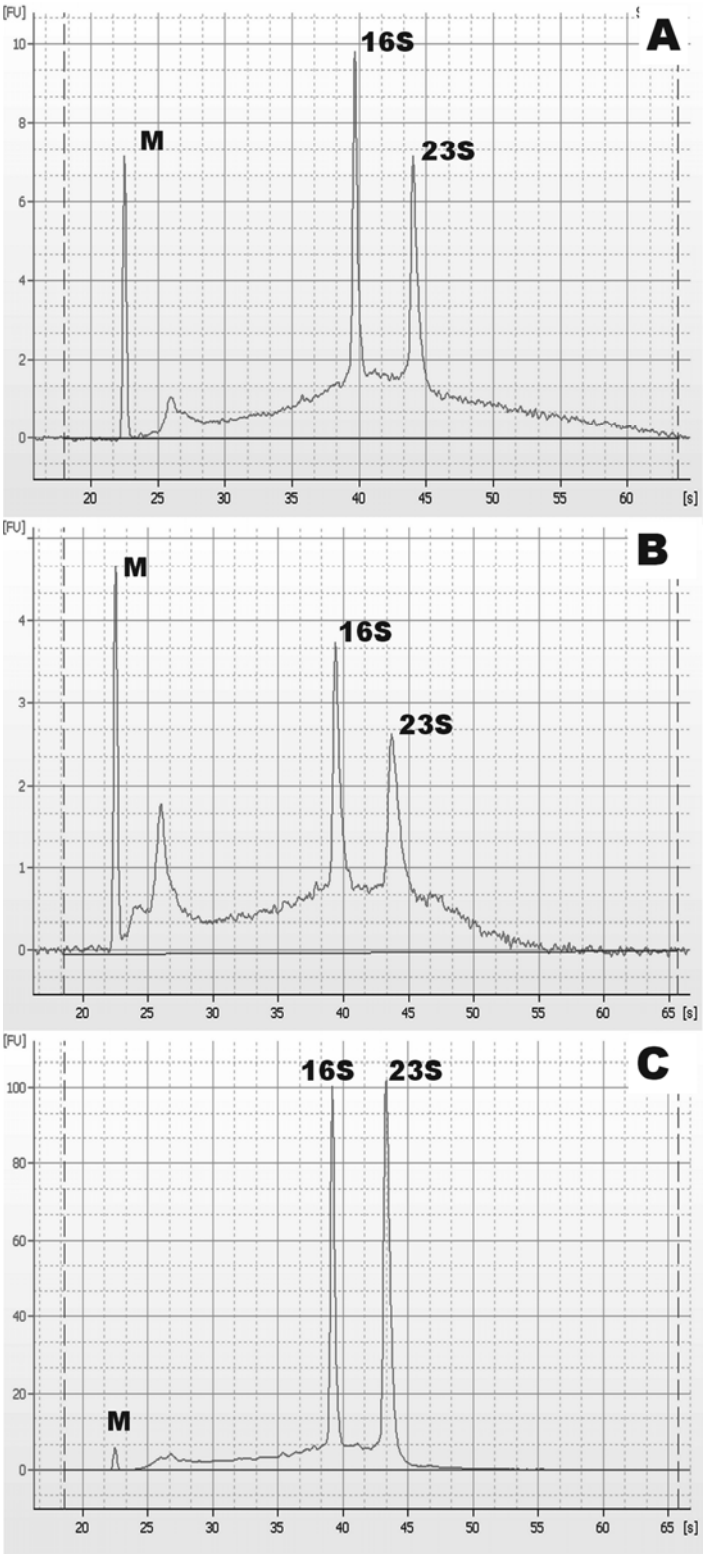

Fig. 2. Analysis of RNA integrity. Electropherograms were obtained using the RNA 6000 *Nanolab*chip and *Agilent* 2100 Bioanalyser. RNA samples were obtained with the improved protocol and three different procedures of DNA digestion. **A**: RNase-Free DNase Set (*Qiagen*) (RIN: 6.9, 23S/16S ratio: 0.8); **B**: RQ1 RNase-Free DNase (*Promega*) (RIN: 6.7, 23S/16S ratio: 0.9); **C**: rDNase, RNase-free (from the Total RNA Isolation Kit; *Macherey Nagel*) (RIN: 8.6, 23S/16S ratio: 1.4). Three main peaks correspond to the marker (M), 16S and 23S rRNA.

of Griffiths *et al.* (2000) and Kang *et al.* (2009) followed by the rDNase on-column digestion were 7.0 and 7.2, respectively. Both methods resulted in 23S-to-16S ratio 0.7 with relatively important noise between the two peaks (*data not shown*).

DISCUSSION

This study aimed at optimizing the total DNA and/or total RNA co-extraction from microbial content of the rumen ecosystem. While a large number of published methods or commercial kits for DNA or RNA extractions are available (Bera-Maillet *et al*. 2009; Griffiths *et al*. 2000; Kang *et al.* 2009; Persôh *et al.* 2008; Saleh-Lakha *et al.* 2005; Schneegurt *et al.* 2003), only few studies (Costa *et al*. 2004; Griffiths *et al.* 2000) reported protocols for total nucleic acids extraction from environmental samples. The chosen method for DNA extraction can have an important influence on the quantitative as well as the qualitative results of the study (Guardia *et al*. 2009). Therefore, for simultaneous study of microbial community structure and gene expression, DNA and/or RNA co-extraction seems to be indispensable. Yields of total nucleic acids and RNA alone obtained in this study were similar in all methods. However, only our improved protocol gave high purity total nucleic acids and RNA, as the absorbance ratios were always near 2. With the other protocols, the elimination of total salts, phenol and organic compounds was incomplete as the absorbance ratio 230/260 remained low. Adding a supplemental chloroform–3-methyl-1-butanol wash in our protocol allowed us to improve the purity of the extract. The saline–alcohol precipitation used has already been shown to give sufficient quantities and good quality RNA (Bera-Maillet *et al*. 2009).

Though the three digestion methods used in this study yielded similar quantities of RNA, the on-column DNA digestion gave better quality RNA. The optimum tempera-

ture for RQ1 RNase-Free DNase (*Promega*) was 37 °C (the enzyme is inactivated at 65 °C). RNase-Free DNase Set (*Qiagen*) can be used at room temperature but inactivation requires a heat shock at 65 °C. On-column digestion is performed at room temperature and the enzyme is chemically denatured and eluted. RNAs are fragile and heat shocks seriously affect extraction quality, as shown on the *Agilent*-generated electropherograms. Moreover, even if 260/280 absorbance ratios for RNA extracts obtained with the three digestion protocols were systematically close to 2, only on-column digestion produced a ratio equal to 2. As the enzyme is eluted from the column, the RNA sample is protein-free.

According to RNA yield and quality we recommend the improved protocol and the on-column DNA digestion for combined DNA/RNA studies on rumen content samples.

To our knowledge, this is the first report on the optimization of DNA/RNA co-extraction from rumen content samples with an additional evaluation of the RNA quality assessed by *Agilent* lab-on-a-chip technology.

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