Protocol

Optimisation 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 analysing parasite infection in *Lutzomyia* spp. by PCR [1] and, for this reason, we evaluated various modifications on a previously published protocol ([2] and Acardi personal communication). The most significant variation was the use of a different lysis buffer [3] to which added Ca2+ (buffer TESCa), because this ion protects proteinase K against autolysis, increases its thermal stability, and could have a regulatory function for its substrate-binding site [4]. Individual sand fly DNA extraction success was confirmed by amplification reactions using internal control primers that amplify a fragment of the *cacophony* gene [5,6]. To the best of our knowledge, this is the first time a lysis buffer containing Ca2+ has been reported for the extraction of DNA from sand flies.

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

1. Introduction

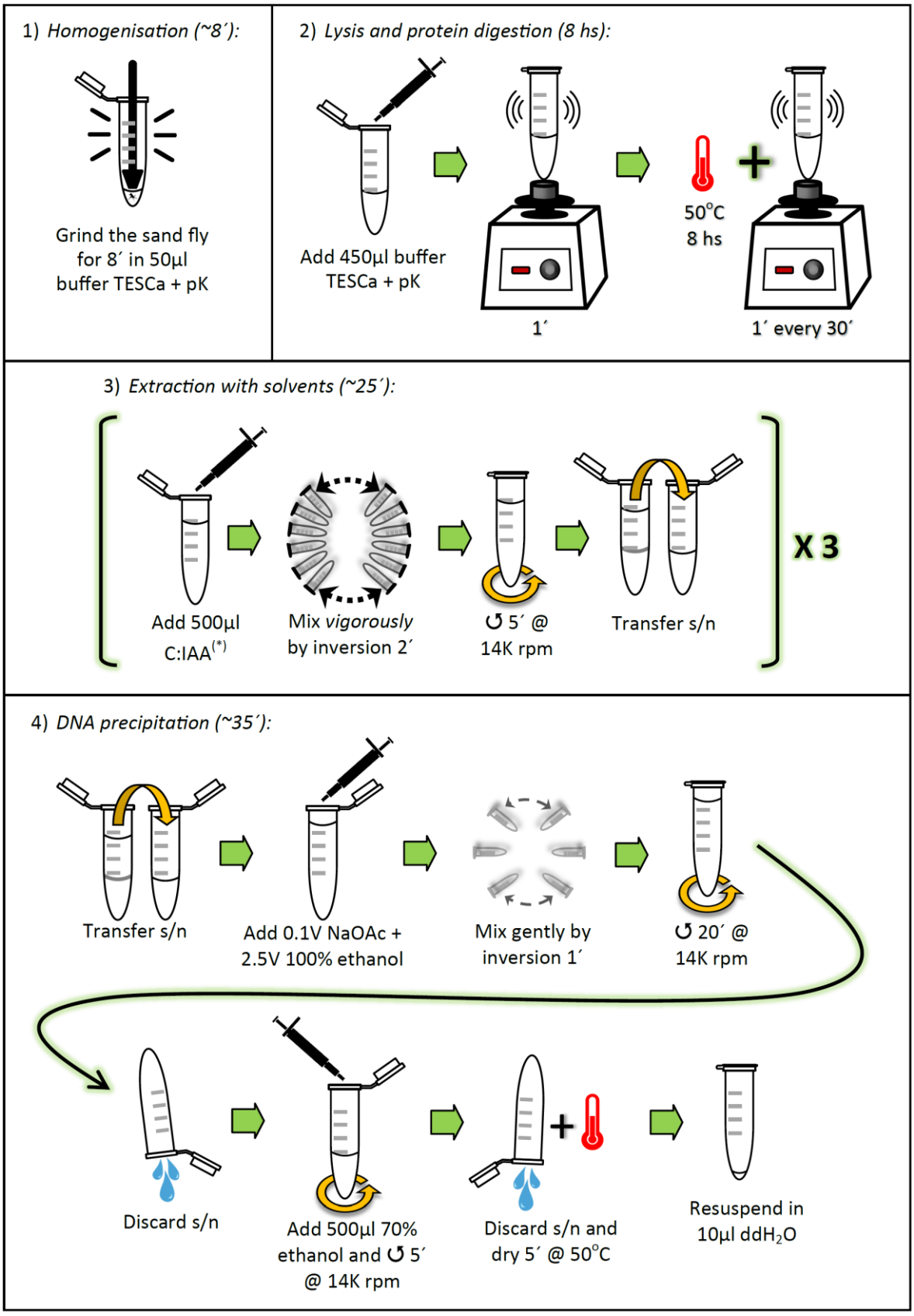
Various protocols have been published for the extraction of DNA from phlebotomines, including methods that eliminate DNA-associated proteins by using detergents and salts [7,8], or with proteinase K and detergents [9], and others that also add extraction steps with phenol-chloroform and precipitation with alcohol [10,11]; commercial DNA extraction kits [12,13]; and the use of Chelex-100 resin [14,15]. 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/or population genetics. In particular, these large-scale individual DNA extractions using traditional methods, usually yield poor results in terms of efficiency, quantity and purity, an

aspect which can be circumvented by the use of commercial kits [16]. Notwithstanding, in developing countries an extensive use of the latter is mostly cost-prohibitive and, consequently, traditional protocols become indispensable.

In our studies we were interested in analysing parasite infection in *Lutzomyia* spp. sand flies by PCR amplification [1]. For this, we previously compared two traditional DNA extraction methods using pools of 5 and 10 field-captured *L. longipalpis* sand flies: one that uses detergents (sodium dodecyl sulfate, SDS) and salt (potassium acetate) to eliminate DNA-associated proteins, and which was reported as an optimisation for the extraction of DNA from smaller sand flies [16]; and another, which we here refer to as pAC, that uses detergent (SDS), proteinase K, and phenol-chloroform extraction ([2] and Acardi personal communication). When this DNA was used as template in PCR reactions, we found that results were more consistent when it was extracted with pAC (*i.e.*, internal control PCRs showed the expected result). Nevertheless, when we used pAC to process individual sand flies (because it had shown better results with the pools of sand flies), we found that amplification was poor and inconsistent (*i.e.*, internal control PCR results were variable). For this reason, we evaluated various modifications on the pAC protocol [2], the most significant of which was the use of a different lysis buffer, which was reported as optimised for the extraction of DNA [3], to which added calcium (here referred to as buffer TESCa). We decided to add Ca2+ to the buffer because in [1-5 mM] it protects proteinase K against autolysis, increases its thermal stability, and could have a regulatory function for its substrate-binding site [4]. Furthermore, even though Ca2+ forms a complex with EDTA in the buffer, it is still capable of interacting with the enzyme [4]. 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 Ca2+ has been reported for the extraction of DNA from sand flies.

2. Experimental Design

The main experimental stages for the DNA extraction protocol are indicated in the following scheme (Scheme I):



Scheme I. Summary of the main experimental stages and steps involved in the DNA extraction protocol (see "3. Procedure" 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. pK, proteinase K; C:IAA, chloroform:isoamyl alcohol (24:1); ↺, centrifuge; s/n, supernatant; V, volumes; @, at; 14K rpm, 14,000 revolutions *per* minute; ddH$_2$O, double-distilled water.

2.1. Materials

- TRIS buffer ($NH_2(CH_2OH)_3$, 121.14g/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, $C_{12}H_{25}NaO_4S$, 288.38 g/mol) (Anedra, Tigre, Argentina; Cat. no.: AN219483180)
- EDTA ($C_{10}H_{16}N_2O_8$, 292.24 g/mol) (Anedra, Tigre, Argentina; Cat. no.: AN00605609)
- Calcium chloride dihydrate ($CaCl_2.2H_2O$, 147 g/mol) (Anedra, Tigre, Argentina; Cat. No.: AN6456)
- Proteinase K (Fermentas-Thermo Fisher Scientific, Waltham, Massachusets, USA; Cat. No.: #EO0491)
- Double-distilled water (ddH2O)
- Chloroform ($CHCl_3$, 119.38 g/mol) (Cicarelli Laboratorios, San Lorenzo, Argentina; Cat. no.: 1116110)
- Isoamyl alcohol (Anedra, Tigre, Argentina; Cat. no.: AN00659925)
- Sodium acetate (CH_3COONa, 82.03 g/mol) (Anedra, Tigre, Argentina; Cat. No.: AN00651808)
- Glacial acetic acid (CH_3COOH, 60,05 g/mol) ((Anedra, Tigre, Argentina; Cat. No.: AN6082)
- Absolute ethanol (C_2H_6O, 46.07 g/mol) (Biopack, Buenos Aires, Argentina; Cat. no.: 1654.08)

2.2. Equipment

- Teflon micropestle (Eppendorf-Fisher Scientific, Leicestershire, UK; Cat. no.: 10683001)
- Vortex (Denville Scientific, Metuchen, New Jersey, 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)

3. Procedure

3.1. Lysis and elimination of proteins. Time for Completion: ~8 hours, 8´.

3.1.1. Homogenisation of sand fly:

1. Aliquot sufficient volume of buffer TESCa (30 mM Tris-HCl pH 8; 10 mM EDTA; 1% SDS, 5 mM $CaCl_2$; see "5. Reagents Setup"), according to the number of samples you will process (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´ with a Teflon micropestle. To avoid cross-contamination between samples, the micropestle must be cleaned and autoclaved after each grinding (*i.e.*, you should have one micropestle ready for each sample you are going to process).

3.1.2. Cell lysis, and protein denaturation and digestion:

4. ⚠ **CRITICAL STEP:** Add 450 µl buffer TESCa + pK (to reach a final volume of 500 µl), vortex for 1´, and incubate at 50ºC during 8 hours, vortexing for 1' every 30 min.

3.2. Extraction with solvents. Time for Completion: ~25´.

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 during 2´. Immediately centrifuge at 14,000 rpm during 5´. 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 during 2´. Immediately centrifuge at 14,000 rpm during 5´. Transfer the supernatant (~460 µl) to a new 1.5 ml microcentrifuge tube.

3.2.3. Third extraction:

7. ⚠ **CRITICAL STEP:** Add 700 µl C:IAA (24:1 v/v) and mix *vigorously* by inversion during 2´. Immediately centrifuge at 14,000 rpm during 5´. Transfer the supernatant (~400 µl) to a new 1.5 ml microcentrifuge tube.

3.3. DNA precipitation. Time for Completion: ~35´

3.3.1. Addition of salt and alcohol:

8. Add 0.1 volumes (~40 µl) 3M Sodium Acetate (NaOAc) pH 5.2 and 2.5 volumes (~1 ml) 100% ethanol, and gently mix by inversion.
   ⏸ **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 (~9 hours), the protocol can be paused here and the sample stored (at least) overnight (ON) at -20ºC.

3.3.2. Centrifugation:

9. Centrifuge at 14,000 rpm for 20´ and discard the supernatant by inversion.
10. Add 500 µl 70% ethanol and centrifuge at 14,000 rpm during 5´. Discard the supernatant by inversion and dry the pellet at 50ºC for 5´. Resuspend the pellet in 10 µl double-distilled water.

3.2. Figures, Tables and Schemes

Table 1. Comparison of the different variations that were assayed for the DNA extraction protocol (see "4. Expected Results" for details). Conditions that improved results are highlighted in **bold**. pK: proteinase K; C:IAA: chloroform:isoamyl alcohol; RT: room temperature; ON: overnight; ddH_2O: double-distilled water.

Step	Variation A [1]	Variation B [2]	Variation C [3]	Variation D [4]
Homogenisation	Grind with micropestle for 8´ in 50 µl buffer.	Grind with micropestle for 8´ in 50 µl buffer.	Grind with micropestle for 8´ in 50 µl buffer.	Grind with micropestle for 8´ in 50 µl buffer.
Lysis and protein digestion	Buffer pAC; Incubation with pK @ 58ºC for 30´, 2, 3, 4 and **8 hs**.	Buffer pAC; Incubation with pK @ 58ºC for 8 hs.	Buffers pAC, TES and **TESCa**; **Incubation with pK @ 50ºC** for 8 hs.	Buffer TESCa **Incubation with pK @ 50ºC** for 1, 2, 3, 4 and **8 hs**.
Extraction with solvents	Gentle mixing by inversion with C:IAA.	Gentle and **vigorous mixing by inversion with C:IAA**.	Vigorous mixing by inversion with C:IAA.	Vigorous mixing by inversion with C:IAA.
DNA precipitation	Incubation with alcohol: ON @ -	Incubation with alcohol @ -20ºC [5]	Incubation with alcohol @ -20ºC [5]	Incubation with alcohol: ON @ -

	20ºC, and **no incubation**			20ºC, and **no incubation**
Final resuspension	20 µl ddH_2O	20 µl ddH_2O	**10 µl ddH_2O**	10 µl ddH_2O

[1] See figure 1; [2] See figure 2; [3] See figures 3 and 4; [4] See figure 5; [5] The protocol was paused in this step due to the length of the previous stages.

4. Expected Results

As we mentioned previously, to optimise DNA extractions from one sand fly we tried various modifications on the pAC protocol. As our main objective was to analyse the DNA in PCR reactions, success was determined by evaluating each sample in amplification reactions using internal control primers that amplify a ~225 bp fragment of the *Lutzomyia* constitutive *cacophony* gene, that includes the IVS6 domain [5,6] (5Llcac and 3Llcac, here referred to as 44F/45R; see Appendix A for detailed PCR conditions). The positive control we used was DNA (1:25 dilution) extracted from a pool of 10 *L. longipalpis* adults from Posadas (Argentina) using the pAC protocol; the negative control was ddH_2O. The variations we assayed and the effects they produced, are mentioned below in chronological order (Table 1); for all these extractions we processed field-captured male adult *L. longipalpis* (Posadas, Argentina):

1. We simultaneously evaluated the effect of different incubation periods (30´, 2, 3, 4 and 8 hours) with pK [0,42 µg/µl] in the original lysis buffer (buffer pAC: 10 mM Tris-HCl pH 8; 200 mM NaCl; 5 mM EDTA; 0.2 % SDS); and, in the DNA precipitation step, we assayed the effect of ON incubation at -20ºC (original protocol) or no incubation (our variation) (Figure 1). The overall results indicated that, as expected, longer incubation times with pK (4 and 8 hours) yielded better results, as did no incubation with 100% ethanol, which was unexpected (Figure 1) (Table 1).

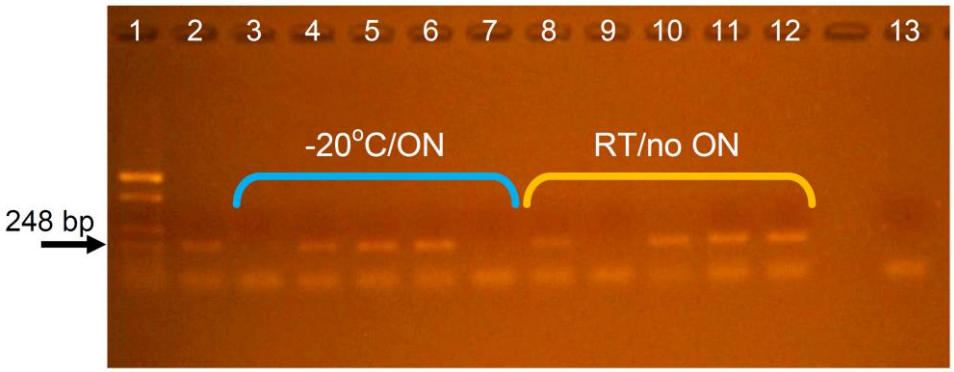


Figure 1. Effects of different incubation periods with pK, and, in the DNA precipitation step, ON incubation at -20ºC or no incubation. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R). Incubation period with pK: 30´, lanes 3 and 8; 2 hs, lanes 4 and 9; 3 hs, lanes 5 and 10; 4 hs, lanes 6 and 11; and 8 hs, lanes 7 and 12; ON incubation at -20ºC (blue bracket): lanes 3-7; No incubation (yellow bracket): lanes 8-12; lane 1: molecular weight (MW) (pZero2/*Hae*II); lane 2: positive control; lane 13: negative control. ON: overnight; RT: room temperature.

2. We then assayed mixing intensity in the extraction with solvents step: mixing by inversion was done gently (standard protocol) and vigorously (our modification) (Figure 2). Results showed an improvement when mixing by inversion was done vigorously (Figure 2) and, due to this, mixing intensity was changed from gentle to vigorous in the extraction with solvents step (Table 1). Due to the length of the first three stages (~9 hours), the protocol was paused in the fourth step (*i.e.*, the sample was incubated ON at -20ºC). We also found that these extractions only yielded positive results when they were used direct (*i.e.*, not diluted) in the PCR reactions (Figure 2).

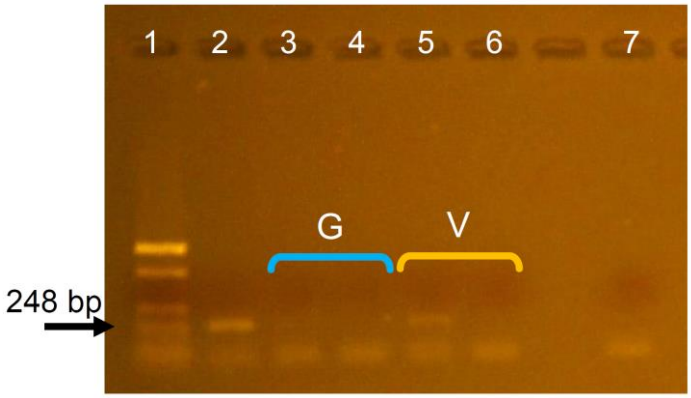


Figure 2. Effect of mixing intensity in the extraction with solvents step. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R). Gentle mixing by inversion (blue bracket, sample G): lanes 3 (direct) and 4 (1:10 dilution); vigorous mixing by inversion (yellow bracket, sample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

3. Nevertheless, as the previous modifications did not determine a clear improvement, we decided to try changes of greater magnitude, yet including the minor modifications that had produced slight improvements (mixing vigorously by inversion during the extractions with solvents, and no incubation in the DNA precipitation step when feasible). We evaluated three different lysis buffers: the original buffer pAC (as control), a lysis buffer that was optimised for DNA extraction, here referred to as buffer TES (30 mM Tris-HCl pH 8, 10 mM EDTA and 1% SDS) [3], and this same buffer TES to which we added Calcium [5 mM], here referred to as buffer TESCa (30 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, and 5 mM $CaCl_2$; see "5. Reagents Setup"). We decided to try this third lysis buffer (TESCa), because Ca2+ at that concentration protects proteinase K against autolysis, increases its thermal stability, and could have a regulatory function for its substrate-binding site [4]. Furthermore, even though Ca2+ forms a complex with EDTA in the buffer, it is still capable of interacting with the enzyme [4]. We also decided to decrease the incubation temperature, from 58° (original pAC) to 50°C (our modification), because the enzyme´s highest activity is found in the range of 50-55ºC, and also to move as far away as possible from its inactivation temperature (65°C) (manufacturer´s recommendation). To evaluate these modifications we processed 3 specimens, one for each of the lysis buffers (pAC, TES, and TESCa). All samples were incubated with pK at 50°C, and resuspended in 10 µl ddH_2O. Due to the length of the first three stages (~9 hours), the protocol was paused in the fourth step (*i.e.*, the samples were precipitated ON at -20ºC). Differently to what we found for extractions with buffer pAC (in which samples had to be used direct for the PCR reactions, Figures 1 and 2), amplification results with buffers TES and TESCa were only positive for diluted extractions (1:5) (Figure 3, Table 1) (*i.e.*, not when used direct, results not shown).

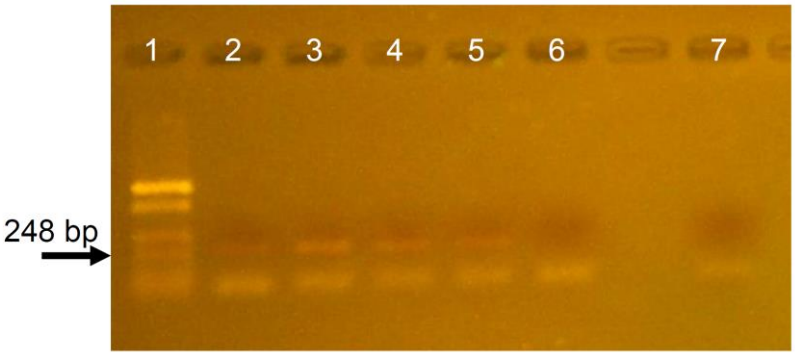


Figure 3. Effect of different lysis buffers. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R). 1: MW (pZero2/*Hae*II); 2 and 3: positive control; 4: lysis buffer TES (1:5 dilution); 5: lysis buffer TESCa (1:5 dilution); 6: buffer pAC (1:5 dilution); 7: negative control.

Due to these results we decided to further evaluate buffers TES and TESCa. For this we processed 8 specimens, 4 with buffer TES, and 4 with Buffer TESCa. All samples were incubated with pK [0.42 µg/µl] at 50°C for 8 hours, mixing by inversion was done vigorously for the three extractions with C:IAA, and pellets were resuspended in 10 µl ddH_2O. Due to the length of the first three stages (~9 hours), the protocol was paused in the fourth step (*i.e.*, the sample was precipitated ON at -20°C). Amplification results were variable for the samples processed with buffer TES (two of the samples did not amplify, and the other two did so with different yields; Figure 4), whereas the ones treated with buffer TESCa showed consistency and greater yields in the amplification reactions (Figure 4) (Table 1).

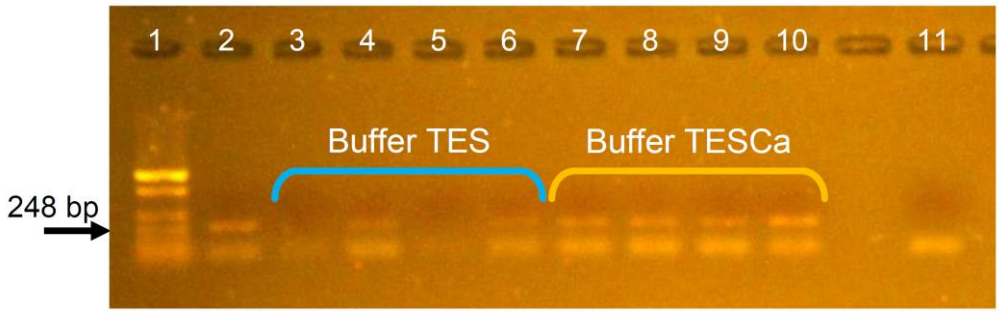


Figure 4. Further analysis of buffers TES and TESCa. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R). 1: MW (pZero2/*Hae*II); 2: positive control; 3-6: lysis buffer TES (1:5 dilution); 7-10: lysis buffer TESCa (1:5 dilution); 11: negative control.

4. 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 were interested in analysing if we could now: 1) reduce the incubation periods with proteinase K in this new lysis buffer; and 2) in the precipitation with alcohol step, eliminate the ON incubation at -20°C. For this, we processed 10 specimens which were incubated with pK at 50°C during 1, 2, 3, 4, and 8 hours (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) (Figure 5). Results with the internal control primers indicated that: 1) 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 5); 2) On the other hand, overall band intensity was greater for the samples that were not precipitated at -20°C (Figure 5) (Table 1).

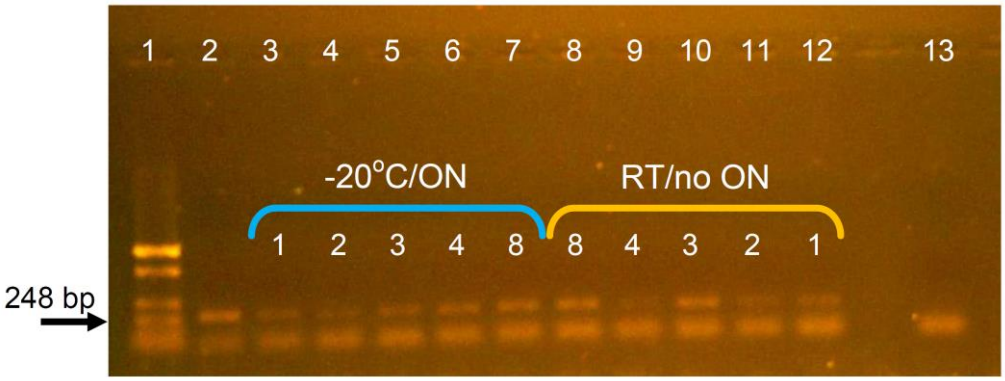


Figure 5. Effects of different incubation periods with pK, and ON or no incubation at -20°C (in the DNA precipitation step), under optimised conditions. DNA extractions were evaluated with an internal control PCR (using primers 44F/44R). The blue bracket includes the samples that were submitted to ON incubation at -20°C (lanes 3-7), and the yellow bracket includes 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: 1h pK + ON @-20°C (1:5 dilution); 4: 2hs pK + ON @-20°C (1:5 dilution); 5: 3hs pK + ON @-20°C (1:5 dilution); 6: 4hs pK + ON @-20°C (1:5 dilution); 7: 8hs pK +

ON @-20°C (1:5 dilution); 8: 8hs pK + no incubation (1:5 dilution); 9: 4hs pK + no incubation (1:5 dilution); 10: 3hs pK + no incubation (1:5 dilution); 11: 2hs pK + no incubation (1:5 dilution); 12: 1h pK + no incubation (1:5 dilution); 13: negative control.

In this way, the main modifications for the final optimised DNA extraction protocol consisted of:

1) an 8-hour 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 I). Pellets were resuspended in 10 µl ddH_2O and a 1:5 dilution was used for the PCR reactions. The complete and detailed optimised protocol is described in "3. Procedure".

Finally, as we have already mentioned, the main reason for optimising the DNA extraction protocol was to analyse parasite infection in *Lutzomyia* spp. by PCR amplification. In this sense, it is important to mention that we used this optimised protocol to extract DNA from individual field-captured *Lutzomyia* spp. from Brazil and Argentina and, as the internal control amplifications were successful (See Appendix B), we were able to perform the aforementioned analysis (results not shown).

5. Reagents Setup

Buffer TESCa:

Composition: 30 mM Tris-HCl pH 8; 10 mM EDTA; 1% SDS; 5 mM $CaCl_2$

To make this buffer you will have to previously prepare the following stock solutions (the indicated molarity is suggested):

1M Tris-HCl pH 8 (autoclaved)

0.5M EDTA (autoclaved)

20% SDS (sterile filter, do NOT autoclave)

100mM $CaCl_2$ (autoclaved)

Calculate the necessary volumes of each stock solution. Add and mix the Tris-HCl pH 8, EDTA, and $CaCl_2$, 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 [1]=12.5 ml)
1M Tris-HCl pH 8	30mM	375 µl
0.5M EDTA	10mM	250 µl
100mM $CaCl_2$	5mM	625 µl
dH_2O [2]	-	10.625 ml
20% SDS	1%	625 µl

Mix and autoclave the reagents highlighted in grey, and then add the SDS. [1] Final volume; [2] Distilled water

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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 the IVS6 domain of the *Lutzomyia* constitutive *cacophony* gene (~225 bp) [5,6]. Amplifications were completed in a GeneMax Thermal Cycler (Bioer Corporation). The reaction mixture contained 1X PCR buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl); 2.5 mM $MgCl_2$; 0.125 mM dNTPs; 0.3 U Taq Pegasus® 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); the negative control was ddH_2O. The following profile was adapted from [5,6]: initial denaturation cycle at 95°C for 30'', followed by 35 cycles with denaturation at 94°C for 30'', annealing at 53°C for 30'' and extension at 72°C for 30'', and a final extension cycle at 72°C for 7'. PCR products were visualised on a 1% agarose gel (Figures 1-5, B1-B3).

Appendix B. Results of the optimised 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* from Argentina (Table B1). DNA was extracted from individual specimens using the protocol we optimised (Scheme I) and, as we did for the optimisation, the success of the extractions was determined by PCR using internal control primers (44F/45R) (Figures B1-B3). Lins *et al*. [6] used these same primers in conjunction with a set of degenerate primers, to amplify a fragment of the *cacophony* gene from all the species we analysed, except for *L. renei*. Similar to what they reported, our amplifications were successful for all species, including *L. renei* (which was not analysed by [6]), but not for *L. migonei*. As Lins *et al*. [6] 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). Alternatively, since *L. migonei* is much smaller than the rest of the species we analysed, it could be that the amount of extracted DNA was insufficient for the PCR amplification. Below we show some of the results we obtained for each of these species (Figures B1-B3).

Table B1. List of *Lutzomyia* spp. that were analysed. Colour-coding for each species coincides with the colour-coding used in Figures B1-B3.

Species [1]	City	State/Province	Country	Figure
L. umbratilis	Presidente Figueiredo	Amazonas	Brazil	B1, B2
L. migonei	Baturite	Ceara	Brazil	B1
L. renei	Lagoa Santa	Minas Gerais	Brazil	B2
L. intermedia	Tancredo Neves	Bahia	Brazil	B3
L. longipalpis (cavunge strain)	Cavunge	Bahia	Brazil	B1, B2

L. longipalpis (jacobina strain)	Jacobina	Bahia	Brazil	B1
L. longipalpis (lapinha strain)	Lagoa Santa	Minas Gerais	Brazil	B1
L. longipalpis	Posadas	Misiones	Argentina	B3

[1] Total number of specimens that were analysed individually = 136

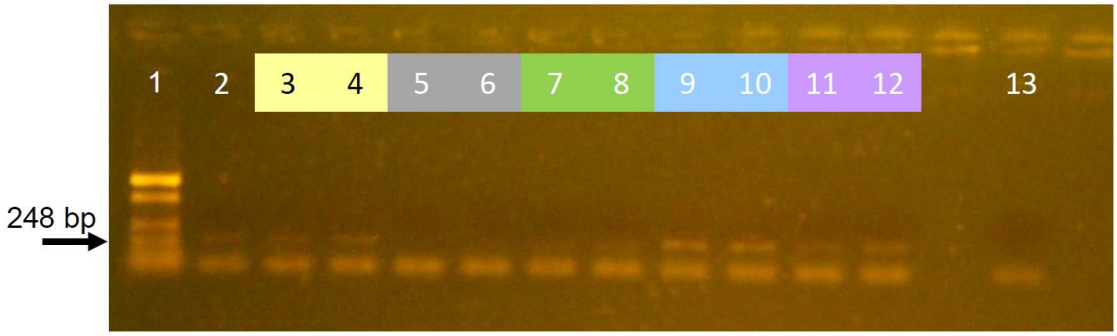

Figure B1. Evaluation of DNA extracted with the optimised protocol from *L. longipalpis* (cavunge, jacobina and lapinha strains), *L. migonei*, and *L. umbratilis*, with an internal control PCR (using primers 44F/44R). Colour-coding coincides with that used for Table B1. 1: MW (pZero2/*Hae*II); 2: positive 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.

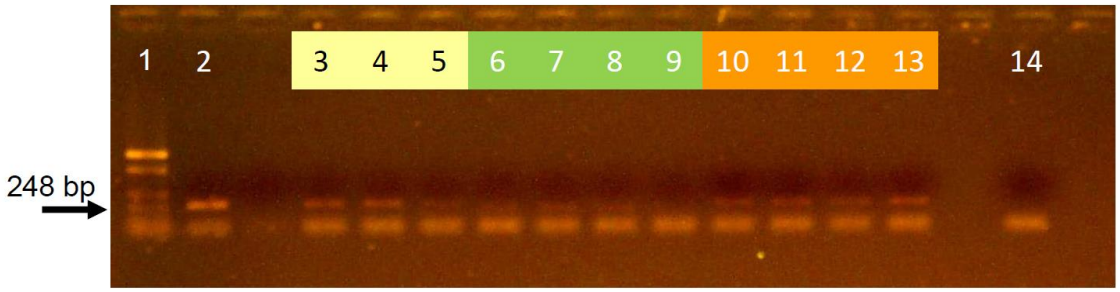

Figure B2. Evaluation of DNA extracted with the optimised protocol from *L. longipalpis* (cavunge strain), *L. umbratilis*, and *L. renei*, with an internal control PCR (using primers 44F/44R). Colour-coding coincides with that used for Table B1. 1: MW (pZero2/*Hae*II); 2: positive control; 3-5: *L. 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.

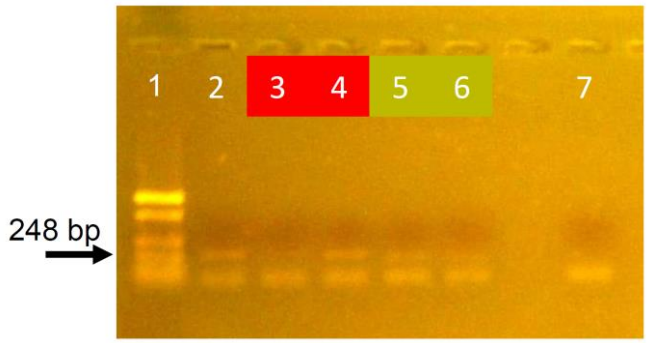

Figure B3. Evaluation of DNA extracted with the optimised protocol from *L. intermedia* and *L. longipalpis* (Argentina), with an internal control PCR (using primers 44F/44R). Colour-coding coincides with that used for Table B1. 1: MW (pZero2/*Hae*II); 2: positive control; 3-4: *L. longipalpis* (Posadas, Misiones, Argentina); 5-6: *L. intermedia* (Tancredo Neves, Bahia, Brazil); 7: negative control.

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