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Full Length Research Paper

Influence of EDTA and magnesium on DNA extraction from blood samples and specificity of polymerase chain reaction

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This study consisting of two trails conducted to examine the impact of initial EDTA level added to blood samples on quantity and quality of genomic DNA isolated from avian fresh blood and the influence of initial EDTA level with various levels of $MgCl_2$ added to polymerase chain reaction (PCR) final volume on amplification pattern. EDTA level added to collected blood samples had no significant impact on quantity as well as quality of extracted genomic DNA. However, higher levels of EDTA increased the OD_{260} and enhanced the OD_{260}/OD_{280} ratio beyond 1.8-1.9 which is broadly accepted as an indicator of high quality DNA. To avoid such an error, EDTA level in initial blood sample must not exceed 9 µg/µl blood. The initial amount of EDTA has no influence on PCR process if a valid DNA isolation protocol is used. Addition of $MgCl_2$ from 1.0 to 2.4 µl in a final volume of 25 µl could support the amplification properly. Low levels of $MgCl_2$ results in incomplete amplification but levels higher than 2.4 µl impedes the amplification by negative interference to the whole reactions.

Key words: EDTA, DNA extraction, Mg^{2+} concentration, PCR.

INTRODUCTION

EDTA (ethylene diamine tetra acetic acid) is a novel molecule to chelate or complex 2 and 3 valent kations such as Mg^{2+} in 1:1 metal-to-EDTA complexes (Figure 1). Because of its strong complexing ability for most metal ions, it is widely used in the food industry (Scott, 2006), in detergents (Oviedo and Rodriguer, 2003) and in molecular biology as an anticoagulant agent and several other applications (Nicole et al., 2004). The choice of a blood anticoagulant is of substantial consequence in molecular genetic research as it has been reported that it may interfere results or even barricade whole process. Beutler et al. (1990) revealed heparin is an unsuitable anticoagulant for many molecular researches since it could inhibit PCR amplification. The confirmation of this results by Jung et al. (1997) and Farnert et al. (1999) created suspicion on other anticoagulants ensuing classical examination of other commonly used agents including EDTA (Nicole et al., 2004).

Magnesium concentration has a significant impact on the efficiency and specificity of PCR reactions in many ways. In addition to its direct influence on Taq polymerase activity and fidelity, magnesium also affects the T_m (annealing temperature) of the various hybrids that form during cycling, including primer-template, template-template, and primer-primer. It is also important that free nucleotides and any EDTA present in the reaction influence the effective magnesium concentration. There is a well known interaction between Mg^{2+} and EDTA in the course of PCR amplification. The presence of EDTA in TE (10 mM Tris, pH 8.0,1 mM EDTA) buffer (up to 1 mM), sometimes used to dissolve genomic DNA, can complex magnesium and reduces its effective concentration. In this case, the magnesium concentration in the reaction should be increased to compensate for this eff-

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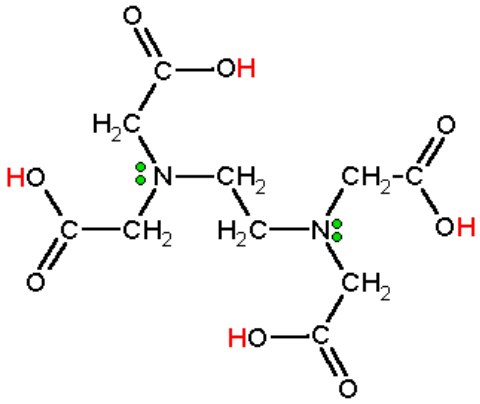

Figure 1. EDTA is a polyprotic acid containing four carboxylic acid groups and two amine groups with lone pair electrons.

ect. The only strategy suggested in the literature to optimize the Mg^{2+} ion requirement is titration of its concentration. The optimal concentration for a particular application should be determined empirically by performing a series of RAPD reactions at different Mg^{2+} concentrations (Rafalski, 1997).

The current study consisting of two trails has two distinct objective as; firstly, to investigate the impact of initial EDTA level added to blood samples on quantity and quality of DNA isolation and secondly, to examine the influence of a wide range of Mg^{2+} concentration in PCR final volume containing DNA samples extracted from blood samples collected in low medium and high levels of EDTA.

MATERILS AND METHODS

Whole blood samples were collected from commercial layer chicken in vials containing 3, 5, 7, 9 and 11 µg EDTA/µl blood (22-25 replicate for each treatment). DNA was extracted using aliquots containing 25 µl whole blood, 500 µl lysis buffer [10 mM Tris-HCl (pH 8.0), 100 mM Nacl, 1 mM EDTA (pH 8.0), .5% SDS] and 0.6 U proteinase K. The mixture was incubated for 90 min at 56°C and then extracted twice with equal volumes of phenol and chloroform. The DNA was precipitated in 100% ethyl alcohol, rinsed in 70% ethyl alcohol, and dissolved in TE (10 mM Tris, pH 8.0,1 mM EDTA). Total extracted DNA was measured at OD_{280} and OD_{260}. OD_{260}/OD_{280} was calculated, extraction efficiency (µg/ µl blood), assay scores for ease of removing the top aqueous phase after first and second spinning was recorded. The data was analyzed using General linear Models of SAS® software (SAS, 1998).

The influence of Mg^{2+} ion concentration on polymerase chain reactions (PCR) was assessed using the genomic DNA samples isolated. PCR reactions were carried out in a final volume of 25 µl containing 2.5 mM Tris-Hcl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, 250 µM of each dNTP, 1.0 µM primer, 1 U of AmpliTaq® DNA polymerase, and 75 ng DNA template. The Thermocycler used was programmed for 10 min initial denaturation at 96°C, then 42 cycles of 30 s at 96°C, 30 s at 35°C, 45 s at 72°C, and 5 min final extension at 72°C. An aliquot of 15 µl was used for electrophoresis on 1.4% agarose gel at a constant voltage of 80 V and visualized by illumination with ultraviolet light after staining with ethidium bromide.

RESULTS

Addition of EDTA in the range of 3 - 9 µg/µl blood had no significant impact on quality as well as quantity of the DNA extracted. EDTA beyond 11 µg/µl blood enhanced the OD_{260} and OD_{260}/OD_{280} significantly. In spite of no significant influence of EDTA volume on total extracted DNA, increasing EDTA levels up to 9 µg/µl resulted in a clear declining trend for total extracted DNA as well as extraction efficiency. However, using 11 µg EDTA/µl blood led to increase in isolated DNA. The objectively assigned scores for ease of removing top aqueous phase after the first spinning in DNA extraction process, was significantly affected by EDTA as the higher levels of EDTA influenced the separation process negatively (Table 1).

The DNA extracted from blood samples collected in 2, 7 and 13 µg EDTA/µl blood were used for PCR. From each DNA sample, 16 PCR experiments were conducted with addition of $MgCl_2$ ranging from 0.2 to 3.2 µl with 0.2 µl incremental interval. There were clear patterns of bands showing successful amplification for all PCR products irrespective of initial EDTA levels in the blood samples used (Figure 1; A, B and C). In contrast, addition of 5.0 mM $MgCl_2$ in the range of 0.2 to 3.2 µl to final volume of 25 µl PCR solution showed a significant effect on band patterns within each PCR sets. The band patterns obtained from gel electrophoresis of amplified products of all three series depicts that, presence of $MgCl_2$ in the range of 1.0 to 2.4 µl $MgCl_2$ in the samples result in efficient amplification in terms of band number and clarity. The $MgCl_2$ levels lower than 1.0 µl were found to cause deficient amplification. Irrespective of initial EDTA level in blood sample, $MgCl_2$ levels of 0.2 to 0.8 µl amplified with less number of non distinct bands. In the contrary, $MgCl_2$ volume beyond 2.4 µl blocked the amplification or caused few irregular bands.

DISCUSSION

EDTA as an anticoagulation agent is widely used to salt away blood sample from agglutination through impeding further enzymatic reactions. There is no absolute recommendation for the amount of the EDTA added to blood sample to ensure agglutination process with no negative impact. However, a survey of the literature shows that it is added to blood samples at 5-9 µg/µl blood. All DNA isolation procedures as well as the protocol used in this study (Khosravinia, 2005) did not result in EDTA contamination of the extracted DNA. The traces of EDTA present in the blood sample might be ent-

Table 1. Effect of various amounts of EDTA on variables pertaining to DNA quality and extraction efficiency.

Variables	EDTA (µg/µl blood)				
	3	5	7	9	11
OD_{280}	0.06±0.00a	0.05±0.00a	0.06±0.00a	0.06±0.00a	0.07±0.01a
OD_{260}	0.10±0.01b	0.10±0.00b	0.10±0.01b	0.11±0.01ab	0.13±0.02a
260/280	1.78±0.01bc	1.75±0.01c	1.80±0.02c	1.80±0.03b	1.90±0.08a
Total extracted DNA (µg)	97.60± 4.30a	80.00±2.64a	78.50±6.30a	79.00±7.00a	87.00±18.20a
Extraction efficiency (µg DNA/µl blood)	3.90±0.15a	3.60± 0.08a	3.50±0.21a	3.50±0.23a	3.70±0.62a

Values are mean ± SE
[a-c]Means with different superscript differ significantly (P<0.05).

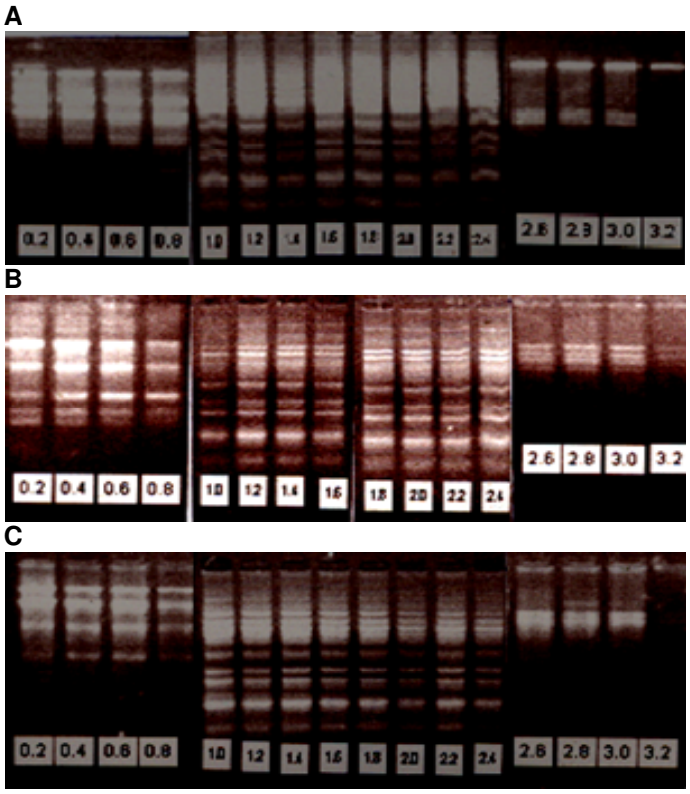

Figure 2. Amplification patterns for DNA samples initially mixed with various volumes of EDTA [2, 7 and 13 µg EDTA/µl blood for parts A, B and C respectively. The numbers demonstrated under each lane is the volume of $MgCl_2$ added to the PCR reaction mixture.

irely washed out following double ether-chloroform washing of digested blood by the lysis buffer. Therefore, the results indicating no significant impact of initial EDTA level (up to 9 µg/µl blood) on quantity as well as quality of isolated genomic DNA are in fair agreement with theoretical expectations. The higher levels of EDTA (beyond 11 µg /µl blood) increased the OD_{260} which in turn could be misleading by enhancing the OD_{260}/OD_{280} ratio beyond 1.8-1.9, which is broadly accepted as an indicator of high quality DNA (Glasel, 1995; Sambrook and Russell, 2001). There is no explanation in the literature for this negative impact of higher levels of EDTA on quality of the DNA isolated. To avoid any doubt on the DNA isolation process, EDTA level in initial blood sample must be kept below 9 µg/µl blood.

One of the most significant components PCR amplification are the Mg^{2+} and whatever is used to stabilize the enzyme. In theory Mg^{2+} should be optimized for each primer/template combination. In practice this is seldom done. In addition to Mg^{2+} ions for the template DNA, the

nucleotides (dNTPs) and the primers, Tag DNA polymerase also requires free Mg^{2+} ions (Dieffenbach et al., 1995). Mg^{2+} concentration has an influence on primer annealing, the melting temperature of the PCR product and product specificity. The *Taq* polymerase, Stoffel fragment of *Taq* polymerase and other polymerases have different requirements for Mg^{2+} ions. On the other hand, both EDTA and dNTPs can strongly chelate with Mg^{2+} lowering its concentration, altering and/or preventing the amplification (OTA, 1978; McPherson et al., 1991).

From our results, the initial amount of EDTA added to blood samples as anticoagulant has no influence on the PCR process if a valid DNA isolation protocol is used. Nonetheless, problems may arise from the presence of up to 1 mM of EDTA in TE buffer, in which genomic DNA is frequently dissolved. EDTA complexes magnesium and reduces the effective concentration of available Mg^{2+}. In the current study, the composition of TE buffer used to dissolve and dilute DNA was 10 mM Tris, pH 8.0,1 mM EDTA. If significant volumes of EDTA-containing DNA solutions are added, the magnesium concentration in the reaction should be increased appropriately. EDTA levels in the DNA extract should never be greater than 0.1 mM. Also, it is not necessary to use a firmly constant level of $MgCl_2$ for PCR amplifications. Figure 2 obviously depicts that addition of $MgCl_2$ in the wide range of 1.0 to 2.4 µl in a final volume of 25 µl could support the amplification properly. Many 10X commercial buffers have Mg^{2+} of 10 mM; this is often too low for optimal amplification. Figure 2 shows that low levels of $MgCl_2$ results in few band or incomplete amplification but extra levels of $MgCl_2$ prevent the amplification. These results are in agreement with many other relevant reports. McPherson et al. (1995) showed that low concentrations of Mg^{2+} produce few bands. Very low Mg^{2+} ions result in a low yield of PCR product, and very high amounts increase the yield of non-specific products and promote misincorporation of nucleotides. Lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. The recommended range of $MgCl_2$ concentration is 1-4 mM, under the standard reaction conditions specified (Gause and Adamovicz, 1994; FLS, 2006). Vernon et al. (2001) revealed that higher Mg^{2+} generally results in higher yield, but if too high amplification of non-specific products will often result.

It has been frequently recommended that the optimal concentration for a PCR reaction component should be determined empirically by performing a series of reactions at different Mg^{2+} concentrations (Rafalski, 1997; FLS, 2006). The concentration of free Mg^{2+} ions should exceed that of the dNTPs concentration by 0.5-2.5 mM. For each new primer pair, it is generally worthwhile to determine the magnesium concentration in 0.5-mM steps over the range of 0.5 mM to 5 mM (Gibson, 1996). If in an experiment a new set of primers are utilized that others have described, the published Mg^{2+}, Mg^{2+}/dNTP must compare with what it is used. If brand new primer pair is used, the Mg^{2+} must be brought up in multiple trials in 0.2 mM increments as has been done in the current study.

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