


Protocol

# Optimization of DNA Extraction from Individual Sand Flies for PCR Amplification

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**Abstract:** Numerous protocols have been published for extracting DNA from phlebotomines. Nevertheless, their small size is generally an issue in terms of yield, efficiency, and purity, for large-scale individual sand fly DNA extractions when using traditional methods. Even though this can be circumvented with commercial kits, these are generally cost-prohibitive for developing countries. We encountered these limitations when analyzing field-collected *Lutzomyia* spp. by polymerase chain reaction (PCR) and, for this reason, we evaluated various modifications on a previously published protocol, the most significant of which was a different lysis buffer that contained Ca<sup>2+</sup> (buffer TESCa). This ion protects proteinase K against autolysis, increases its thermal stability, and could have a regulatory function for its substrate-binding site. Individual sand fly DNA extraction success was confirmed by amplification reactions using internal control primers that amplify a fragment of the *cacophony* gene. To the best of our knowledge, this is the first time a lysis buffer containing Ca<sup>2+</sup> has been reported for the extraction of DNA from sand flies.

**Keywords:** sand fly; DNA extraction; calcium; PCR; lysis buffer; *Lutzomyia*

## 1. Introduction

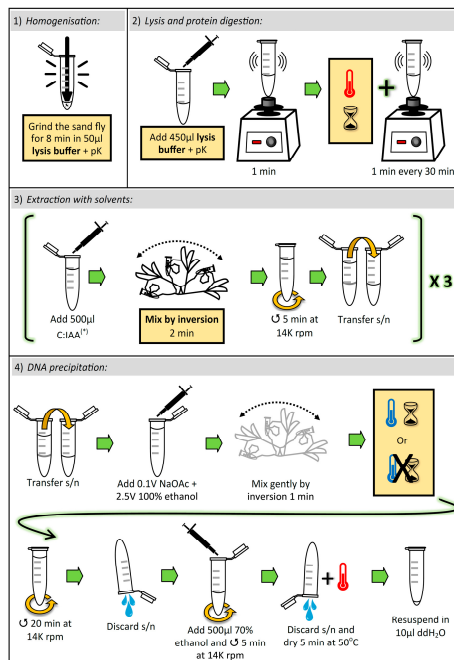
Various protocols have been published for the extraction of DNA from phlebotomines, including methods that eliminate DNA-associated proteins by using detergents and salts [1–3], or with proteinase K and detergents [4], and others that also add extraction steps with phenol-chloroform and precipitation with alcohol [5,6]; commercial DNA extraction kits [7,8]; and the use of Chelex-100 resin [9,10]. Nevertheless, the small size of the sand flies (around 3 mm long) can be an issue,

especially in studies that require analysis on an individual basis, such as parasite infection, variability, and population genetics. In particular, these large-scale individual DNA extractions using traditional methods usually yield poor results in terms of efficiency, quantity and purity, which in turn affect PCR success and DNA conservation. This can be circumvented by the use of commercial kits [3], particularly in terms of purity. Notwithstanding, in developing countries, an extensive use of the latter is cost-prohibitive and, consequently, traditional protocols become indispensable.

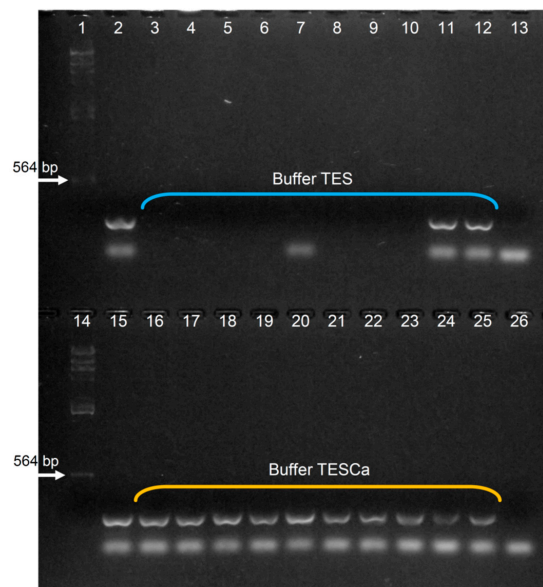
In our studies we were interested in detecting parasite infection in *Lutzomyia* spp. sand flies by PCR amplification [11]. Nevertheless, as parasite DNA is not necessarily expected, we first needed to confirm that the DNA extraction had been successful, to ensure that negative results were not due to a poor extraction. In our studies this was done with internal control primers that amplify a fragment of the constitutive *Lutzomyia cacophony* gene [12,13]. We previously used a traditional DNA extraction method with pools of 5 and 10 field-captured *L. longipalpis* sand flies. The protocol, which we here refer to as pAC, uses detergent (SDS), proteinase K and phenol-chloroform extraction ([14] and Acardi personal communication). The DNA extracted from these pools of sand flies yielded the expected results consistently when subjected to the internal control PCR. Nevertheless, when we used pAC to process individual sand flies, we found that amplification was poor and inconsistent (i.e., internal control PCR results were variable). For this reason, we decided to evaluate various modifications and, as this method eliminates DNA-associated proteins with proteinase K, we focused on this first crucial step. A number of researchers have reported that calcium ions activate proteinase K and that they are required for the enzymatic action of the protein [15–17]. Even though another study disputes the reduction in proteolytic activity of proteinase K when calcium is absent, it concedes that calcium-free proteinase K precipitates irreversibly in the presence of EDTA, leading to a reduced effective concentration [18]. Because of this, we decided to add calcium to the lysis buffer (here referred to as buffer TESCa). DNA extracted from individual sand flies using this and other variations we implemented, produced consistent and successful results in the amplification reactions. To the best of our knowledge, this is the first time a lysis buffer containing  $\text{Ca}^{2+}$  has been reported for the extraction of DNA from sand flies.

## 2. Experimental Design

In the following scheme (Scheme 1) we show the main variations that were assayed to optimize DNA extractions from one sand fly (for details see “Section 5”, Appendices B–E, and Table 1):



**Scheme 1.** This scheme summarizes the different variations that were analyzed for the DNA extraction protocol. The assayed modifications are indicated by yellow boxes and bold text (for details see “Section 5”, Appendices B–E, and Table 1). Figures are schematic (i.e., not an exact representation) and are not drawn to scale. (\*) In the third extraction with solvents, add 700 µL C:IAA. min, minutes; hs, hours; pK, proteinase K; C:IAA, chloroform:isoamyl alcohol (24:1); ∪, centrifuge; s/n, supernatant; V, volumes; 14K rpm, 14,000 revolutions per minute; ddH<sub>2</sub>O, double-distilled water.



**Figure 1.** Analysis of samples processed with buffers TES and TESCa. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R) and were diluted (1:5). 1: Molecular weight (MW) (Lambda/HindIII); 2 and 15: positive control; 3–12: lysis buffer TES; 16–25: lysis buffer TESCa; 13 and 26: negative control.

**Table 1.** Comparison of the different variations that were assayed for the DNA extraction protocol (for details see “Section 5” and Appendices B–E). Conditions that improved results are highlighted in **bold**. pK: proteinase K; C:IAA: chloroform:isoamyl alcohol; RT: room temperature; ON: overnight; ddH<sub>2</sub>O: double-distilled water.

Step	Variation A <sup>1</sup>	Variation B <sup>2</sup>	Variation C <sup>3</sup>	Variation D <sup>4</sup>
Homogenization	Grind with micropestle for 8 min in 50 µL buffer.	Grind with micropestle for 8 min in 50 µL buffer.	Grind with micropestle for 8 min in 50 µL buffer.	Grind with micropestle for 8 min in 50 µL buffer.
Lysis and protein digestion	Buffer pAC; Incubation with pK at 58 °C for 0.5, 2, 3, 4 and 8 h.	Buffer pAC; Incubation with pK at 58 °C for 8 h.	Buffers pAC, TES and TESCa; <b>Incubation with pK at 50 °C</b> for 8 h.	Buffer TESCa <b>Incubation with pK at 50 °C</b> for 1, 2, 3, 4 and 8 h.
Extraction with solvents	Gentle mixing by inversion with C:IAA.	Gentle and <b>vigorous mixing by inversion with C:IAA.</b>	Vigorous mixing by inversion with C:IAA.	Vigorous mixing by inversion with C:IAA.
DNA precipitation	Incubation with alcohol: ON at –20 °C, and <b>no incubation</b>	Incubation with alcohol at –20 °C <sup>5</sup>	Incubation with alcohol at –20 °C <sup>5</sup>	Incubation with alcohol: ON at –20 °C and <b>no incubation</b>
Final resuspension	20 µL ddH <sub>2</sub> O	20 µL ddH <sub>2</sub> O	<b>10 µL ddH<sub>2</sub>O</b>	10 µL ddH <sub>2</sub> O

<sup>1</sup> See Figure A1; <sup>2</sup> See Figure A2; <sup>3</sup> See Figures 1 and A3; <sup>4</sup> See Figure A4; <sup>5</sup> The protocol was paused in this step due to the length of the previous stages.

### 3. Final Procedure

See also Scheme 2 in Section 5.2.

#### 3.1. Lysis and Elimination of Proteins. Time for Completion: ~8 h, 8 min

##### 3.1.1. Homogenization of Sand Fly

1. Aliquot sufficient volume of buffer TESCa (30 mM Tris-HCl pH 8; 10 mM EDTA; 1% SDS, 5 mM CaCl<sub>2</sub>; see “Section 4.3”), i.e., 500 µL per sample, and add proteinase K (pK) (to the aliquot) to a final concentration of (0.42 µg/µL).
2. Place one adult sand fly in a 1.5 mL microcentrifuge tube, and add 50 µL of buffer TESCa + pK.
3. **▲ CRITICAL STEP:** Grind the sand fly thoroughly for 8 min with a Teflon micropestle. To avoid cross-contamination between samples, the micropestle must be cleaned and autoclaved after each grinding (i.e., one micropestle per sample).

##### 3.1.2. Cell Lysis, and Protein Denaturation and Digestion

4. **▲ CRITICAL STEP:** Add 450 µL buffer TESCa + pK (to a final volume of 500 µL), vortex for 1 min, and incubate at 50 °C for 8 h, vortexing for 1 min every 30 min.

#### 3.2. Extraction with Solvents. Time for Completion: ~25 min

##### 3.2.1. First Extraction

5. **▲ CRITICAL STEP:** Add 500 µL chloroform:isoamyl alcohol (C:IAA) (24:1 *v/v*) and mix vigorously by inversion for 2 min. Immediately centrifuge at 14,000 rpm for 5 min. Transfer the supernatant (~480 µL) to a new 1.5 mL microcentrifuge tube.

##### 3.2.2. Second Extraction

6. **▲ CRITICAL STEP:** Add 500 µL C:IAA (24:1 *v/v*) and mix vigorously by inversion for 2 min. Immediately centrifuge at 14,000 rpm for 5 min. Transfer the supernatant (~460 µL) to a new 1.5 mL microcentrifuge tube.

### 3.2.3. Third Extraction

7. **▲ CRITICAL STEP:** Add 700  $\mu\text{L}$  C:IAA (24:1 *v/v*) and mix vigorously by inversion for 2 min. Immediately centrifuge at 14,000 rpm for 5 min. Transfer the supernatant ( $\sim 400 \mu\text{L}$ ) to a new 1.5 mL microcentrifuge tube.

### 3.3. DNA Precipitation. Time for Completion: $\sim 35$ min

#### 3.3.1. Addition of Salt and Alcohol

8. Add 0.1 volumes ( $\sim 40 \mu\text{L}$ ) 3 M Sodium Acetate (NaOAc) pH 5.2 and 2.5 volumes ( $\sim 1 \text{ mL}$ ) 100% ethanol, and gently mix by inversion for 1 min.

**Ⓜ PAUSE STEP and OPTIONAL STEP:** We found that after adding NaOAc and ethanol, results improved when the sample was immediately centrifuged (i.e., was not incubated at all). Nevertheless, due to the length of the previous stages ( $\sim 9$  h), the protocol can be paused here and the sample stored overnight (ON) at  $-20^\circ\text{C}$ .

#### 3.3.2. Centrifugation

9. Centrifuge at 14,000 rpm for 20 min and discard the supernatant by inversion.
10. Add 500  $\mu\text{L}$  70% ethanol and centrifuge at 14,000 rpm for 5 min. Discard the supernatant by inversion and dry the pellet at  $50^\circ\text{C}$  for 5 min. Resuspend the pellet in 10  $\mu\text{L}$  double-distilled water.

## 4. Materials, Equipment, and Reagents Setup

### 4.1. Materials

- TRIS buffer ( $\text{NH}_2(\text{CH}_2\text{OH})_3$ , 121.14 g/mol) (Anedra, Tigre, Argentina; Cat. no.: AN00915709)
- Hydrochloric acid (HCl, 36.46 g/mol) (Biopack, Buenos Aires, Argentina; Cat. no.: 9632.08)
- Sodium Dodecyl Sulfate (SDS,  $\text{C}_{12}\text{H}_{25}\text{NaO}_4\text{S}$ , 288.38 g/mol) (Anedra, Tigre, Argentina; Cat. no.: AN219483180)
- EDTA ( $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$ , 292.24 g/mol) (Anedra, Tigre, Argentina; Cat. no.: AN00605609)
- Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 147 g/mol) (Anedra, Tigre, Argentina; Cat. No.: AN6456)
- Proteinase K (Fermentas-Thermo Fisher Scientific, Waltham, MA, USA; Cat. No.: #EO0491)
- Double-distilled water ( $\text{ddH}_2\text{O}$ )
- Chloroform ( $\text{CHCl}_3$ , 119.38 g/mol) (Cicarelli Laboratorios, San Lorenzo, Argentina; Cat. no.: 1116110)
- Isoamyl alcohol (Anedra, Tigre, Argentina; Cat. no.: AN00659925)
- Sodium acetate ( $\text{CH}_3\text{COONa}$ , 82.03 g/mol) (Anedra, Tigre, Argentina; Cat. No.: AN00651808)
- Glacial acetic acid ( $\text{CH}_3\text{COOH}$ , 60,05 g/mol) (Anedra, Tigre, Argentina; Cat. No.: AN6082)
- Absolute ethanol ( $\text{C}_2\text{H}_6\text{O}$ , 46.07 g/mol) (Biopack, Buenos Aires, Argentina; Cat. no.: 1654.08)

### 4.2. Equipment

- Teflon micropestle (Eppendorf-Fisher Scientific, Leicestershire, UK; Cat. no.: 10683001)
- Vortex (Denville Scientific, Metuchen, NJ, USA; Cat. no.: Vortexer S7030)
- Water bath (Jiangsu Jinyi Instrument Technology Company Limited, Shanghai, China; Cat. no.: SHZ-88)
- High-speed bench-top centrifuge (Heal Force, Shanghai, China; Cat. no.: Neofuge 15)
- Micropipettes p1000, p200, p20 (Gilson, Middleton, WI, USA; Cat. nos.: F144566, F144565, and F144563)

### 4.3. Reagents Setup

#### Buffer TESCa

Composition: 30 mM Tris-HCl pH 8; 10 mM EDTA; 1% SDS; 5 mM CaCl<sub>2</sub>.

Calculate the necessary volumes for each stock solution. Add and mix the Tris-HCl pH 8, EDTA, and CaCl<sub>2</sub>, autoclave, and then add the SDS.

Below we give an example (Table 2):

**Table 2.** Example of how to prepare an adequate volume of Buffer TESCa.

Reagent	Final Concentration	Volume (Vf <sup>1</sup> = 12.5 mL)
1 M Tris-HCl pH 8	30 mM	375 µL
0.5 M EDTA	10 mM	250 µL
100 mM CaCl <sub>2</sub>	5 mM	625 µL
dH <sub>2</sub> O <sup>2</sup>	-	10.625 mL
20% SDS	1%	625 µL

Mix and autoclave all the reagents except the SDS, and then add the SDS. <sup>1</sup> Final volume; <sup>2</sup> Distilled water.

## 5. Results

As previously mentioned, we found that internal control PCR results were variable for individual sand flies processed with the pAC protocol (results not shown). For this reason, we assayed various modifications to optimize DNA extractions from one sand fly (Scheme 1). The quality and quantity of the DNA extracts were measured using an AmpliQuant AQ-07 Spectrophotometer, but we found there was no correlation between the amount of DNA quantitated and the success of the PCR reactions. Similarly, a previous study describing the optimization of a DNA extraction procedure from individual human hairs (which poses similar difficulties to those faced by the extraction of DNA from individual sand flies), showed that there was no correlation between the amount of DNA quantitated and the success of STR genotyping, i.e., some extracts were correctly genotyped when quantitation failed to detect any DNA [19]. Hence, and as our main objective was to analyze the DNA extracts in PCR reactions for field studies, success was determined by evaluating each sample in amplification reactions using internal control primers that amplify the IVS6 domain of the *Lutzomyia* constitutive *cacophony* gene (~225 bp) [12,13] (5Llac and 3Llac, here referred to as 44F/45R; see Appendix A for detailed PCR conditions). The positive control we used was DNA extracted from a pool of 10 *L. longipalpis* adults from Posadas (Argentina) using the pAC protocol; the negative control was ddH<sub>2</sub>O. The variations we assayed and the effects they produced are mentioned below (see also Scheme 1 and Table 1); for all these extractions we processed field-captured male adult *L. longipalpis* (Posadas, Argentina).

### 5.1. Assayed Modifications

We first analyzed the effect of minor modifications on the pAC protocol and found that longer incubation times with pK and no incubation at −20 °C (in the DNA precipitation step) in general yielded better results (Appendix B; Table 1). We also found that results improved when mixing by inversion was done vigorously in the extraction with solvents step (Appendix C; Table 1). Nevertheless, as the previous modifications did not determine a consistent improvement, we decided to evaluate changes of greater magnitude (yet including these minor modifications that had produced slight improvements). We decreased the incubation temperature from 58° (original pAC) to 50 °C (our modification) because pK digestion is routinely performed at 50 °C [20], and to move as far away as possible from its inactivation temperature (65 °C) (manufacturer's recommendation). More importantly, we assayed three different lysis buffers: the original buffer pAC (as control; 10 mM Tris-HCl pH 8; 200 mM NaCl; 5 mM EDTA; 0.2% SDS) (according to Acardi personal communication), another commonly used lysis buffer, here referred to as buffer TES (30 mM Tris-HCl pH 8, 10 mM EDTA and 1% SDS),

and this same buffer TES to which we added  $\text{Ca}^{2+}$  (5 mM), here referred to as buffer TESCa (30 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, and 5 mM  $\text{CaCl}_2$ ; see “Section 4.3”). There were various reasons for evaluating the addition of calcium to the lysis buffer (buffer TESCa). Different studies have reported that calcium ions greatly affect the enzymatic activity of pK [15–17] and that enzymatic activity is significantly reduced when they are removed (up to 80%) [15]. Even though another study suggested that calcium ions stabilize the native conformation of the enzyme but do not affect proteolytic activity [18], it showed that  $\text{Ca}^{2+}$ -free pK precipitated irreversibly in the presence of EDTA leading to a much reduced effective concentration [18]. Furthermore, even though  $\text{Ca}^{2+}$  forms a complex with EDTA in the buffer, it is still capable of interacting with the enzyme [15]. In addition, a previous study found that activation of proteinase K by calcium improved the extraction of DNA from individual human hairs [19]. This same study showed that pK suffered loss of activity when the lysis buffer contained EDTA but no calcium [19].

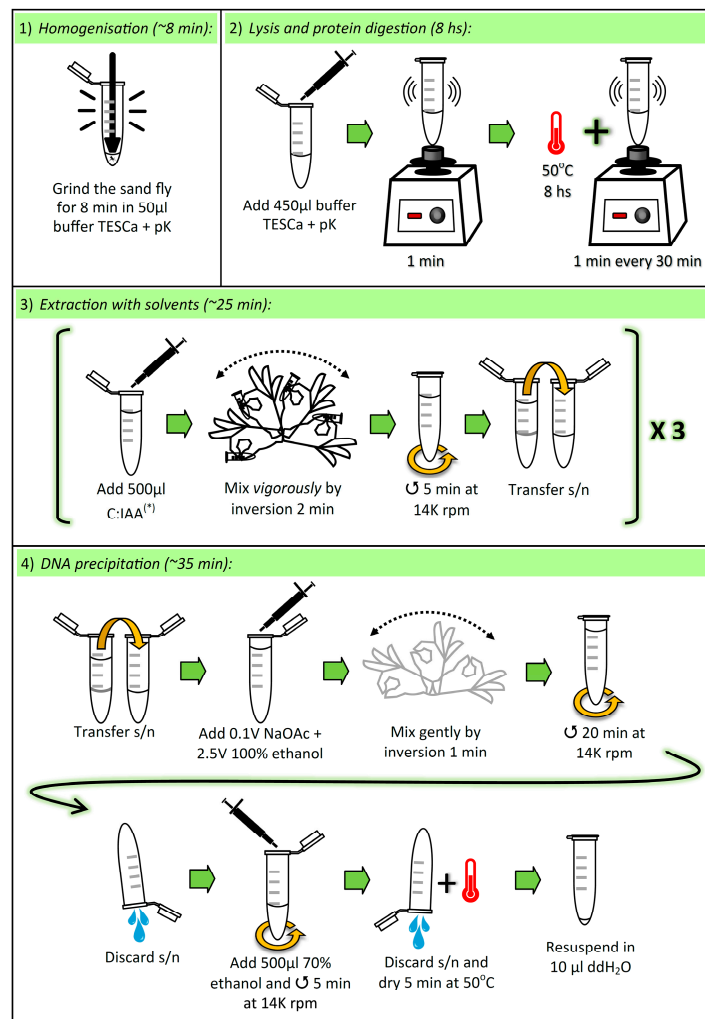
To evaluate these modifications, we processed specimens with the different lysis buffers (pAC, TES, and TESCa) (Table 1) and found that amplification was only positive when the samples were processed with buffers TES and TESCa (Appendix D). Due to these results we decided to further evaluate buffers TES and TESCa and processed more specimens with these buffers. All samples were incubated with pK (0.42  $\mu\text{g}/\mu\text{L}$ ) at 50 °C for 8 h, mixing by inversion was done vigorously for the three extractions with C:IAA, and pellets were resuspended in 10  $\mu\text{L}$  ddH<sub>2</sub>O. Due to the length of the first three stages (~9 h), the protocol was paused in the fourth step (i.e., the sample was precipitated ON at –20 °C). Amplification was variable for the samples processed with buffer TES (results were positive for only 2 of the 10 samples; Figure 1), whereas amplification was successful for all the samples treated with buffer TESCa (Figure 1). Chi-square analysis indicated that the association between both treatments (buffers TES and TESCa) and their outcomes was very significant (two-tailed  $p$  value = 0.0019).

Having determined that buffer TESCa and the previous modifications (incubation with pK at 50 °C, and vigorous mixing during the extraction with solvents), consistently improved DNA extractions, we also analyzed if we could reduce the incubation periods with proteinase K in this new lysis buffer, and eliminate the ON incubation at –20 °C. As we had found previously, longer incubation periods with pK improved PCR amplification, and incubation at –20 °C seemed to have little effect (Appendix E; Table 1).

## 5.2. Conclusions

To summarize, the main modifications for the final optimized DNA extraction protocol consisted of:

- (1) an 8-h incubation with proteinase K in buffer TESCa at 50 °C;
- (2) vigorous mixing by inversion during the extraction with solvents step; and
- (3) precipitation with alcohol with no ON incubation at –20 °C (Scheme 2). Pellets were resuspended in 10  $\mu\text{L}$  ddH<sub>2</sub>O and a 1:5 dilution was used for the PCR reactions. The complete and detailed optimized protocol is described in “Section 3”.



**Scheme 2.** Summary of the main experimental stages and steps involved in the optimized DNA extraction protocol (see “Section 3” for details). The approximate time needed to complete every stage is indicated. Figures are schematic (i.e., not an exact representation) and are not drawn to scale. (\*) In the third extraction with solvents, add 700 μL C:IAA. min, minutes; hs, hours; pK, proteinase K; C:IAA, chloroform:isoamyl alcohol (24:1); ∪, centrifuge; s/n, supernatant; V, volumes; 14K rpm, 14,000 revolutions per minute; ddH<sub>2</sub>O, double-distilled water.

DNA obtained using our method is suitable for long-term conservation, since individual sand fly DNA extracts were stored at  $-20\text{ }^{\circ}\text{C}$  and used as a template as much as 6 years later in PCR reactions which yielded positive results.

In conclusion, the above-mentioned changes (the most significant of which was the addition of calcium ions to the lysis buffer) optimized DNA extraction from individual sand flies when compared to the original pAC protocol, and enabled us to consistently obtain positive amplification results with the internal control primers. Moreover, we used this optimized protocol to extract DNA from individual field-captured *Lutzomyia* spp. from Brazil and Argentina, and the internal control amplifications were successful (Appendix F).

Our results also suggest that pK activity is reduced when the lysis buffer contains EDTA but no calcium (Figure 1), in accordance with a previous study that explored this same solution for optimizing the extraction of DNA from individual human hairs [19], and supporting previous evidence on the dependence of pK activity on the presence of calcium ions [15–17]. Furthermore, pK digestion is routinely performed at  $50\text{ }^{\circ}\text{C}$  [20], and it has been reported that pK’s activity can increase several fold within the  $50\text{ }^{\circ}\text{C}$  to  $60\text{ }^{\circ}\text{C}$  range [21]. In this context, buffers pAC, TES and TESCa were tested within



pK's optimal temperature range (50–60 °C), and we only obtained consistently successful results with the lysis buffer that contained calcium (buffer TESCa). Similarly, McNevin et al. [19] used a different lysis buffer (at 56 °C) and also found that DNA extraction only improved when calcium was added to the buffer [19]. This would suggest that, when working within the enzyme's optimal temperature range, it is the addition of Ca<sup>2+</sup> to the lysis buffer that improves DNA extraction.

**Author Contributions:** Conceptualization, C.B.M.; methodology, C.B.M. and L.G.C.; investigation, L.G.C. and C.B.M.; validation, L.G.C. and C.B.M.; formal analysis, L.G.C. and C.B.M.; resources, C.B.M., A.E.S., J.C.M., F.A.P., N.F.C.S., M.S.S. and O.D.S.; writing—original draft preparation, C.B.M.; writing—review and editing, C.B.M., L.G.C., N.F.C.S., O.D.S., M.S.S. and J.C.M.; visualization, C.B.M.; supervision, C.B.M.; project administration, C.B.M.; funding acquisition, C.B.M.

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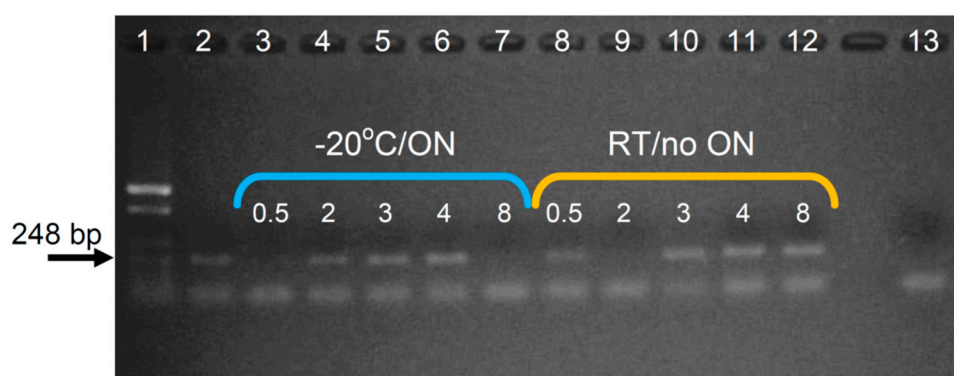
**Conflicts of Interest:** The authors declare no conflict of interest.

## Appendix A Internal PCR Control Conditions

An internal control PCR was implemented to confirm the efficiency and quality of the DNA extractions using published primers 5LlcaC and 3LlcaC (here referred to as 44F and 45R), that amplify a ~225 bp fragment of the *Lutzomyia* constitutive *cacophony* gene which includes the IVS6 domain [12,13]. Amplifications were completed in a GeneMax Thermal Cycler (Bioer Corporation). The reaction mixture contained 1× PCR buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl); 2.5 mM MgCl<sub>2</sub>; 0.125 mM dNTPs; 0.3 U Taq Pegasus<sup>®</sup> DNA polymerase (Productos Bio-Lógicos, Argentina); 0.5 μM of each primer (44F and 45R); 0.1 mg/mL bovine serum albumin (BSA), and 1 μL template, in a final volume of 10 μL. DNA extractions were diluted (1:5), the positive control was a (1:25) dilution of a previous DNA extraction (using the pAC protocol) from a pool of 10 *L. longipalpis* adults from Posadas (Argentina), and the negative control was ddH<sub>2</sub>O. The following profile was adapted from [12,13]: initial denaturation cycle at 95 °C for 30 s; followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s; and a final extension cycle at 72 °C for 7 min. PCR products were visualized on 1.5% (Figure 1) and 1% agarose gels (Figures A1–A7).

## Appendix B Assaying Different Incubation Periods with pK in Buffer pAC, and ON or No Incubation at –20 °C (in the DNA Precipitation Step)

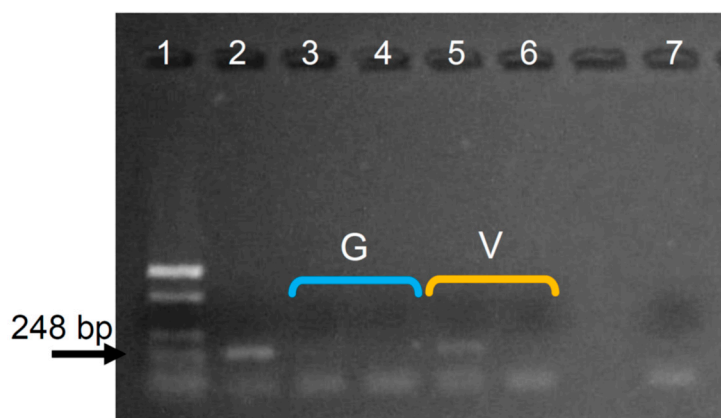
We simultaneously evaluated the effect of different incubation periods (0.5, 2, 3, 4 and 8 h) with pK (0.42 μg/μL) in the original lysis buffer (pAC); and, in the DNA precipitation step, we assayed the effect of ON incubation at –20 °C (original protocol) or no incubation (our variation) (Figure A1; Scheme 1). The overall results indicated that, as expected, longer incubation times with pK (4 and 8 h) in general yielded better results, as did no incubation with 100% ethanol, which was unexpected (Figure A1; Table 1). They also suggested that, when precipitating with 100% ethanol at –20 °C, incubation with pK should only occur for 2–4 h and, when precipitating with 100% ethanol at room temperature (no incubation), incubation with pK should occur for more than 3 h (Figure A1; Table 1).



**Figure A1.** Effects of different incubation periods with pK in buffer pAC, and ON or no incubation at  $-20\text{ }^{\circ}\text{C}$  (in the DNA precipitation step). DNA extractions were evaluated with an internal control PCR (using primers 44F/44R), and were undiluted. The blue bracket indicates the samples that were subjected to ON incubation at  $-20\text{ }^{\circ}\text{C}$  (lanes 3–7), and the yellow bracket indicates the samples that were not incubated before centrifugation (lanes 8–12); in both cases, the numbers below the brackets indicate the hour/s of incubation with pK. 1: MW (pZero2/*Hae*II); 2: positive control; 3: 0.5 h (30 min) pK + ON @  $-20\text{ }^{\circ}\text{C}$ ; 4: 2 h pK + ON @  $-20\text{ }^{\circ}\text{C}$ ; 5: 3 h pK + ON @  $-20\text{ }^{\circ}\text{C}$ ; 6: 4 h pK + ON @  $-20\text{ }^{\circ}\text{C}$ ; 7: 8 h pK + ON @  $-20\text{ }^{\circ}\text{C}$ ; 8: 0.5 h (30 min) pK + no incubation; 9: 2 h pK + no incubation; 10: 3 h pK + no incubation; 11: 4 h pK + no incubation; 12: 8 h pK + no incubation; 13: negative control. MW: molecular weight; ON: overnight; RT: room temperature.

### Appendix C Assaying the Intensity When Mixing by Inversion in the Extraction with Solvents Step

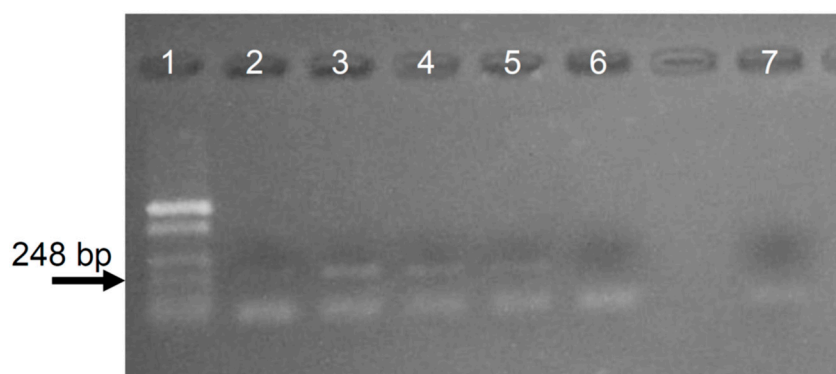
Samples were incubated with pK in buffer pAC for 8 h and, in the extraction with solvents step, mixing by inversion was done gently (standard protocol) and vigorously (our modification) (Figure A2; Scheme 1). Due to the length of the first three stages (~9 h), the protocol was paused in the fourth step (i.e., samples were incubated ON at  $-20\text{ }^{\circ}\text{C}$ ). Results showed there was an improvement when the samples were mixed vigorously (Figure A2; Table 1), and we implemented this minor modification to the protocol.



**Figure A2.** Effect of mixing intensity in the extraction with solvents step. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R). Gentle mixing by inversion (blue bracket, sample G): lanes 3 (undiluted) and 4 (1:10 dilution); vigorous mixing by inversion (yellow bracket, sample V): lanes 5 (undiluted) and 6 (1:10 dilution); lane 1: MW (pZero2/*Hae*II); lane 2: positive control; lane 7: negative control. G: gentle; V: vigorous; MW: molecular weight.

## Appendix D Assaying Different Lysis Buffers

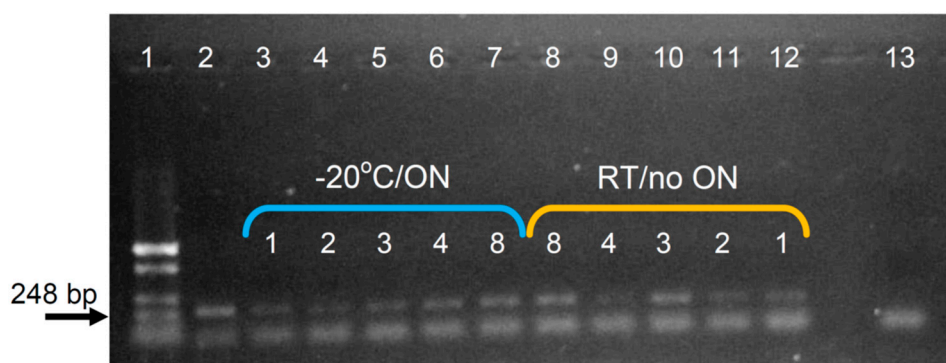
As indicated in “Section 5”, we processed specimens with the three lysis buffers (pAC, TES, and TESCa). Samples were incubated with pK (0.42  $\mu\text{g}/\mu\text{L}$ ) at 50 °C for 8 h, mixing by inversion was done vigorously for the three extractions with C:IAA, and pellets were resuspended in 10  $\mu\text{L}$  ddH<sub>2</sub>O (Scheme 1, Table 1). Due to the length of the first three stages (~9 h), the protocol was paused in the fourth step (i.e., the sample was precipitated ON at –20 °C). We found that amplification was only successful for the samples processed with buffers TES and TESCa (Figure A3).



**Figure A3.** Effect of different lysis buffers. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R), and were diluted (1:5). 1: MW (pZero2/*Hae*II); 2 and 3: positive control; 4: lysis buffer TES; 5: lysis buffer TESCa; 6: buffer pAC; 7: negative control. MW: molecular weight.

## Appendix E Assaying Different Incubation Periods with pK in Buffer TESCa, and ON or No Incubation at –20 °C (in the DNA Precipitation Step)

To analyze if we could (1) reduce the incubation periods with proteinase K in the new lysis buffer (TESCa), and (2) in the precipitation with alcohol step, eliminate the ON incubation at –20 °C, we processed specimens which were incubated with pK at 50 °C during 1, 2, 3, 4, and 8 h (2 specimens *per* condition). One sample of each incubation period was precipitated with alcohol ON at –20 °C, and the other without (i.e., the sample was centrifuged immediately after adding NaOAc and alcohol) (Scheme 1, Table 1). Results with the internal control primers indicated that: (1) Overall, and as we had found before, for both treatments (with and without ON precipitation at –20 °C), band intensity decreased as incubation time with proteinase K decreased (Figure A4), even though the faintly visible PCR product in lane 9 (4 h incubation with pK) suggested that longer incubation times do not always yield more DNA; (2) On the other hand, overall band intensity was greater for the samples that were not precipitated at –20 °C, barring the aforementioned exception (lane 9, 4 h incubation with pK) (Figure A4; Table 1).



**Figure A4.** Effects of different incubation periods with pK in buffer TESCa, and ON or no incubation at  $-20^{\circ}\text{C}$  (in the DNA precipitation step). DNA extractions were evaluated with an internal control PCR (using primers 44F/44R) and were diluted (1:5). The blue bracket indicates the samples that were subjected to ON incubation at  $-20^{\circ}\text{C}$  (lanes 3–7), and the yellow bracket indicates the samples that were not incubated before centrifugation (lanes 8–12); in both cases, the numbers below the brackets indicate the hour/s of incubation with pK. 1: MW (pZero2/HaeII); 2: positive control; 13: negative control.

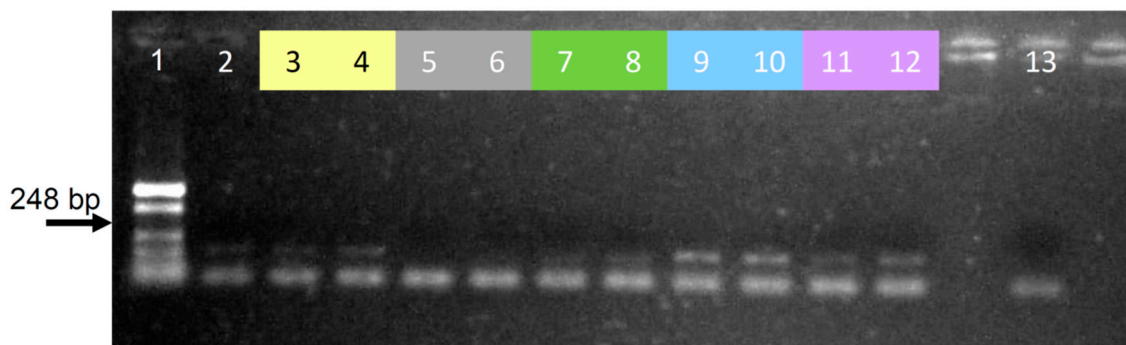
#### Appendix F Results Using the Optimized Protocol with Different *Lutzomyia* Spp.

As previously mentioned, we extracted DNA from various *Lutzomyia* spp. captured in different regions of Brazil, and from *L. longipalpis* collected in Argentina (Table A1). DNA was extracted from individual specimens using the protocol we optimized (Scheme 2) and, as we did for the optimization, DNA extracts were analyzed by PCR using internal control primers (44F/45R) (Figures A5–A7). Lins et al. [13] used these same primers, in conjunction with a set of degenerate primers, to analyze the *cacophony* gene from all the species we analyzed, except for *L. renei*. Similar to what they reported, our amplifications were successful for all species, including *L. renei* (which was not analyzed by [13]), but not for *L. migonei*. As Lins et al. [13] did not specify which primers they used for each of the species (44F/45R or the degenerate primers), it is possible that the *cacophony* fragment from *L. migonei* was previously amplified using the degenerate primers (i.e., not 44F/45R). Below we show some of the results we obtained for each of these species (Figures A5–A7).

**Table A1.** List of the *Lutzomyia* spp. that were analyzed. Color-coding for each species coincides with the color-coding used in Figures A5–A7.

Species <sup>1</sup>	City	State/Province	Country	Figure
<i>L. umbratilis</i>	Presidente Figueiredo	Amazonas	Brazil	A5, A6
<i>L. migonei</i>	Baturite	Ceara	Brazil	A5
<i>L. renei</i>	Lagoa Santa	Minas Gerais	Brazil	A6
<i>L. intermedia</i>	Tancredo Neves	Bahia	Brazil	A7
<i>L. longipalpis</i> (cavunge strain)	Cavunge	Bahia	Brazil	A5, A6
<i>L. longipalpis</i> (jacobina strain)	Jacobina	Bahia	Brazil	A5
<i>L. longipalpis</i> (lapinha strain)	Lagoa Santa	Minas Gerais	Brazil	A5
<i>L. longipalpis</i>	Posadas	Misiones	Argentina	A7

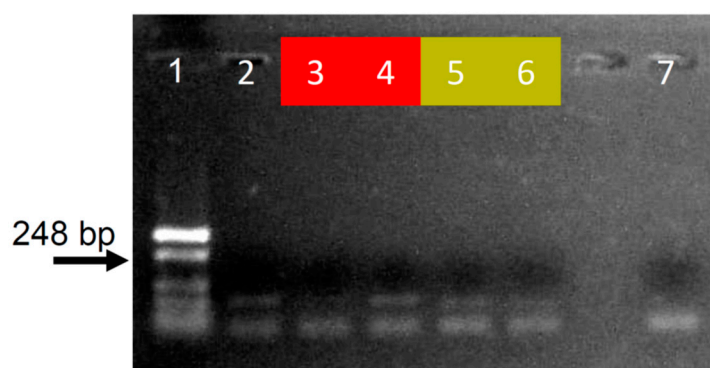
<sup>1</sup> Total number of specimens that were analyzed individually = 136.



**Figure A5.** PCR amplifications using DNA extracted with the optimized protocol from *L. longipalpis* (cavunge, jacobina and lapinha strains), *L. migonei*, and *L. umbratilis*. DNA extracts were evaluated with an internal control PCR (using primers 44F/44R) and were diluted (1:5). Color-coding coincides with that used for Table A1. 1: MW (pZero2/*Hae*II); 2: positive control; 3–4: *L. longipalpis* cavunge strain (Cavunge, Bahia, Brazil); 5–6: *L. migonei* (Baturite, Ceara, Brazil); 7–8: *L. umbratilis* (Presidente Figueiredo, Amazonas, Brazil); 9–10: *L. longipalpis* jacobina strain (Jacobina, Bahia, Brazil); 11–12: *L. longipalpis* lapinha strain (Lagoa Santa, Minas Gerais, Brazil); 13: negative control.



**Figure A6.** PCR amplifications using DNA extracted with the optimized protocol from *L. longipalpis* (cavunge strain), *L. umbratilis*, and *L. renei*. DNA extracts were evaluated with an internal control PCR (using primers 44F/44R) and were diluted (1:5). Color-coding coincides with that used for Table A1. 1: MW (pZero2/*Hae*II); 2: positive control; 3–5: *L. longipalpis* cavunge strain (Cavunge, Bahia, Brazil); 6–9: *L. umbratilis* (Presidente Figueiredo, Amazonas, Brazil); 10–13: *L. renei* lapinha (Lagoa Santa, Minas Gerais, Brazil); 14: negative control.



**Figure A7.** PCR amplifications using DNA extracted with the optimized protocol from *L. longipalpis* (Argentina) and *L. intermedia*. DNA extracts were evaluated with an internal control PCR (using primers 44F/44R) and were diluted (1:5). Color-coding coincides with that used for Table A1. 1: MW (pZero2/*Hae*II); 2: positive control; 3–4: *L. longipalpis* (Posadas, Misiones, Argentina); 5–6: *L. intermedia* (Tancredo Neves, Bahia, Brazil); 7: negative control.

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