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Technical note

Incubation, pre-lysis and post-purification on the yield and purity of nucleic acids extracted from blood of domestic goats contained in FTA cards

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Abstract:

Molecular techniques require extractions of nucleic acids in adequate quantity and purity. This work describes a generalized linear model (GLM) of an adjusted factor with fixed effects on nucleic acid yield (ng/µl) and purity (A_{260}/A_{280} and A_{260}/A_{230}), for five methods of DNA extraction using FTA cards with goat (*Capra aegagrus hircus*) blood. Two commercial methods based on silica columns (Invitrogen and Macherey Nagel; MN), the chelating resin method (Chelex), the CTAB method and the phenol-chloroform-isoamyl alcohol (PCI) method were tested. Additionally, for MN, an incubation step with PBS (Phosphate Buffered Saline) buffer at high temperature prior to lysis and a purification step post extraction were

evaluated using a fixed-effect model of two factors with interaction. DNA concentrations and purity ratios were variable; the highest concentration was obtained with the MN kit (170.45 ng/µl), but with deficiencies in purity (0.32 of A_{260}/A_{230}, 0.34 of A_{260}/A_{280}). Despite this, all extraction methods generated PCR products with specific D-loop primers (mtDNA). The combined effect of the pre-incubation and post-purification stages yielded satisfactory purity values (1.89 for A_{260}/A_{230} and 1.65 for A_{260}/A_{280}), as well as concentration ratios (476.78 ng/µl) with low variability. In conclusion, the concentration and purity of DNA from blood samples is greatly improved when using a commercial kit in combination with pre-lysis incubation and post-extraction purification. These nucleic acids are suggested for use in potential molecular applications *a posteriori*.

Key words: DNA, Silica columns, CTAB, Phenol-chloroform, Chelex, PCR, Small ruminants.

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Blood is commonly used for clinical studies and research, as it is an important source of genomic DNA (gDNA) in its fraction of white blood cells[1]. For applications in zootechnics and veterinary medicines, it is collected and stored in FTA cards (Whatman® FTA® Cards), due to the convenience and long-term storage[2]. FTA cards are then subsequently used in a variety of genomic applications, such as molecular markers and next-generation sequencing[3].

There are different DNA extraction techniques, with different results and implications related to costs, inputs and risks for the user. DNA extractions by commercial kits through silica or cellulose columns are usually easy-to-use and moderately expensive (0.40 to 0.44 dollars)[4] procedures, require less time, amount of reagents and pose a lower health risk, compared to traditional methods that use salts (sodium chloride, guanidine salts), resins (chelex) and organic compounds (phenol, chloroform), allow the extraction of nucleic acids at a lower cost (0.27 dollars), however, they usually have longer execution times, limiting the number of samples that can be processed[5,6].

Phenol-chloroform-isoamyl alcohol (PCI) is a commonly used method, based on organic compounds. A large amount of gDNA[7], while the phenol inactivates any potential nuclease and contaminating proteins of the DNA can be obtained[8]. This process involves numerous steps with toxic and corrosive substances and prolonged incubations[6]. Chelex resin is a chelating agent that purifies compounds through the exchange of ions, generally involves

simple and fast procedures, does not include organic solvents and does not require multiple tube transfers, however, DNA is obtained in reduced quantity and quality[3]. The cetyltrimethylammonium bromide (CTAB) method has been widely used for DNA extraction in plant tissue, seeds[9,10], in animal tissues[5,11]. Just as the PCI method, it uses hazardous chemicals and requires numerous steps that increase its execution time, affecting its applicability on a large scale[5,12].

The main parameters analyzed after a nucleic acid extraction are purity, concentration and integrity. Purity and concentration are usually assessed by UV-VIS spectrophotometry and fluorometry. The absorbance profile measured by spectrophotometry allows the detection of contaminants such as proteins, salts and polysaccharides. On the other hand, agarose gel electrophoresis is a commonly used method to evaluate DNA integrity, either directly from DNA (striking band of DNA of high molecular weight) or by visualizing PCR products from the extracted nucleic acids[13].

The objective of the present study was to compare five methods of extraction from goat blood samples kept in FTA cards, according to the concentration and purity of the resulting DNA. In addition, in the most promising method of extraction, it was evaluated the effect of a pre-incubation with PBS before cell lysis and post-purification of the eluate with organic solvents.

Fifty-seven dairy goats (*Capra aegagrus hircus*) located in the Central, Caribbean, North, Chorotega and Central Pacific Regions of Costa Rica were sampled. The blood collection was carried out following the protocol of Berumen *et al*[14], placing approximately 200 µl of blood on a Whatman FTA® card (Flinders Technology Associates, UK). The cards were stored at room temperature (RT), in a cool (moisture-free) place, in the dark and inside airtight plastic bags.

Five extraction methods were used: two of them were PureLink™ (Invitrogen, USA) and NucleoSpin® Blood (Mackerey Nagel, Germany) commercial kits; which were carried out following the manufacturer's protocol. The DNA was resuspended in 100 ul of nuclease-free double deionized water. The third method used was that of CTAB by Lodhi *et al*, adapted[15], the fourth was by means of Chelex-100 resin (Bio Rad Laboratories, Inc, USA) and the last method was by means of phenol/chloroform/isoamyl-alcohol (PCI). Approximately one quarter of the circle of the FTA card was used in all extractions performed with the different methods. Additionally, prior to treatment with the lysis buffer (typical of each of the protocols evaluated), the effect of adding an additional incubation (pre-incubation) was tested in all extraction methods, with 200 µL of PBS buffer[16], for 1 h at 42 °C, stirring by inversion every 10 min. Once the incubation with PBS was finished, each of the extraction protocols was carried out. Three repetitions per individual were used, for a total of 174 repetitions.

In the extraction of DNA from Chelex resin, once the hour of incubation with PBS had finished, 500 µL of 10 % Chelex-100® resin was added and it was incubated at 70 °C with proteinase K (Thermo Scientific, USA) for 1 h. Subsequently, the samples were centrifuged at 14,000 rpm at 4 °C, an approximate volume of 70 µL of supernatant was isolated and transferred to a new tube. The sample was precipitated by adding 70 µL of 3M sodium acetate (NaOAc) (Ambion, USA) and 180 µL of 96 % ethanol (Sigma-Aldrich, Germany) and incubated for 20 min at -20 °C. Subsequently, the sample was centrifuged at 14,000 rpm for 15 min. The supernatant was discarded, and the resulting pellet was washed twice with 200 µL of 70 % ethanol, allowing it to dry completely at 42 ºC for 5 min using a Savant™ DNA SpeedVac™ concentrator (Thermo Scientific, USA). Finally, 50 µl of TE buffer was added and it was incubated at 37 °C for 30 min to facilitate resuspension.

After incubation with PBS, 750 µL of lysis buffer (20 mM Na-EDTA, Tris-HCl, pH 8.0 with HCl, 1.4 M NaCl, 2.0 % (m/v) PVP and 0.2 % (v/v) beta-mercaptoethanol) and 8 µL of proteinase K (20 mg/ml) (Thermo Scientific, USA) were added the sample and was incubated at 70 °C for 1 h. Subsequently, 750 µL of chloroform:octanol (24:1) was added, mixing by inversion. The sample was centrifuged at 13,000 rpm by 5 min at RT and 300 µL of supernatant was transferred to a new tube, adding one volume of cold isopropanol (-20 °C). An incubation was performed at -20 °C for 20 min and the tube was centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was discarded, and the sample was completely dried at 42 °C for 5 min with the use of a SpeedVac concentrator (Thermo Scientific, USA). The pellet was resuspended in 50 µL of TE buffer and incubated at 37 °C for 30 min.

In the extraction of DNA with PCI[17], after incubation with PBS, an incubation was performed at 70 °C with 600 µL of STES buffer (0.5 M NaCl, 0.2 M Tris-Hcl, 0.01 M EDTA and 0.1 % SDS) and 8 µL of proteinase K (20 mg/mL). Subsequently, 600 µL of PCI (phenol:chloroform:isoamyl alcohol 25:24:1) was added and it was centrifuged at 10,000 rpm for 10 min at RT. Subsequently, the aqueous phase was isolated and a mixture of chloroform/isoamyl alcohol (24:1) (USB Corporation, USA) was added in a 1:1 ratio. The tubes were centrifuged at 10,000 rpm for 1 min, the upper phase was isolated and 100 µL of 3M NaOAc (pH 5.2) and 750 µL of absolute ethanol (Sigma-Aldrich, Germany) were added. The sample was incubated for 20 min at -20 ºC and centrifuged at 14,000 rpm for 10 min at 4 ºC to recover nucleic acids. The supernatant and pellet of the precipitated DNA were removed, washed with 500 µL of 70 % ethanol, then centrifuged at 18,000 rpm for 2 min at RT, and the supernatant was decanted. The pellet was completely dried at 42 °C for 5 min in a thermal block. Once the alcohol evaporated, the sample was resuspended with 100 µL of nuclease-free double deionized water and incubated at 37 °C for 30 min.

All extracted genomic DNA samples were evaluated by electrophoretic mobilization in 1 % agarose gels (TBE buffer 0.5 %), at 80 V, 400 mA for 45 min. The purity (coefficients

A_{260}/A_{280} and A_{260}/A_{230}) and the concentration of the samples were obtained using a NanoDrop 2000™ UV-visible microvolume spectrophotometer (Thermo Scientific, USA).

Once all extractions were carried out, the method that yielded the highest amounts of recovered DNA (ng/µL) was selected, and an additional purification was carried out by phenol-chloroform[17] with an initial volume of 100 µL. Fifty microliters of phenol (pH 8.0) and 50 µL of chloroform:isoamyl alcohol (24:1) (USB Corporation, USA) were added. The sample was centrifuged at 12,000 rpm for 15 min at RT. The aqueous phase was transferred to another tube. One tenth of volume of 3M NaOAc (pH 5.2) (Ambion, USA) and 2.5 volumes of cold absolute ethanol (Sigma-Aldrich, Germany) were added and mixed by inversion. The sample was incubated at -20 °C for 20 min and centrifuged for 10 min at 12,000 rpm. The supernatant was removed, and the sample was resuspended in 70 µL of nuclease-free double deionized water.

The integrity of the total DNA and the possible effect of inhibition by trace contaminants of the extraction method were evaluated by means of an end-time PCR (final volume: 20 µL), in triplicate and composed of 1X of PCR Master Mix (Thermo Scientific, USA), 0.8 µM of each primer and 1 µL total DNA (but not with equivalent concentrations). The selection of the samples was random. The D-loop region of the caprine mitochondrial DNA (mtDNA) was amplified using the primers DAF (5´TTCTTCAGGGCCATCTCATC3´) and DGR (3´GCGGATGCATGGTGAAAT5´)[18], synthesized by MACROGEN (Korea). The PCR was performed under the following cycling conditions: 94 °C for 3 min (initial denaturation), 35 cycles of: 94 °C for 30 sec (denaturation), 55 °C for 30 sec (annealing), 72 °C for 45 sec (extension) and finally 72 °C for 10 min as final extension. PCR products were resolved by 1.5 % agarose gel electrophoresis (TBE 1X), 80 V, 400 mA for 60 min in TBE 1X buffer solution (pH 8.0, Invitrogen, USA).

The statistical analysis was performed in PROC GLM of SAS. A one-way fixed-effect model for the comparison of the five methods and a two-way fixed-effect model with interaction for the evaluation of incubation and purification by a Levene test were fitted, looking for evidence of homogeneity of variance. Multiple comparison tests between treatments were performed using the Tukey procedure. In all cases, the values of the statistic associated with $\alpha<0.05$ were considered significant.

The results obtained in this study showed that the five extraction protocols performed (Invitrogen, MN, Chelex, CTAB and PCI) differed in purity (A_{260}/A_{230} and A_{260}/A_{280}) and amount of DNA (ng/µL). When assessing the concentration by absorbance at 260 nm, the highest concentrations and dispersion measurements were obtained with the MN (170.45 ng/µL ± 74.82) and Invitrogen (29.70 ng/µL ± 25.31) methods. Regarding the protocols with organic solvents, the CTAB method yielded the highest values of extracted DNA, followed

by Chelex and finally that of PCI (10.35 ng/µL, 2.96 ng/µL and 2.23 ng/µL, respectively) (Table 1).

Table 1: Measurement of ratios A_{260}/A_{230}, A_{260}/A_{280} and quantification of DNA (ng/µL) obtained by Nanodrop spectrophotometry from goat blood samples subjected to five nucleic acid isolation methods

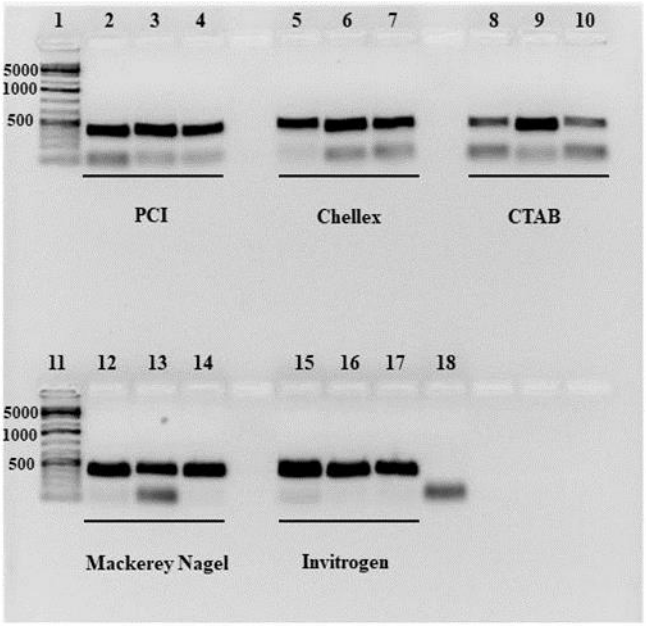
		A_{260}/A_{230}		A_{260}/A_{280}		DNA (ng/µl)	
Method	**n**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Invitrogen	9	0.38[a]	0.09	0.45[a]	0.22	29.70[b]	25.31
CTAB	10	0.14[b]	0.02	0.74[a]	0.09	10.35[c]	8.30
PCI	17	0.37[a]	0.22	1.23[b]	0.53	2.23[d]	1.39
Chelex	10	0.26[a]	0.15	1.17[c]	0.69	2.96[e]	2.04
MN	36	0.32[a]	0.02	0.34[a]	0.02	170.45[a]	74.82

n= number of samples analyzed; SD= standard deviation; CTAB= hexadecyltrimethylammonium bromide; PCI= phenol-chloroform-isoamyl alcohol; MN= Mackerey Nagel.

[abcde] Different letters correspond to significant differences ($\alpha<0.05$). Different letters by column correspond to significant differences with $\alpha<0.05$.

Regarding contamination due to proteins (A_{260}/A_{280}), no significant differences were found between the commercial methods, while in the organic solvent protocols, statistical differences were detected between the three, with PCI showing the best yield, followed by Chelex and CTAB (1.23, 1.17 and 0.74), respectively. The purity values associated with the coefficients of A_{260}/A_{280} were below 1.8 in all extractions, with the commercial methods showing the lowest values, MN (0.34) and Invitrogen (0.45). In addition, all A_{260}/A_{230} ratios showed values well below 1.5. However, the lowest values of A_{260}/A_{230} corresponded to DNAs extracted by CTAB (0.14), followed by the Chelex method (0.26) (Table 1). Despite this, the DNA concentration and purity coefficients did not affect the obtaining of partial amplifications of the D-loop region from a PCR (294 bp, genbank accessions: MW514310 and MW514311), since amplicons were generated for all the samples analyzed, regardless of the extraction method used (Figure 1).

Figure 1: Partial amplification of the mitochondrial D-loop gene by PCR of the five different methods of DNA extraction from *Capra aegagrus hircus* blood



Lanes 1 and 11: molecular size marker (1Kb Thermo Fisher Scientific). 2-4: amplifications of samples extracted by the PCI method. 5-7: Chellex method. 8-10: CTAB method. 12-14: MN method. 15-17: Invitrogen method. 18: negative control (reaction mixture without DNA).

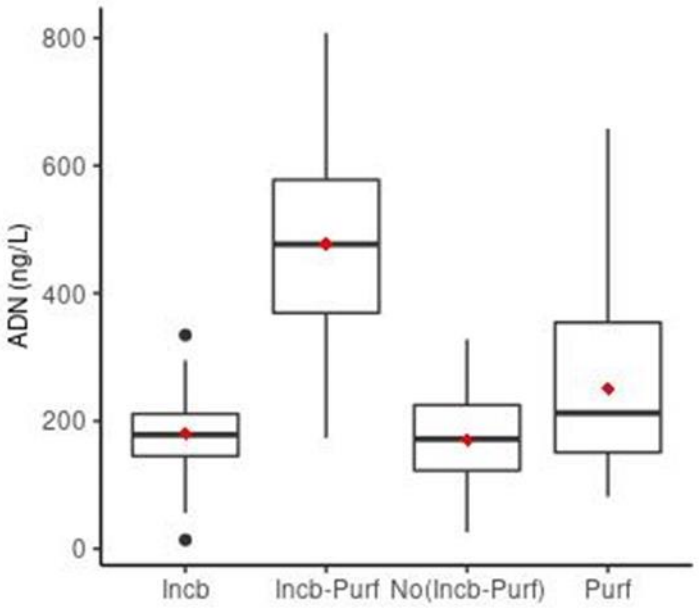
Based on the promising yield of the DNA concentration obtained by the MN method, and the execution of additional steps as an assumption of an improvement in yield and quality of nucleic acids, it was observed that incubation-purification step significantly increased the DNA concentration (476.78 ng/μL), as well as the purity of the extractions performed (Table 2, Figure 2). The magnitude of the difference between the means of the concentration of nucleic acids, from the method without modification (170.45 ng/μL) with respect to incubation-purification was 306.33 ng/μL, while when it was only purified, the increase was 80.63 ng/μL, and finally of 10.53 ng/μL when it was only incubated. On the other hand, the purity reflected in the A_{260}/A_{230} and A_{260}/A_{280} ratios for the MN method showed an improvement (1.89 and 1.65, respectively) in terms of the values obtained from no incubation – no purification (0.32 and 0.34, respectively).

Table 2: Effect of preincubation with PBS prior to cell lysis and post-purification of the eluate with phenol:chloroform on DNA extraction with the MN method from blood contained in FTA cards

		A_{260}/A_{230}		A_{260}/A_{280}		DNA (ng/µl)	
	n	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Incubation-purification	36	1.89[a]	0.21	1.65[a]	0.20	476.78[a]	164.37
Incubation	36	0.33[b]	0.03	0.35[b]	0.03	180.98[b]	62.40
Purification	36	2.02[c]	0.16	1.66[a]	0.13	251.08[c]	126.47
No incubation-No purification	36	0.32[d]	0.02	0.34[c]	0.02	170.45[d]	74.82

n= number of samples analyzed; SD= standard deviation.
[abcde] Different letters correspond to significant differences ($\alpha<0.05$).

Figure 2: Distribution of nucleic acid concentrations (ng/extractedµL) according to the modifications made to the MN protocol



Incb= incubation only; Incb-Purf= incubation and purification combined as modifications in the method; No(Incb-Purf)= no incubation and no purification; Purf= purification only.
Horizontal lines in the boxes represent the median, vertical lines represent the upper and lower limits, rhombuses inside the boxes represent the mean and circles outside the boxes represent outliers below or above the mean.

Preliminary results regarding the concentration of DNA obtained in the methods without a pre-incubation step (2 to 170 ng/µL) could indicate that the white blood cells retained in the FTA card were not released from the solid support or that the optimal digestion of the cell membrane did not occur. Therefore, the addition of a purification step together with a pre-incubation step in the MN method generated significant increases in extraction yields in terms

of concentration and purity. Other authors, as in this research, reported little variability and small deviations in DNA concentration when using a commercial kit[19].

The results of this work showed that all the extractions conducted had A_{260}/A_{280} purity values below the recommended one (1.8 to 2.0)[20]. However, despite these non-optimal values (residual proteins and trace contaminants) obtained in the five DNA extraction methods, no inhibition of enzymatic reactions by PCR was observed. More sensitive molecular applications such as Sanger sequencing, PCR-RFLP genotyping, microarrays or NGS could be affected by the presence of salts, organic solvents, EDTA, nucleases and contaminating proteins that are carried in the DNAs[21-24]. The low values obtained with the A_{260}/A_{230} coefficient in samples extracted from commercial kits could be due to compounds with absorption at 230 nm acting as trace contaminants, which include chaotropic salts such as guanidine thiocyanate[25], EDTA, non-ionic detergents such as Triton™ X-100 and Tween®, proteins, amino acids[20,25], phenol, polysaccharides and other floating solid particles such as silica fibers. In the case of DNA extraction based on protocols with organic solvents (PCI and CTAB), values of A_{260}/A_{230} below what was expected could be due to factors such as errors when separating the aqueous phase from the interphase or the carrying of contaminants such as phenol, chloroform in the successive steps in the extraction. In the case of ion exchange resins (Chelex), the lower values could be due to protein contamination[26].

The results demonstrate the limitations of extracting DNA from FTA cards that retain goat blood samples, for their use in later genomic applications. However, positive results in the isolation and purification of total DNA using the commercial NucleoSpin® Blood kit of Mackerey Nagel (MN) for molecular analysis in small ruminants, with additional steps that ensure the quality and purity of nucleic acids for use in techniques with high concentration and DNA integrity requirements.

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Conflicts of interest

The authors declare no conflict of interest.

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