DNA Extraction from Blood and Forensic Samples

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10.1 Introduction and Purpose

DNA is usually extracted from fresh blood. However, successful individual-specific DNA profiles are routinely obtained from any biological source (saliva, hair, semen, etc.) containing nucleated cells, even from the cellular debris left on a touched object [1]. All this makes DNA analysis an irreplaceable tool for personal identification in Forensic Medicine and allows, in the Clinical Laboratory, the resolution of real or suspected specimen mislabelling. In the same manner, genetic data can be gathered from aged samples, such as skeletal remains or museum specimens [2]. Nevertheless, since a higher number of PCR cycles is usually required to produce amplicons from such samples, the risk of contamination has to be always considered [1–3]. Thus, rigorous precautions have to be adopted alongside the extraction procedures both to prevent and identify the exogenous contamination which can be inadvertently introduced. Reagents, disposables, pipettes, gloves, etc. can be important sources of contamination and even the operator can contaminate the sample by his/her DNA (by breathing, for example). For all these reasons, particular precautions have to be adopted when handling forensic/aged samples. Moreover, the size of the forensic specimen is usually very small so that the risk of mistyping due to exogenous contamination increases exponentially [1, 2].

10.2 Precautions

The usual precautions adopted in each laboratory are enough in case of DNA extraction from fresh blood, while particular precautions need to be introduced when handling both forensic and aged specimens.

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The particular precautions suggested for DNA extraction from minute/aged samples are the following:

- DNA extraction should be performed in a separate room dedicated solely for this purpose.
- Reagents, pipettes and disposables employed in that room should be dedicated solely to DNA extraction.
- Pipettes have to be cleaned often with a solution of bleach in water (10% v/v).
- Reagents are intended to be autoclaved or UV treated.
- Disposables should be UV treated.
- Nuclease-free aerosol-resistant pipette tips are compulsory.
- Avoid handling large volumes of working solutions: it is preferable to handle small-volume solutions.
- Wear disposable latex gloves (to be changed often), mask and white coat. The white coat should be left in the extraction room and dedicated solely for this purpose. In particular cases, wear a surgical cap and a mono-use white coat.
- Discard the tips into a mono-use plastic container to which 10% of bleach in water has been added.
- Do not extract the forensic sample (e.g. hair) and the reference samples (e.g. fresh blood) simultaneously.
- Before opening the Eppendorf tubes containing DNA, centrifuge them briefly to spin down any trace of the sample from the tube cover: your thumb could be an important source of cross-contamination.
- After use, clean the working desk and the inside part of the centrifuge with 10% of bleach.
- Metallic instruments (forceps, scissors etc.) can be sterilized on the Bunsen flame.
- Alongside the extraction procedure, always introduce at least one blank extraction control (BEC). Most simply, you have to perform a step-by-step extraction from a lysis buffer in which no biological components have been added (remember that the BEC validates your PCR-based results; see also Sect. 10.5.
- Record in a database the genetic profiles of all the samples analysed in your laboratory as well as the genotypes of the operators.

10.3 DNA Isolation from Blood Samples

This protocol is suitable for EDTA/blood. This protocol allows recovery of up to 30–40 μg of DNA, so it is suitable for long-term replicate uses of the samples (population genetics, for example). If the sample is a clot, put a small part of it into an Eppendorf tube and start from step 7 of Method 10.4.1.3.

10.3.1 Reagents

- *Absolute ethanol*: Fluka (02,860) and ethanol (70%) in water (v/v).
- *10 mg/ml Proteinase K* (stock solution): Sigma (P2308). Dissolve 100 mg of Proteinase K in 10 ml of autoclaved 50% glycerol. Store at −20°C.
- *3 M Sodium acetate solution*: Fluka (71,196). Dilute to 0.2 M with water (Milli Q) to provide the working solution.
- *Sodium dodecyl sulphate* (SDS): Sigma (L-71,725). Prepare a 10% (w/v) solution in water. Weigh the SDS in a fume cupboard, wearing the mask.
- *8-Hidroxy-quinoline*: Sigma (H 6,752).
- *Phenol*: Sigma (P9346).
- *Chloroform* ($CHCl_3$): Fluka (25,670).
- *Isoamyl alcohol* (3-methylbutanol): Sigma (I9392).
- *Phenol/CHCl$_3$/isoamyl alcohol* (25:24:1 v/v/v) solution. Mix 25 ml of Phenol, 24 ml of Chloform, 1 ml of Isoamyl alcohol, and 50 mg of 8-Hydroxy-quinoline. Remember that these substances are toxic: follow the recommendations reported on the bottles. The mixture is then equilibrated by forming an emulsion with an equal volume of 10 mM Tris pH 7.5. Allow the phases to separate, remove the upper phase and repeat the process. Store at 4°C in a light-tight bottle.

10.3.2 Equipment

- *Disinfected adjustable pipettes* with the following ranges: 0.5–10 μl, 20–200 μl, and100–1,000 μl
- *Nuclease-free aerosol-resistant pipette* tips
- *Eppendorf tubes*
- *Centrifuge* suitable for centrifugation of Eppendorf tubes at full speed (12,000–14,000 rpm)

10.3.3 Method

Red Cells Lysis

1. Flip the blood tubes several times to ensure homogeneity. Transfer a 0.7 ml aliquot into an Eppendorf tube.

2. Adjust to 1.5 ml with sterile water; then centrifuge the tubes at full speed (12,000–14,000 rpm) for 1 min in a micro-centrifuge.
3. Discard 1 ml from the supernatant of each tube without disturbing the leukocyte pellet.
4. Add 1 ml of sterile water to each tube and gently flip them. Centrifuge as described above. Remove and discard the supernatant.
5. Add to each leukocyte pellet 375 µl of 0.2 M Na-Acetate pH 7.0, 25 µl of 10% SDS, and 15 µl of Proteinase K solution.
6. Resuspend the pellet by vortexing. Incubate at 37°C for at least 1 h (overnight incubation is suggested for a higher recovery of DNA).

Phenol/Chloroform/isoamyl alcohol purification

7. Spin down the condensate from the tube walls.
8. Add 200 µl of the Phenol/$CHCl_3$/Isoamyl alcohol (25:24:1 v/v/v) solution and shake for about 10–20 s to obtain an emulsion.
9. Centrifuge the tubes for 2 min at full speed. The upper aqueous phase[1] is transferred into a new Eppendorf tube.
10. Precipitate the DNA by adding 1 ml of 100% ethanol. Close the tube and gently invert it until the DNA is visible as a clot.
11. Spin down the precipitate. Remove and discard the supernatant without disturbing the pellet.
12. Resuspend the pellet in 200 µl of 0.2 M Na-Acetate. Incubate in a water bath at 55°C for about 10 min or until suspended.
13. Precipitate the DNA with 500 µl of 100% ethanol as described in step 10 and repeat step 11.
14. Wash the sample by adding 1 ml of 70% ethanol and repeat point 11. Dry the pellet at 37 °C in an oven.
15. Add 200–400 µl of sterile water. Redissolve the sample at 37 °C for about 2–6 h. Store at −20°C until use.

10.4 DNA Isolation from Forensic Samples

Forensic analysis requires procedures which enable the isolation of genomic DNA from a big variety of biological samples usually adhering to solid (wood, glass, metal, plastic) or soft substrata (cloth, paper, cardboard, carpet, etc.). In addition, in some circumstances the biological specimens can be so tiny that they are not visually appreciable.

One of most relevant concern in handling all these samples is represented by the chemical composition of the substrata themselves which can contain unknown substances inhibiting *Taq I* polymerase, so leading to PCR failure. Empirically, the smaller the amount of the treated substratum, the higher the chance of a successful outcome of the PCR.

The real case-work offers a virtually infinite set of substrata. However, from a practical point of view, they can be divided into:

- Absorbing substrata (AS): cloth, paper, wood, etc.
- Non-absorbing substrata (NAS): metal, plastic, nylon, etc.

As a basic strategy, while for AS it is preferable to put a small part of the whole specimen into the extraction solution, for NAS it is more convenient to remove the biological component from the substratum before DNA extraction. DNA extraction from bones, soft tissues, saliva swabs and post coital swabs will be considered separately.

10.4.1 DNA Extraction from Absorbing Substrata

This protocol is suitable for blood stains, semen stains,[2] saliva stains, cigarette butts,[3] etc. It is always recommended that at least half of the specimen is stored for confirming tests.

10.4.1.1 Reagents

Note: In addition to Reagents of Sect. 10.3.1

- *Physiological solution*: (145 mM NaCl)
- *1× Lysis buffer (LB)*: (2% SDS, 100 mM NaCl, 40 mM DTT, 10 mM Tris pH 7.5, 10 mM Na_2EDTA pH 8.0). Aliquot small volumes of this solution

[1] Always take care not to remove any of the interface material or the lower organic phase.

[2] For a differential lysis see Sect. 10.4.7

[3] For cigarette butts, remove the outer part of the filter using a blade.

(5–10 ml) and store them at −20°C. Before use, melt at 37°C

- *$CHCl_3$/Isoamyl alcohol* (24:1 v/v) solution. Mix 24 ml of Chloroform and 1 ml of Isoamyl alcohol. The mixture is equilibrated by forming an emulsion with 10 mM Tris pH 7.5 (equal volume). Allow the phases to separate, remove the upper phase and repeat the process. Store at 4°C in a light-tight bottle
- *Glycogen*: 10 mg/ml in water (Bioline, BIO-37,077)

10.4.1.2 Equipment

Note: In addition to Equipment of Sect. 10.3.2

- *Sterile surgical blades and tweezers*

10.4.1.3 Method

1. Cut a piece of the stain (about 10–15 mm^2) on a Petri dish and put it into an Eppendorf tube.[4]
2. Add 1 ml of physiological solution.
3. Incubate the sample at room temperature for about 30 min, with occasional shaking.
4. Using a tip remove the substratum, squeezing it well.
5. Centrifuge the sample at full speed for 5 min (12,000–14,000 rpm).
6. Remove the supernatant without disturbing the pellet.
7. Resuspend the pellet in 0.5–0.75 ml of LB and 20–30 µl of Proteinase K.
8. Incubate at 37 °C for at least 3–6 h. Centrifuge briefly.
9. Add 300 µl of Phenol/$CHCl_3$/Isoamyl alcohol (25:24:1 v/v/v).
10. Vortex for about 10 s.
11. Centrifuge for 2 min at full speed (12,000–14,000 rpm).
12. Transfer the supernatant in a new tube.
13. Repeat steps 9–12.
14. Add 300 µl of $CHCl_3$/Isoamyl alcohol(24:1)
15. Vortex and centrifuge as described previously.
16. Transfer the supernatant in a new tube.
17. Precipitate the samples by adding 1/10 volume of 3 M Na-acetate pH 7.5, 2.5 volumes of absolute ethanol and 1 µl of (10 mg/ml) glycogen. Leave at −20°C for at least 3 h.
18. Centrifuge for 15 min at full speed[5].
19. Discard the supernatant by a tip.
20. Wash by adding 1 ml of 70% ethanol; invert the tube a few times and centrifuge at full speed for 30 s.
21. Discard the supernatant as above.
22. Dry the DNA pellet in an oven at 37°C.
23. Add appropriate volume (20–50 µl) of sterile water, resuspend the sample and store it at −20°C until use.

10.4.2 DNA Extraction from Non-absorbing Substrata

This protocol is suitable for DNA extraction from any kind of biological material left on non-absorbing substrata such as plastic, nylon, glass, etc.

10.4.2.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1

- *2× Lysis buffer* (4% SDS, 200 mM NaCl, 80 mM DTT, 20 mM Tris pH 7.5, 20 mM Na_2EDTA pH 8.0)

10.4.2.2 Equipment

Note: In addition to Equipment of Sect. 10.3.2
None

10.4.2.3 Method

1. Using a tipped pipette, dissolve the stain on the surface using 150 µl of water (see footnote 4).

[4]If the stain is actually small (or not visible), put the substratum directly into 500 µl of LB to which 30 µl of Proteinase K has been added and start from point 8 of this method. Remember to remove the substratum before phenol purification.

[5]When inserting the Eppendorf tube into the rotor, mark (or remember) the position of the cover hinge. This will help you in identifying the location of the pellet.

2. Transfer this solution into an Eppendorf tube.
3. Repeat step 1 on the surface of the substratum.
4. To about 300 µl of the recovered solution, add an equal volume of 2× Lysis buffer and 30 µl of Proteinase K.
5. Proceed as described from step 8 onwards in Method Sect. 10.4.1.

10.4.3 DNA Extraction from Saliva Swabs

In the forensic practice, it is a validated procedure to collect a saliva swab as reference sample. This is a safe and robust protocol which allows obtaining enough DNA to perform tens of genetic tests.

10.4.3.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1
None

10.4.3.2 Equipment

Note: In addition to Equipment of Sect. 10.3.2
None

10.4.3.3 Method

1. Isolate the swab from the stick used to collect the saliva from the mouth and put it into an Eppendorf tube.[6]
2. Add 1 ml of physiological solution and incubate at room temperature for 5–10 min, stirring occasionally to remove the cells from the swab.
3. With a sterile tip, remove the cotton swab from the stick, squeezing it well.
4. Centrifuge for 2 min at full speed and discard the supernatant.
5. Add to the pellet 500 µl of 0.2 M Na-Acetate, 25 µl of 10% SDS and 30 µl of Proteinase K (10 mg/ml). Resuspend the pellet. Incubate at 37°C for at least 2–3 h.
6. Purify the sample by adding 250 µl of phenol/chloroform/isoamyl alcohol (25:24:1) as usual.
7. Precipitate by adding 1 ml of 100% ethanol and 1 µl of (10 mg/ml) glycogen. Leave at −20°C for at least 3 h.
8. Centrifuge at full speed for 10 min. Discard the supernatant and wash the pellet with 1 ml of 70% ethanol without disturbing the pellet.
9. Centrifuge for 10 s.
10. Discard the ethanol without disturbing the pellet.
11. Air dry and resuspend in an appropriate volume (20–40 µl) of water. Store at −20°C until use.

10.4.4 DNA Extraction from Soft Tissues

Each biological tissue contains DNA suitable for genetic typing. However, from decomposed bodies it is preferable to perform DNA extraction from connective tissues, as ligaments, cartilages, nails [4], etc.

10.4.4.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1
None

10.4.4.2 Equipment

Note: In addition to Equipment of Sect. 10.3.2

- Ten millilitre tubes

10.4.4.3 Method

1. Put the sample (about 20–40 mg) in a 10 ml tube containing 5 ml of physiological solution.[7]
2. Shake briefly and discard the solution.

[6]Use no more than half a swab and store the remaining part at −20° C.

[7]If the sample is mummified, before this step it is preferable to incubate the sample in water at room temperature for at least 12-18 h.

3. Repeat steps 1 and 2 twice.
4. Place the sample on a Petri dish.
5. Cut the sample into small pieces.
6. Put the fragments into an Eppendorf tube.
7. Add 0.5–0.75 ml of 1 X Lysis buffer and 40 µl of Proteinase K.
8. Incubate at 37°C for 2–3 h.[8]
9. Centrifuge briefly.
10. Add 20 µl of Proteinase K and proceed as described from step 8 onwards in Method "DNA extraction from absorbing substrata"

10.4.5 DNA Extraction from Hairs

Hair roots are a suitable source of nuclear DNA while only mitochondrial DNA (mDNA) typing is expected from hair shafts. However, an extensive SNP analysis from a large ≈4,000-year-old-permafrost-preserved hair tuft was recently reported [6].

10.4.5.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1
None

10.4.5.2 Equipment

Note: In addition to Equipments of Sect. 10.3.2
None

10.4.5.3 Method

1. Identify the hair root by a magnifying lens.
2. Cut about 8–10 mm of that end with a blade.
3. Place the sample into an Eppendorf tube.
4. Add 0.5–0.75 ml of 1 X Lysis buffer and 40 µl of Proteinase K.
5. Proceed as described from step 8 onwards in Method "DNA extraction from absorbing substrata" Sect. 10.4.1.

[8]As even a temperature of 37°C causes DNA damage [3], working at room temperature is suggested for ancient samples [5].

10.4.6 DNA Extraction from Bone

Bones usually provide the last source of genetic material of any individual. Before DNA extraction, their surface needs to be cleaned carefully to remove both soil and other several sources of contamination. In addition, a decalcification step by the chelating Na_2EDTA permits a more efficient recovery of the genetic material still present in the sample [7]. Long bones (femur, for example) are usually preferable.[9]

10.4.6.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1

- *EDTA solution*: Na_2EDTA 0.5 M pH 8.0

10.4.6.2 Equipment

Note: In addition to Equipments of Sect. 10.3.2

- *A large base (ϕ about 4–5 cm) tube*
- *A saw* (sterilized by UV radiation)
- *Glass paper* (sterilized by UV radiation)
- *Ultra-filtration devices*: Amicon® Ultra 100 kDa filter units (Millipore; cod. UFC 810,024 for volumes <4 ml and cod. UFC510, 024 for volumes <0.5 ml)
- *Centrifuge*: for UFC 810, 024, a suitable centrifuge allowing 4,000 rpm is required

10.4.6.3 Method

1. Remove the external surface of a section of the bone by the glass paper.
2. Slice a section of about 3–5 mm thickness with the saw.
3. Put the section in the large base tube and add about 20 ml of EDTA solution.
4. Incubate at room temperature for 18–48 h, with gentle shaking.
5. Remove the bone section and put it into a Petri dish.
6. With a blazer, remove small fragments of the inner part (medulla) of the section.

[9]The same protocol can be used for teeth. In this case, after cleaning the surface using a sterile drill, the tooth has to be divided by a longitudinal sawing and one of the two parts processed as described for bones.

7. Put them into an Eppendorf tube.
8. Add 0.5–0.75 ml of 1 X Lysis buffer and 40 µl of Proteinase K.
9. Incubate at 37°C for 2–3 h.[10]
10. Centrifuge briefly. Add 20 µl of Proteinase K and incubate at 37°C for another 8–12 h.
11. Proceed from step 9 to step 16 of Method of Sect. 10.4.1.
12. Purify the sample by ultra-filtration following the manufactures protocols (at least three washes are recommended).

10.4.7 Differential Lysis Between Sperm and Epithelial Cells

Vaginal or anal swabs can contain a mixture of sperm and epithelial cells. Although the employment of Y-specific STR markers allows the identification of the male compound, it is however preferable to separate the two genetic fractions. This is usually performable by a preferential lysis of the epithelial cells whose cellular membrane is more sensitive to the SDS/Proteinase K treatment. Indeed, by this procedure the more robust sperm cells can be preferentially recovered by centrifugation.

10.4.7.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1
None

10.4.7.2 Equipment

Note: In addition to Equipment of Sect. 10.3.2
None

10.4.7.3 Method

Differential Lysis

1. Isolate the swab from the stick (see footnote 6) and put it into an Eppendorf tube.
2. Add 1 ml of physiological solution and incubate at room temperature for 15 min, mixing occasionally to remove the cells from the substratum.
3. With a sterile tip remove the cotton swab squeezing it well.
4. Centrifuge for 3 min at full speed and discard the supernatant without disturbing 50 µl of the pellet.[11]
5. Resuspend the pellet in 500 µl of 1× LB supplemented with 15 µl Proteinase K (10 mg/ml). Mix gently. Incubate for at least one hour at 56°C in order to lyse the epithelial cells, but for less than 2 h to minimize the lysis of the sperm cells.
6. Centrifuge for 5 min.
7. Transfer all the supernatant, except 50 µl of the pellet, into a clean Eppendorf tube (SN tube)[12]
8. Resuspend the pellet gently in 200 µl of physiological solution.
9. Centrifuge for 5 min at full speed.
10. Transfer the supernatant into the SN tube leaving 50 µl of the pellet in the original tube (OT).
11. Repeat step 8 twice by adding 200 µl of physiological solution.

DNA Extraction from the Pellet (Sperm Fraction)

Proceed from step 7 in Method of Sect. 10.4.1.3 using the pellet of the original tube (OT).

DNA Extraction from the Supernant (Epithelial Fraction of the SN Tube)

Add 30 µl of Proteinase K and 20 µl of 10% SDS to half volume of the supernatant and proceed from step 8 in Method of Sect. 10.4.1.3. Store the remaining supernatant at –20°C.

10.4.8 DNA Extraction from Urine

Also urine is a source of DNA suitable for identification purposes. Note, however, that a high amount of bacterial contamination is expected.

[10] As even a temperature of 37°C causes DNA damage [3], working at room temperature is suggested for ancient samples [5].

[11] The pellet can be composed both by sperm and apithelial cells. Thus, it is strongly recommended to analyze 1-2 µl microscopically.

[12] The supernatant (SN) is employed for DNA extraction from the epithelial fraction.

10.4.8.1 Reagents

Note: In addition to Reagents of Sect. 10.4.1.1
None

10.4.8.2 Equipment

Note: In addition to Equipment of Sect. 10.3.2
None

10.4.8.3 Method

1. Transfer 1 ml of urine into an Eppendorf tube.
2. Centrifuge at full speed for 2 min.
3. Remove the supernatant.
4. Add to the pellet 1 ml of physiological solution.
5. Resuspend the pellet gently and centrifuge at full speed for 2 min.
6. Remove the supernatant.
7. Proceed from step 7 in Method of Sect. 10.4.1

10.5 Remarks and Troubleshooting

By the employment of the protocols described above, your samples should be ready for PCR amplification. If the extraction protocol worked successfully on a suitable biological sample, the amplification of a locus with low molecular weight (no more than 200–300 bp) through 30–32 cycles of PCR should give the following results at the checking mini-gel (see Chap. 17, Sect. 17.1):

- Positive PCR control; +
- Negative PCR control: –
- Blank extraction control (BEC): –
- Unknown sample (e.g. your sample): +

If the PCR controls (both + and –) are consistent, note that a positive signal in "blank extraction control" (BEC) indicates that contamination occurred alongside the extraction procedure. In this case, it is mandatory to perform a new extraction by freshly prepared solutions and new consumables. However, genotype the amplicons obtained from the BEC in any case: this could help in the identification of the source of contamination. In addition, these undesirable results are often represented by non-specific PCR products, so a correct interpretation of the genetic profile of the unknown sample could be however performed.

Regrettably, it is quite common that aged/forensic samples do not provide amplicons at all (– at checking mini-gel). This unsuccessful outcome of amplification is usually due to one of these two main reasons: (1) no suitable template; (2) inhibition of the PCR.

No Suitable Template

Successful amplification outcomes require minimal amounts of suitable template [3]. Nevertheless, the genetic material obtained from aged/forensic samples can be both degraded (e.g. of low molecular weight) and chemically damaged [2, 8]. This means that no (or low copy number of) entire templates are available for PCR amplification. In addition, a high degree of exogenous (fungal/bacterial) contamination can be present. All these features make the sample not or scarcely sensitive to PCR amplification. If this is the case (see Sect. 10.5.1) both a higher number of PCR cycles and a higher amount of template could be enough. As last chance, a new extraction can be performed employing a much higher amount of the original specimen.

Inhibition of the PCR

A forensic or aged biological sample can be contaminated by several compounds which are co-purified with the nucleic acids in spite of the accuracy of the protocol employed. Out of these contaminating substances, several (for example, humic acid, tannins, urea, etc.) are known to act as inhibitors of the DNA polymerases. Sometimes, the presence of such substances is revealed simply by a visual check, being the sample heavily coloured or cloudy. In other cases, a particular fluorescence is appreciable at UV transillumination (Fig. 10.1).

More often, no particular feature characterises these contaminated samples, so a logical approach has to be adopted to distinguish an actual inhibition of the polymerisation [9, 10] from an unsuitable template (no or low copy amount of entire templates).

If inhibition is identified (see the following section), several strategies can be carried out. The first attempt could be a further treatment of the sample by Proteinase K treatment, phenol extraction and ethanol

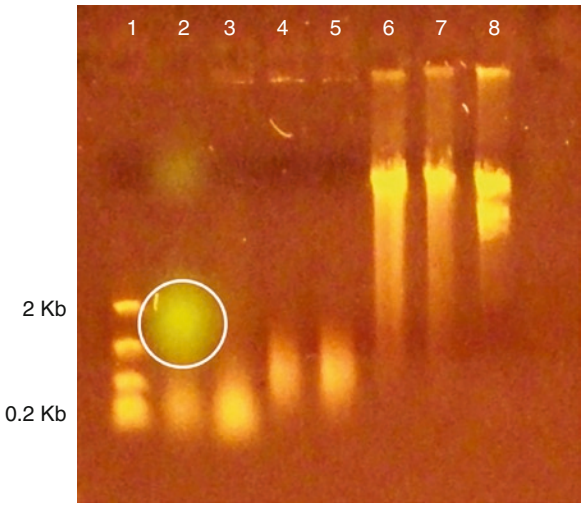

Fig. 10.1 UV transillumination of a 1% agarose gel (containing EtBr) after electrophoresis. The circle in lane 2 shows a grey-blue fluorescence contaminating the DNA preparation from a 2,100 year-old mummified tissue. This unknown contaminating substance was removed by ultra-filtration (lane 3). Also note the low molecular weight of this sample. Lanes 4 and 5: low molecular weight control DNA; lanes 6 and 7: high molecular weight control DNA; lane 1: easy ladder (Bioline); lane 8: λ/Bam HI

precipitation. Nevertheless, this is sample-consuming and often unsuccessful. Therefore, the employment of centrifugal filter devices is more convenient because these allow purification of the samples (see Equipment of Sect. 10.4.6). Recently, the employment of *Taq I* polymerases, which are less sensitive to inhibition, has also been described [11].

10.5.1 Distinguishing Between No Suitable Template and Inhibition of the PCR

The detection of PCR inhibitors can be performed by several methods, among which Real-Time PCR is preferred [9, 10]. Although this approach is both sensitive and accurate, it requires expensive equipments and often the availability of commercial kits. Here we describe a simple and inexpensive PCR-based method routinely employed in our Laboratory as screening assay. It is based upon the presence of an internal positive control (IPC) which is able to point out the presence of PCR inhibitors within the unknown sample. In addition, since repeated *Alu* sequences are the target of this PCR assay, a high sensitivity is also assured. The described method allows the synthesis of a 262 bp long fragment [12] from a sub-cellular amount (<1 pg) of human control DNA.

10.5.1.1 Primers, Additional Reagents and Standards

- *Primers*:P1:5'GCCTGTAATCCCAGCACTTT3';P: 5'GAGACAGGGTCTCGCTCTG3'.
- *Mineral Oil*[13]: (Sigma, M5904).
- *High molecular weight human DNA* (CTRL sample) at the concentration of 10 pg/μl (as determined at DO_{260}/DO_{280}).

10.5.1.2 Method

PCRs are performed in a final volume of 15 μl through 28 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s.

Set up the samples as follows:

- For *n* samples to test, prepare 13 μl × (2 *n* + 3) of a PCR mix containing 115 nM of each primer, 230 μM of dNTP, 1.7 mM of $MgCl_2$, 1.15 × buffer and 1.15 U of *TaqI* polymerase. In a standard assay (supposing that a single unknown sample has to be analysed) the protocol of amplification should be the following, as described in Table 10.1.

Assuming the consistency of the results from tubes 1 and 2, negative results both in tube 3 and 4 indicate PCR inhibition while PCR failure only in tube 3 shows

Table 10.1 Description of the amplification protocol

Tube	PCR mix (μl)	H_2O (μl)	CTRL sample (μl)	Unknown (μl)
1 (+PCR)	13	1	1	–
2 (–PCR)	13	2	–	–
3 Unknown	13	1	–	1
4 Competition assay	13	–	1	1

[13]In some thermal-cyclers mineral oil must be added to the samples to prevent their evaporation. However, when handling forensic/ancient samples it is always preferable to employ it to prevent dispersions during PCR cycles.

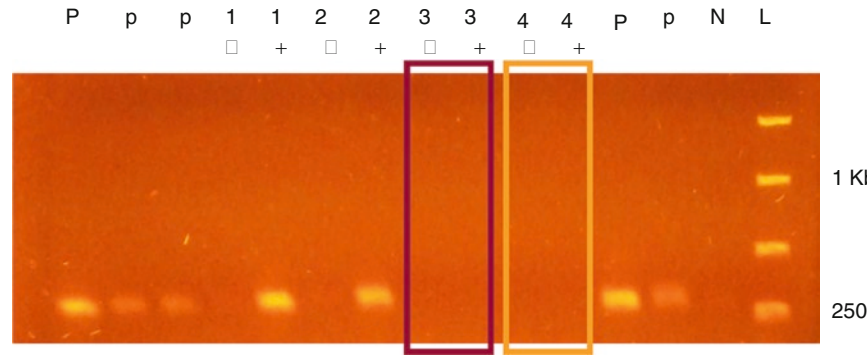

Fig. 10.2 Agarose gel electrophoresis of the *Alu* assay performed to detect PCR inhibition (30 cycles). P: positive PCR control (10 pg of high molecular weight DNA); p: positive control (1 pg of high molecular weight DNA); N: negative PCR control; 1, 2, 3 and 4 indicate four aged forensic samples amplified as alone (−) and in the presence (+) of the internal positive control (P). In samples 1 and 2 amplifications occurred only when added with the internal positive control. This indicates no or low copy numbers of entire templates. For samples 3 and 4 (indicated by the rectangles) amplification never occurred. In these cases, the presence of inhibitors has to be considered. L: molecular weight ladder

unsuitable template (no or low copy number of entire template) (Fig. 10.2).

- Mix well and dispense 13 µl of this solution into the PCR tubes (discarding the remaining volume of about 13 µl).
- Cover this solution by a drop of mineral oil.
- Add the water into the tubes that require it.
- Finally add the DNA.
- Run the cycles as described above.
- Analyse 4 µl of each reaction by electrophoresis through a 2% agarose gel containing EtBr as usual.

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