Short Technical Reports

Extraction of PCR-Quality Plant and Microbial DNA from Total Rumen Contents

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ABSTRACT

DNA from rumen digesta has several diagnostic applications such as studying microbial community dynamics, transgene/DNA stability, and population typing of various rumen bacteria. Several DNA extraction procedures are described in the literature for rumen digesta, which describe the removal of tannins, polysaccharides, and other PCR inhibitors. Some of these protocols are time-consuming and impractical when handling a large number of samples routinely. Here we describe a rapid method for the extraction of PCR-quality plant and microbial DNA from total rumen contents that is based on modifications in the cetyltrimethylammonium bromide procedure followed by cleanup using a Qiagen column. This procedure is highly reproducible and relatively short, once the initial grinding of the samples is performed, and it consistently yields PCR-quality DNA.

INTRODUCTION

Several DNA extraction procedures for isolation of genomic DNA from various sources have been described previously, including the cetyltrimethylammonium bromide (CTAB) method (3) and its modifications (4,11). However, while using the modified CTAB procedures, it is difficult to obtain PCR-quality DNA from samples that are inherently rich in polysaccharides, proteins, tannins, humic acid, etc. (5,8,15). These contaminants are known to inhibit PCR amplification, resulting in false-negative results. Phenolics have been previously shown to induce DNA degradation as well (12), thereby reducing PCR efficiency. Agarose embedding of DNA to remove PCR inhibitors has been recommended (8); however, this procedure is not practical for a large number of samples because of the excessive processing time involved.

Table 1. Protocol for Isolating Genomic DNA from Rumen Digesta

1. Weigh out 2 g rumen digesta. Grind the sample to a fine powder using liquid nitrogen.
2. Transfer the ground sample to a 50-mL Falcon® tube and add 10 mL CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (v/v) 2-mercaptoethanol, and 100 µg/mL (v/v) proteinase K]. Note that 2-mercaptoethanol and proteinase K are added to the buffer after pre-warming it to 65°C. Incubate at 65°C for 2 h with occasional mixing.
3. Cool the solution to room temperature and add 30 µL RNase A (10 mg/mL) and incubate for 30–40 min at 37°C.
4. Extract the homogenate with an equal volume of 24:1 chloroform:isoamyl alcohol. Mix gently and centrifuge at 10 000× *g* for 10 min at 4°C. Recover the top aqueous phase and repeat the chloroform:isoamyl alcohol extraction twice.
5. Precipitate the DNA from the recovered aqueous phase with 0.6 volumes of isopropyl alcohol. Recover the DNA by centrifugation at 7500× *g* for 15 min at 4°C.
6. Resuspend the DNA pellet in 800 µL AP1 buffer and RNase A from the DNeasy Plant kit. Incubate the sample for 10 min at 55°C, coupled with gentle mixing 2–3 times.
7. Add 260 µL AP2 buffer and incubate on ice for 15 min. Spin the solution, discard the pellet (if any), and either store the supernatant at 4°C or proceed to Step 8.
8. To a 200-µL aliquot of the supernatant, add 100 µL AP3 buffer and 200 µL ethanol and mix by inverting a few times. Apply 500 µL lysate obtained onto a DNeasy mini spin column (6000× *g* for 1 min). Discard flow-through and wash the column with 500 µL supplied AW buffer (three times). Leave the buffer for up to 5 min before spinning. Elute the DNA with pre-warmed (65°C) AE buffer (50 µL) twice. Note that if higher amounts of DNA are desired, total lysate obtained (1060 µL) from Step 7 can be processed.
9. Estimate the DNA concentration and use the required amounts for downstream processing (e.g., PCR, restriction digestion, etc.).

Modifications to the original protocol are underlined.

Our laboratory is investigating the stability of transgenes and rate of ruminal DNA degradation from diets fed to animals. Obtaining PCR-quality DNA from rumen digesta posed a significant procedural challenge. To our knowledge, there is only one procedure described in the literature for isolation of microbial DNA from rumen contents (5), which is a modified phenol:chloroform extraction and uses PEG to remove tannins. For our purposes, we desired a simple, relatively fast procedure for obtaining plant and microbial genomic DNA, and several procedures were examined, including (*i*) glass bead extraction (1), (*ii*) classical lysis using SDS and proteinase K followed by phenol:chloroform extraction (16), (*iii*) CTAB method (10), (*iv*) Wizard® genomic DNA purification system (Promega, Madison, WI, USA), and (*v*) DNeasy® Plant kit (Qiagen, Mississauga, ON, Canada). These procedures proved unsuccessful and were not time efficient for consistently obtaining PCR-quality DNA from rumen digesta. Procedures such as CsCl gradients and dialysis (7), as well as immunomagnetic (14) and chromatographic separation (2), may be helpful in isolating genomic DNA from rumen contents but are time-consuming and impractical when handling a large number of samples.

We have developed a procedure that combines modifications to the CTAB method (10) with subsequent cleanup

Table 2. Primer Design and Thermal Cycling Conditions Used for Detection of a 1.363-kb EPSPS Transgene and Its Fragments, Plant (Rubisco), and Microbial (BC) Controls

	Fragment Name	Primer Design (5'→3')	Expected Fragment Size (bp)	PCR conditions
Plant Control	Rubisco	F: GCGTGACGTCGTCACGTAG R: CGTTGCCTGCCACAGGATTAAGG	540	30 cycles of 94°C for 1 min, 55°C for 1min, and 72°C for 1 min[a]
Bacterial Control	BC	F: TCCTACGGGAGGCAGCAGT R: GGA CTA CCA GGG TAT CTA ATC CTG TT	466	22 cycles of 95°C for 15 s, 68.5°C for 30 s, and 72°C for 30 s[b]
Transgene	Fragment 1	Promoter-CTP	179	34 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 1 min[a]
	Fragment 2	Promoter-Gene	527	
	Fragment 3	CTP-Gene	300	
	Fragment 4	Gene-Gene	300	
	Fragment 5	Gene-Gene	278	
	Fragment 6	Gene-Terminator	420	
	Fragment 7	Gene-Gene	270	
	EPSP Synthase	Whole Gene	1363	33 cycles of 94°C for 1 min and 74°C for 3 min[b]

[a]Begin with 95°C for 5 min; final extension at 72°C for 5 min

[b]Begin with 95°C for 10 min; final extension at 72°C for 10 min

CTP, chloroplast transit peptide. Seven different EPSPS fragments were amplified.

of the DNA using the DNeasy Plant kit to obtain PCR-quality DNA from rumen digesta. Using the developed procedure, we obtain consistently good yields, and the DNA is useful for amplifying both plant and microbial DNA fragments and could potentially be used for other procedures such as restriction digestion and blotting purposes. This procedure is simple, rapid, and convenient and consistently produces nonsheared DNA.

MATERIALS AND METHODS

Sample Collection

RoundUp Ready® canola (Monsanto, St. Louis, MO, USA) is glyphosate tolerant because of the expression of a recombinant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). To test the developed procedure, genomic DNA was isolated from rumen digesta of four ruminally cannulated sheep fed mixed genetically modified diets (containing 6.5% RoundUp Ready canola). The rumen contents were collected via a ruminal cannula and processed for DNA extraction.

DNA Extraction

Two grams of rumen digesta were processed according to the procedure described in Table 1. The developed procedure combines cell lysis with protein precipitation using CTAB, and the DNA thus obtained is precipitated following organic extractions. The precipitated DNA was solubilized in AP1 (provided with the DNeasy Plant kit) and further processed using the DNeasy Plant kit with several modifications, as indicated in Table 1.

Amplification Protocols

PCR amplification of a 540-bp fragment of *Brassica napus* Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco; GenBank® accession no. X75334) was used as the plant control. Universal primers were used to detect a 466-bp bacterial 16S rDNA fragment (9). EPSPS transgene (1.363 kb) as well as four different construct-specific and three EPSPS fragments ranging from 179 to 527 bp (Fragments 1–7) were used to detect the transgene within rumen digesta. Table 2 outlines the primers and PCR conditions.

All PCRs (50 µL) contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP mixture, 0.2 µM each forward and reverse primer, and 1.25 U *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada). DNA (100 ng) was used as a template for PCR, and RoundUp Ready leaf DNA was used as positive control. PCR was performed using a PTC-100™ thermal cycler (MJ Research, Watertown, MA, USA).

RESULTS AND DISCUSSION

We attempted the CTAB and DNeasy Plant extraction individually for genomic DNA isolation but found the best results when the two protocols were adapted as described. Here we provide a comparison of isolation of

genomic DNA using the classical CTAB procedure (10) to our developed procedure.

Using the developed method, DNA concentrations obtained were between 50 and 60 µg/2 g sample (wet weight) and absorbance ratios (A_{260}/A_{280}) were between 1.8 and 2.0. Figure 1 shows a representative gel of high-molecular-weight DNA obtained. Using the developed procedure, the PCR-amplifiable genomic DNA was consistently obtained from all the samples tested, whereas, when CTAB procedure alone was used, we could amplify only two of the seven EPSPS gene and construct-specific fragments (Figure 2A, lanes 1). Though the EPSPS whole gene (1.363 kb) could be amplified using the CTAB and the outlined procedures, it was visualized as a very faint band from the former, even though the same amount of DNA template was used for amplification (Figure 2B).

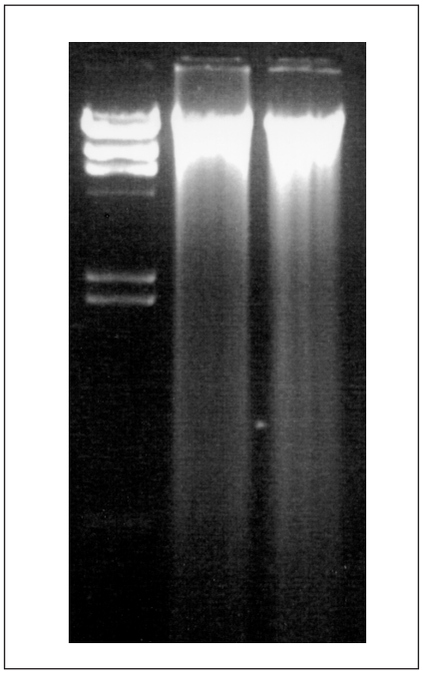

Figure 1. Representative gel electrophoresis of genomic DNA from two independent extractions (0.7% agarose gel) isolated from rumen contents, as described in Table 1. Lane M, Lambda DNA/*Hin*dIII molecular mass marker; lanes 1 and 2, genomic DNA (5 µL DNA/lane) used for PCR amplification.

We could also successfully amplify the 540-bp low-copy plant (Rubisco; Figure 3) as well as the bacterial (466 bp) control (Figure 4) using our procedure. However, we could not detect the Rubisco gene fragment while the bacterial control was visualized as a low-intensity band by the CTAB extraction alone (Figure 4). The high number of bacterial cells could account for the amplification of 16S rDNA from bacteria as compared to the inability to amplify the single-copy plant control (Rubisco) using the CTAB procedure. At least two different primer combinations were tried for amplifying rumen bacteria, which included bacterial *rec A* gene and primer sets designed for Gram-positive and recalcitrant bacteria (data not shown) described previously for characterization of different rumen bacteria (13).

To further test the CTAB procedure alone, we performed spiking experiments for EPSPS gene fragments with positive control (RoundUp Ready canola leaf DNA) and found residual inhibitors in the preparation. Here we were unable to reproducibly PCR-amplify for EPSPS gene fragments, as well as plant and bacterial controls using some of the extraction protocols mentioned earlier (glass bead extraction, classical lysis, Wizard, and DNeasy Plant kits), suggesting that either the DNA template was not good quality or inhibitors precluded PCR. Furthermore, the intensity of bands on gels with our procedure was consistently high, which circumvented the need for fine-tuning the PCR conditions for various reaction sets of transgene fragments.

Extraction of DNA from rumen contents has several applications and provides the means to address the microbial community dynamics, characterization, and shifts in molecular eco-

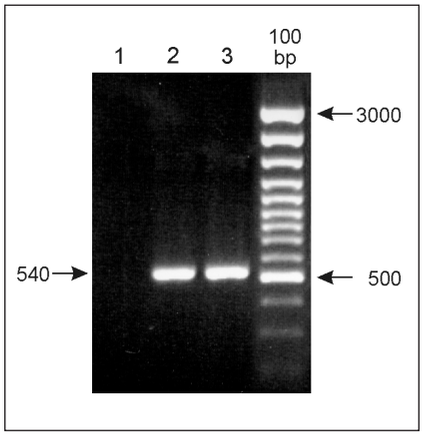

Figure 3. PCR amplification of a 540-bp *B. napus* Rubisco fragment using the CTAB extraction procedure (lane 1), the developed procedure (lane 2), and positive control (lane 3). A 100-bp DNA ladder is used as marker.

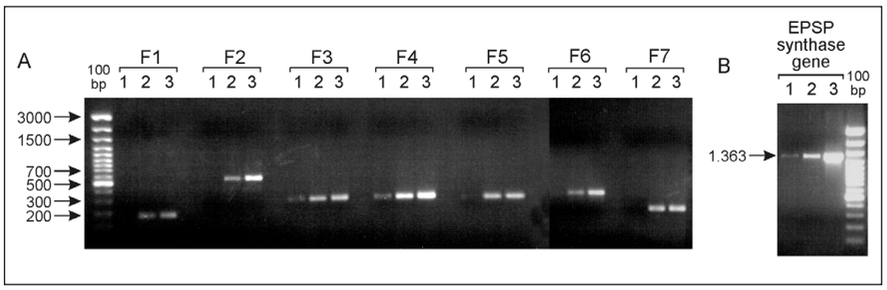

Figure 2. PCR amplification of EPSPS. (A) Gene fragments (F1–F7). (B) Whole gene (1.363 kb) using the CTAB extraction procedure (lane 1), the developed procedure (lane 2), and positive control (RoundUp Ready leaf, lane 3). A 100-bp DNA ladder plus (MBI Fermentas, Burlington, ON, Canada) is used as marker. Fragments 1–7 are detailed in Table 2. Twenty microliters of sample were loaded on a 1.5% agarose gel. Each fragment (F1–F7) is separated by a blank lane.

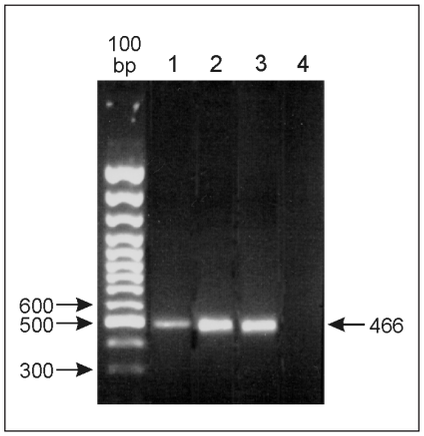

Figure 4. PCR amplification of a 466-bp bacterial 16S rDNA fragment using the CTAB extraction procedure (lane 1), the developed procedure (lane 2), and bacterial positive control (lane 3). Lane 4 is the negative control and has all the components of PCR except the DNA template. A 100-bp DNA ladder is used as marker.

logical populations in the rumen. An important prerequisite for such studies where a large number of samples are handled is the ability to isolate genomic DNA reproducibly. We have developed an improved procedure that is relatively fast, efficient, and consistently yields good amount of genomic DNA for further downstream processing (e.g., PCR, restriction digestion, etc.).

To our knowledge, this is the first described procedure that can detect both plant and microbial DNA from total rumen digesta. Several procedures were tried, and, ideally, no one procedure would provide both high yield and low proteins and inhibitor(s). Previous investigations have shown that polyvinylpyrrolidone and PEG bind phenolics and are highly effectively in their removal from plant tissue (6); however, these procedures did not work well for us, nor did attempts to clean up the DNA template from other procedures seem time efficient.

The developed procedure combines the high DNA yield from the CTAB method with the high DNA purity from the Qiagen column. Among other advantages, it is phenol-free, yields predominantly high-molecular-weight DNA, and has comparatively fewer steps than some of the other genomic DNA extraction protocols; thus, it reduces the chances of contamination. The DNeasy Plant kit is also available in 96-well format; thus, DNA upon CTAB extraction (described) can be adapted for high-throughput sample processing.

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Ranjana Sharma, S. Jacob John, Dana M. Damgaard, and Tim A. McAllister
Agriculture and Agri-Food Canada Research Centre
Lethbridge, Alberta, Canada