

DNA Extraction and Quantitation of Forensic Samples Using the Phenol–Chloroform Method and Real-Time PCR

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Summary

Forensic laboratories are increasingly confronted with problematic samples from the scene of crime, containing only minute amounts of deoxyribonucleic acid (DNA), which may include polymerase chain reaction (PCR)-inhibiting substances. Efficient DNA extraction procedures, as well as accurate DNA quantification methods, are critical steps involved in the process of successful DNA analysis of such samples. The phenol–chloroform method is a sensitive method for the extraction of DNA from a wide variety of forensic samples, although it is known to be laborious compared with single-tube extraction methods. The relatively high DNA recovery and the quality of the extracted DNA speak for itself. For reliable and sensitive DNA quantitation, the application of real-time PCR is described. We modified a published real-time PCR assay, which allows for the combined analysis of nuclear and mitochondrial DNA, by introducing 1) improved hybridization probes with the use of minor groove binders; 2) an internal positive control (for both nuclear and mitochondrial DNA) for the detection of PCR inhibitors; and 3) different amplicon lengths for the determination of the degradation state of the DNA. The internal positive controls were constructed by site directed mutagenesis by overlap extension of the wild-type mitochondrial and nuclear DNA target with the advantage that no additional probes, which are cost-intensive, are required. The quantitation system is accomplished as a modular concept, which allows for the combined determination of the above-mentioned features (quantity/inhibition or quantity/degradation) depending on the situation,

Key Words: Real-time PCR (rtPCR); phenol–chloroform method; forensic DNA extraction.

1. Introduction

Successful deoxyribonucleic acid (DNA) profiling of forensic samples is largely dependent on the quality and the amount of DNA that is recovered from the sample in question. This is of particular importance when casework samples are to be analyzed, which frequently involve difficult specimen that contain only minute amounts of DNA and are likely to have suffered environmental stress (DNA degradation). Apart from that, the quality of a forensic trace is impacted by the nature of the substrate on which it was deposited (e.g., blood on denim). When the DNA is extracted from the source, trace compounds of the substrate may be coextracted, which can further influence the typing process. As a consequence, the efficiency and sensitivity of the extraction procedure are critical parameters that define the suitability of an extraction method for forensic samples.

The phenol–chloroform method (*1*) is a well-established forensic extraction procedure even though it is known to be laborious compared with alternative approaches and involves toxic reagents, such as phenol, which requires special safety precautions in the laboratory (laminar flow fume hood). As a matter of fact, phenol–chloroform extraction is still the method of choice for samples containing only very little amounts of DNA (e.g., hair shafts, bone samples, decomposed samples) or samples suspected to contain polymerase chain reaction (PCR)-inhibiting substances. Although this method involves intensive manual interaction—the sample is handled through at least three generations of reaction tubes—the DNA recovery of the phenol–chloroform extraction is known to be relatively high.

To control the success of the extraction step, DNA quantitation usually is performed for forensic casework material, although the usability of a sample in the forensic context is determined by the ability to generate a DNA profile after all. The information on the DNA quantity is mostly used to estimate the appropriate amount of DNA extract to be added to the PCR master mix to avoid overloading with excess of DNA. Common quantitation techniques involve photometric/fluorometric determination of the DNA amount, which are insensitive to the source of DNA, in contrast to specific DNA-based hybridization assays (*2–4*). Both of these are less sensitive to the detection of DNA compared with the subsequent PCR assay, that is, extracts, in which quantitation failed, may still bring a useful DNA profile. A negative quantitation result does not necessarily indicate the absence of DNA—in contrast, the actual DNA amount may be masked as a result of substances included in the extract that interfere with the detection method. This is why some laboratories refrain from quantitation per se and directly apply an aliquot of the extract to PCR (mostly singleplex or small multiplexes) to estimate the quantity by analyzing the

peak heights of the resulting DNA profile. Note that the latter method is not an explicit DNA quantitation method, but the estimate is based on the experience of the individual laboratory.

In contrast to the above-mentioned quantitative techniques, real-time PCR (rtPCR) is a very sensitive, stable, reproducible, and specific DNA quantitation method and leads to directly applicable results because the amount of DNA is inferred by the same process (i.e. PCR), which is then used for DNA profiling.

Here, we describe a rtPCR assay that is based on the coamplification of a nuclear DNA (nDNA) target (Retinoblastoma gene) and a mitochondrial DNA (mtDNA) target (spans over the genes for transfer ribonucleic acid lysine and ATP synthase 8) modifying the method published by Andreasson et al. (5). We added the following three features to the assay. First, fluorescent probes with a minor groove binder (MGB; ref. 6) are used, which enhance the sensitivity of the hybridization and the PCR efficiency for the amplification of longer fragments. Second, an internal positive control (IPC) is coamplified with the genome-specific target (either nDNA or mtDNA), which allows for the detection of PCR inhibitor present in a sample. This information is useful for the subsequent processing of the sample as (additional) purification or a dilution of the DNA extract may be the consequence to overcome inhibition. Third, the degradation stage of the DNA can be determined by application of different amplicon lengths used for the quantitation process. This serves as basis for further selection of amplification kits or locus-specific primer pairs and which helps with the interpretation of DNA profiles deduced from that extract.

1.1. Phenol–Chloroform Method

In general, this method involves disruption and lysis of the stain material, digestion of cell components and removal of contaminants by organic solvents. The DNA is finally recovered by alcohol and salt precipitation and subsequent rehydration. An alternative protocol involves the purification of the extracted DNA by means of column based methods instead of the alcohol precipitation (7).

Cell lysis is performed using an enzyme- and detergent-based buffer (Proteinase K with sodium dodecyl sulfate). Organic extraction is performed by adding an equal volume of water-saturated, buffered phenol to the aqueous DNA sample, vigorously vortexing the mixture, and centrifugation to allow phase separation. The upper, aqueous layer is carefully removed to a new tube, avoiding the phenol interface. This step is followed by the addition of chloroform to extract residual phenol from the aqueous phase. The DNA is concentrated by ethanol precipitation in the presence of salt. After washing with 70% ethanol, the pellet DNA is dried in a speed vac and dissolved in low salt buffer. This method is suitable for the extraction of DNA from a wide range of cell types and stain materials.

If the stain material consists of a mixture of sperm and nonsperm cells, such as epithelial cells, the phenol–chloroform extraction is preceded by a step called differential extraction (8). Differential extraction is a procedure in which sperms are separated from the other cells before lysis and DNA extraction. This is accomplished by selective digestion of the nonsperm cell fraction and separation of intact sperms by centrifugation. The nonsperm cell DNA is isolated under mild conditions that break only the epithelial cells but leave the sperm cells intact.

For the extraction of DNA from hairs, a buffer system containing Proteinase K and Ca^{2+} instead of ethylene diamine tetraacetic acid (EDTA) improves the efficiency of hair digestion significantly, resulting in an enhanced success rate in DNA typing (9).

1.2. *rtPCR Quantitation Using the TaqMan Assay*

The TaqMan assay is a real-time, homogeneous PCR system in which the sample is amplified and typed simultaneously without the need for additional manipulations post-PCR. The assay uses standard PCR primers to generate an amplicon and an internal fluorescent hybridization probe (10), which is specific for a sequence region within the amplicon. The assay is run on an instrument that is capable of measuring fluorescence directly through the lid of the reaction tube at each cycle of PCR—so that detection occurs online. A quencher molecule is attached to the 3' end of the probe, so that the probe does not emit fluorescence in its normal state. During PCR, the amplicon accumulates and the probe specifically binds to the product during the annealing phase. In the extension phase, Taq polymerase cleaves the probe via its 5'-exonuclease activity and thus separates the fluorochrome and quencher molecules (11–14). At this point, fluorescent signal is emitted and detected. The course of the reaction can be displayed by an amplification plot (**Fig. 1A**). The initial cycles of PCR, in which only little change in fluorescence occurs, are used to define the baseline for the amplification plot. The threshold fluorescence signal is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the cycle threshold (C_t ; refs. 15 and 16). The ΔR_n -value (baseline-corrected endpoint reporter signal) is a measure of the amount of PCR product amplified in the course of real time PCR. When C_t values are plotted against the decade log of the initial target copy number, a straight line is obtained (**Fig. 1B**). This line can be used to identify the dynamic range of the assay and can be used to quantify the amount of initial target DNA from an unknown sample by calculating the actual template copy number from the derived C_t value (calibration with known DNA standards required; see **Note 1**).

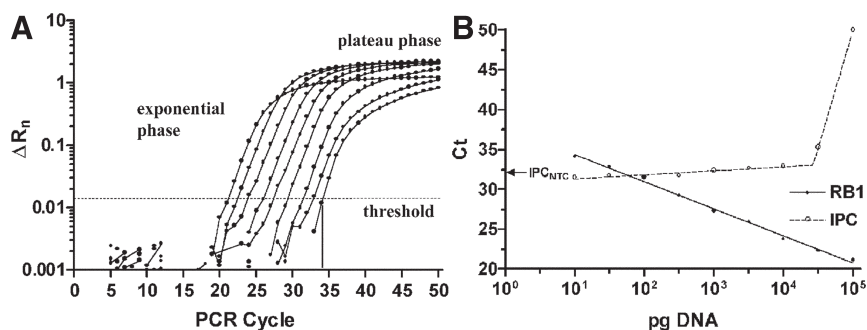


Fig. 1. (A) Amplification plots of a dilution series of the DNA standard (genomic DNA) showing the changes in fluorescence vs PCR cycle number (logarithmic view). (B) Standard curve derived from the data shown in (A); the threshold cycle C_t is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. C_t values are plotted against the amount of template DNA. Also shown are C_t values of the IPC, which is included in the assay for the detection of PCR inhibitors; note that elevated C_t values of the IPC indicate the loss of amplification resulting from the competitive consumption of PCR components by elevated template concentrations (10^4 – 10^5).

1.2.1. MGBs

The modified probes are characterized by a MGB moiety that fits into the minor groove of double-stranded DNA, which increases the stability and specificity of probe hybridization and finally enhances the PCR efficiency for longer DNA fragments (Table 1; *see Note 2*). Because of the increased stability, TaqMan MGB probes are very short (approx 13 to 20 bases long) compared with standard TaqMan probes.

Furthermore, MGB probes are labeled with a nonfluorescent quencher (NFQ) in place of the previous standard TAMRA quencher dye. The NFQ, also called “dark quencher,” does not emit detectable fluorescence, leading to a less complex signal with lower fluorescent background, which improves spectral discrimination and makes data interpretation easier.

1.2.2. IPC

To evaluate the performance of the rtPCR assay and to include an objective measure of potential PCR inhibition, we introduced an IPC that is amplified simultaneously in the same assay with the sample to be quantified. The IPC brings an amplification result in another dye layer and can therefore be evaluated independently from the signal of the unknown sample. The C_t value for the IPC ($C_{t_{IPC}}$) is set to 25–32 cycles (by limiting the primer concentration or

Table 1
Apparent rtPCR Efficiency Using BHQ and MGB TaqMan Probes
for Different Amplicon Lengths

Fragment lengths for RB1	BHQ	MGB
79-bp fragment	0.9896 (0.9496–1.0331)	0.9852 (0.9417–1.0332)
156-bp fragment	0.8604 (0.8257–0.8981)	0.9795 (0.9576–1.0025)
246-bp fragment	0.7222 (0.6919–0.7554)	0.8583 (0.7950–0.9318).

The rtPCR efficiency can be calculated from the slope of the standard curve by the formula $E = (10^{-1/\text{slope}}) - 1$. The numbers in brackets show 95% confidence intervals.

the copy number), to avoid troublesome competition between the amplification of the IPC and the genuine sample (*see Note 3*). PCR inhibitors in the DNA extract would be recorded by increased Ct values. Partial inhibition would result in Ct values between that of the negative controls (no template controls) and the total amount of cycles used for PCR (*see Note 4*).

The IPCs were constructed by site-directed PCR mutagenesis by overlap extension (SOE; refs. **17–19**) of the wild-type mtDNA and nuclear RB1 target. SOE results in the introduction of specific mutations in the modified template differing from the original sequence in a way that the modified template would not be amplified with the conventional primers, whereas the modified primers would only amplify the SOE product. This is true for both the nDNA and the mtDNA target. This is why the mtDNA–SOE product can be used as IPC for the quantitation of nDNA and the IPC_{nDNA} as control for the quantitation of mtDNA. SOE is a fast and technically simple approach for manipulating DNA-sequences. In general, four primers are needed to introduce site-specific mutation(s) (**Fig. 2**). Two PCRs are performed, each using a perfectly complementary primer at the end of the sequence and a mismatched primer designed to introduce a mutation at a specific position. This results in overlapping fragments, in which the mutation is located in the region of the overlap (*see Notes 5,6*). The overlapping fragments are then annealed to each other in a second round of PCR where the entire mutated DNA fragment is amplified by means of the two complementary primers at the end of this DNA fragment.

The advantages of this procedure are that IPCs can be easily designed and kept in house, minimizing the costs of the assay. Alternative IPCs would require additional probes, which are cost-intensive.

1.2.3. Checking for DNA Degradation

The use of various fragment lengths for the rtPCR quantitation of a specific DNA target allows for the determination of DNA degradation. The latter can

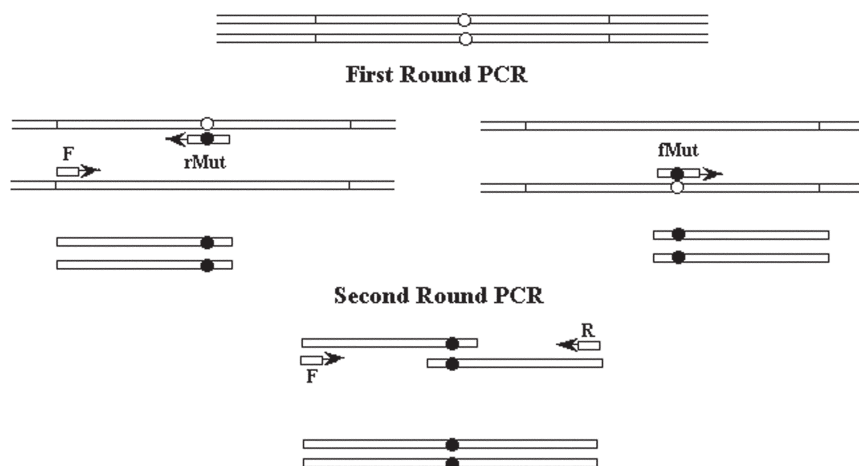


Fig. 2. First-round PCR: in two separate PCR reactions, two partially overlapping DNA fragments are amplified. The first primer pair is used to amplify the DNA that contains the mutation site together with upstream sequences. In this reaction, the reverse SOE primer (rMut) contains the mutation(s) to be introduced into the wild-type DNA-template. The second primer pair is used to amplify the DNA that contains the mutation site together with downstream sequences. In this reaction, the forward SOE primer (fMut) contains the mutation(s) to be introduced into the wild-type DNA-template. Second-round PCR: The overlapping fragments are mixed, denatured, annealed, and extended using the flanking primers F and R.

be assessed by comparing the amplification results of the different amplicons. These modifications upgrade the original rtPCR protocol to a modular concept which, depending on the combination of the individual modules, can provide information both about the quantity and the quality of DNA by using only two fluorogenic oligoprobes. In **Fig. 3**, a selection of some of these possible combinations is displayed.

2. Materials

2.1. Phenol–Chloroform Method

1. Sterile distilled water.
2. Hydrogen peroxide 30%.
3. 1 M Tris-HCl solution, pH 8.0.
4. 1 M Tris-HCl solution, pH 9.0.
5. 0.5 M EDTA disodium salt.
6. 5 M Sodium chloride.
7. Ethanol 100%.

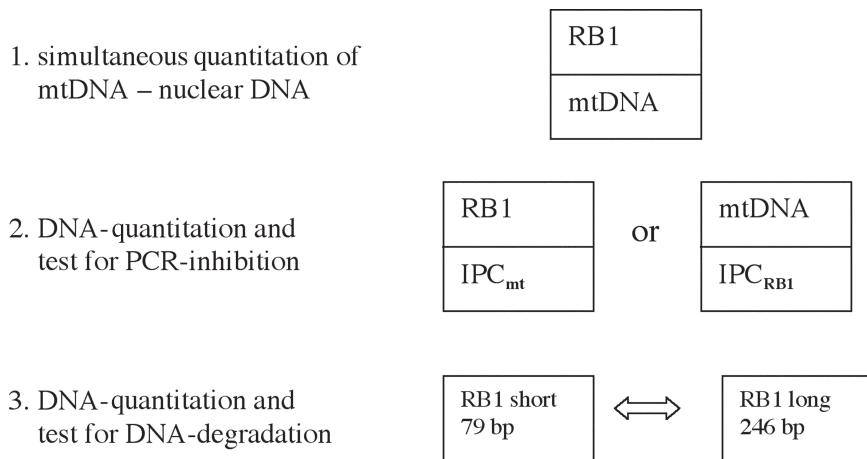


Fig. 3. Schematic representation of how the modular concept of rt PCR can be used to check besides DNA quantity for PCR inhibition or DNA degradation. Setup 1 allows simultaneous quantitation of nuclear and mtDNA as published by Andreasson et al. (5). Setup 2 allows simultaneous quantitation of nuclear or mtDNA and detection of PCR inhibitors. Setup 3 allows quantitation of nuclear DNA and detection of fragmentation but needs two separate reactions.

8. Ethanol 70%.
9. Proteinase K stock solution: 20 mg/mL (dissolve 100 mg of proteinase K in 5 mL of sterile distilled water; store frozen in 1-mL aliquots).
10. 1 M dithiothreitol (DTT) solution.
11. Phenol solution (+ bottle of equilibration buffer for pH 8.0).
12. Chloroform:isoamylalcohol solution 24:1.
13. 3 M sodium acetate buffer solution, pH 5.2.
14. 10% sodium dodecyl sulfate solution.
15. Linear polyacrylamide(LPA; 5 mg/mL, Ambion).
16. 1 M Calcium chloride solution.
17. Extraction buffer (EB): 10 mM Tris-HCl, pH 8.0, 10 mM EDTA disodium salt, pH 8.0; 100 mM sodium chloride; and 2% sodium dodecyl sulfate.
18. Extraction buffer for hairs (EBH): 10 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 100 mM sodium chloride, 2% sodium dodecyl sulfate.
19. Sterile Petri dishes.

All extractions are performed in sterile 1.5-mL Sarstedt tubes with screw caps.

2.2. rtPCR Quantitation

1. ExoSapIT Amersham/Pharmacia.
2. TOPO TA Cloning Kit, Invitrogen Life Technologies.
3. TaqMan® Universal PCR Master Mix, No AmpErase® UNG.

2.3. Sequence of Primers and Fluorogenic Probes

Nuclear Target: *RB1 Gene*

Primers for cloning:

pRB1 F 5'-AGGTTGCTAACTATGAAACACTGGC-3'

pRB1 R 5'-CCATCTCAGCTACTGGAAAACATTC-3'

Primers for 79-bp amplicon: (5)

RB1-2672 F 5'-CCAGAAAAATAATCAGATGGTATGTAACA-3'

RB1-2750 R 5'-TGGTTTAGGAGGGTTGCTTCC-3'

Primers for 156-bp amplicon:

pRB1 F 5'-AGGTTGCTAACTATGAAACACTGGC-3'

RB1-2750 R 5'-TGGTTTAGGAGGGTTGCTTCC-3'

Primers for 246-bp amplicon:

pRB1 F 5'-AGGTTGCTAACTATGAAACACTGGC-3'

pRB1 R 5'-CCATCTCAGCTACTGGAAAACATTC-3'

BHQ probe:

RB1-2727 BHQ 5'-FAM-CAGCACTTCTTTTGAGCACACGGTCG-BHQ1 - 3'

MGB probe:

RB1-2727 MGB: 5'-FAM-CAGCACTTCTTTTGAGCAC-MGBNFQ-3'

Primers for SOE:

RB1 2750 SOE F 5'-GTGCTGAACTAACCAACGCTCCGAAACGACTGAA -3'

RB1 2750 SOE R 5'-TTCAGTCGTTTCGGAGCGTTGGTTAGTTCAGCACTTC-3'

pRB1 F 5'-AGGTTGCTAACTATGAAACACTGGC-3'

pRB1 R 5'-CCATCTCAGCTACTGGAAAACATTC-3'

Primers for RB1-IPC

RB1 2750 IPC R 5'-TCGTTTCGGAGCGTTGGTTAG 3'

RB1-2672 F 5'-CCAGAAAAATAATCAGATGGTATGTAACA-3'

Mitochondrial Target: *Spans Over the Genes for Transfer Ribonucleic Acid Lysine and ATP Synthase 8*

Primers for cloning:

pMt F 5'-GGGTATACTACGGTCAATGCCTCTGA-3'

pMt R 5'-CAATGAATGAAGCGAACAGATTTTC-3'

Primers for mt target: (5)

mt-8294 F 5'-CCACTGTAAAGCTAACTTAGCATTAAACC-3'

mt-8436 R 5'-GTGATGAGGAATAGTGTAAGGAGTATGG-3'

BHQ-Probe:

mt-8345 BHQ 5'-JOE-CCAACACCTCTTTACAGTGAAATGCCCA-BHQ1 - 3'

MGB-Probe:

mt-8345 MGB 5'-VIC - CCA ACA CCT CTT TAC AGT GAA-MGBDQ-3'

Primers for SOE:

mt8294 SOE F 5'- GCCCAGTG TAGAGCTATGTTAGCATTTAGGTTTTAAGTTAA-3'

mt8294 SOE R 5'- TAAAACCTAAAATGCTAACATAGCTCTACACTGGGCTCTAGAG-3'

pMt F 5'-GGGTATACTACGGTCAATGCCTCTGA-3'

pMt R 5'-CAATGAATGAAGCGAACAGATTTTC-3'

Primers for mt-IPC

mt8294 IPC F 5'-CCAGTG TAGAGCTATGTTAGCATTTAGG-3'

mt-8436 R 5'- GTGATGAGGAATAGTGTAAGGAGTATGG-3'

4. Bovine serum albumin fraction V.
5. Sterile distilled water.
6. DNA standards.

3. Methods

3.1. Phenol–Chloroform Method

3.1.1. Extraction of DNA From All Types of Cells and Stain Materials Except Hairs and Sperm-Cell/Nonsperm-Cell Mixtures

1. To the sample add 500 μL of extraction buffer plus 20 μL of proteinase K (20 mg/mL).
2. Vortex and incubate at 56°C overnight with agitation in a Thermomixer.
3. Add an equal volume of buffered Phenol (pH 8.0; *see* **Notes 7, 8**), vortex, and spin for 10 min at maximum speed in a microcentrifuge.
4. Transfer the aqueous (upper) phase to a new microcentrifuge tube with 0.5 mL of chloroform (*see* **Note 9**).
5. Vortex and spin for 10 mins at maximum speed in a microcentrifuge.
6. Transfer the aqueous (upper) phase to a new microcentrifuge tube with 50 μL of 3 M NaAc, pH 5.2.
7. Add 0.8 mL of 100% EtOH, vortex, and precipitate at –20°C for at least 1.5 h (*see* **Notes 10, 11**).
8. Recover DNA by centrifugation for 30 min at maximum speed in a microcentrifuge and decant the supernatant.
9. To the pellet add 1 mL of 70% EtOH and spin for 20 min at maximum speed in a microcentrifuge.
10. Dry pellet in a vacuum centrifuge for 30 min (*see* **Note 12**).
11. Dissolve pellet in 50 μL of Tris buffer (10 mM, pH 9.0).

3.1.2. Extraction of DNA From Hairs

1. Cut hair in 1.5-mL Sarstedt tube already containing 500 μL of extraction buffer for hairs (EBH).
2. Add 20 μL of proteinase K solution (20 mg/mL) and 20 μL of DTT solution.
3. Proceed with **Subheading 3.1.1.** at **step 2**.

3.1.3. Extraction of DNA From Sperm-Cell/Nonsperm-Cell Mixtures

1. To the stain add 500 μL of extraction buffer plus 20 μL of proteinase K (20 mg/mL).
2. Vortex and incubate at 37°C for 30 min with agitation in a Thermomixer.
3. Remove stain material with tweezers sterilized in 10% H_2O_2 and spin for 5 min at maximum speed in a microcentrifuge (*see* **Note 13**).
4. Transfer supernatant (= nonsperm-cell fraction) to a new tube and proceed with **Subheading 3.1.1., step 2**.
5. Add 1 mL of sterile water to the pellet, vortex and spin for 5 min at maximum speed in a microcentrifuge, and remove the supernatant.

- 6. Repeat **step 5** twice.
- 7. Add 500 μL of extraction buffer plus 20 μL of proteinase K (20 mg/mL) plus 20 μL DTT (1 M) and proceed with **Subheading 3.1.1., step 2.**

3.2. rtPCR

3.2.1. Generation of IPCs by Site-Directed Mutagenesis by Overlap Extension (SOE) of the RB1 and mtDNA Target

- 1. Set up PCR for amplification of target sequence to be mutagenized by mixing the following reagents:

	RB-1	mtDNA
H ₂ O, sterile	12.8 μL	12.8 μL
10X PCR buffer	2 μL	2 μL
dNTP mix (2.5 mM each)	1.6 μL	1.6 μL
pRB1 F (10 μM)	0.6 μL	–
pRB1 R (10 μM)	0.6 μL	–
pMt F (10 μM)	–	0.6 μL
pMt R (10 μM)	–	0.6 μL
Ampli Taq Gold (5 u/ μL)	0.4 μL	0.4 μL
Template DNA (5 ng/ μL)	2 μL	2 μL
	20 μL	20 μL

- 2. Amplify using the denaturation, annealing, and extension times and temperatures listed in the table below

Cycle number	Denaturation	Annealing	Extension
Initial hold	10 min at 95°C		
30 cycles	15 s at 95°C	30 s at 61°C	45 s at 72°C
Final hold	10 min 72°C		

- 3. Set up first-round SOE-PCR 1 by mixing the following reagents:

	RB-1	mtDNA
H ₂ O, sterile	12.9 μL	12.9 μL
10X PCR buffer	2 μL	2 μL
dNTP mix (2.5 mM each)	1.6 μL	1.6 μL
pMt F (10 μM)	–	1 μL
mt8294 SOE R (10 μM)	–	1 μL
pRB1 F (10 μM)	1 μL	–
RB1 2750 SOE R (10 μM)	1 μL	–
Ampli Taq Gold (5 u/ μL)	0.5 μL	0.5 μL
Amplicon RB1 (1:10.000)	1 μL	–
Amplicon mtDNA (1:100.000)	–	1 μL
	20 μL	20 mL

4. Set up first round SOE-PCR 2 by mixing the following reagents:

	RB-1	mtDNA
H ₂ O, sterile	12.9 mL	12.9 mL
10X PCR buffer	2 µL	2 µL
dNTP mix (2.5 mM each)	1.6 µL	1.6 µL
mt8294 SOE F (10 µM)	–	1 µL
pMt R (10 µM)	–	1 µL
RB1 2750 SOE F (10 µM)	1 µL	–
pRB1 R (10 µM)	1 µL	–
Ampli Taq Gold (5 u/µL)	0.5 µL	0.5 µL
Amplicon RB1 (1:10.000)	1 µL	–
Amplicon mtDNA (1:100.000)	–	1 µL
	20 µL	20 µL

5. Amplify using the denaturation, annealing, and extension times, and temperatures listed in the table below:

Cycle number	Denaturation	Annealing	Extension
Initial hold	10 min at 95°C		
15 cycles	15 s at 95°C	30 s at 56°C	30 s at 72°C
26 cycles	15 s at 95°C	30 s at 60°C	30 s at 72°C

6. Pool equal volumes of the amplification products of SOE PCR 1 and SOE PCR 2 for the individual targets and purify the PCR products using the ExoSapIT Kit from Amersham/Pharmacia according to the manufacturer's instructions (*see Note 14*).

7. Set up the second-round SOE-PCR by mixing the following reagents:

Pre-Mastermix

	RB-1	mtDNA
H ₂ O, sterile	4.8 µL	4.8 µL
10X PCR buffer	2 µL	2 µL
dNTP mix (2.5 mM each)	1.6 µL	1.6 µL
pMt F (10 µM)	–	0.6 µL
pMt R (10 µM)	–	0.6 µL
pRB1 F (10 µM)	0.6 µL	–
pRB1 R (10 µM)	0.6 µL	–
50X Advantage2 PolMix	0.4 µL	0.4 µL
	10 µL	10 µL

Final Mastermix

	RB-1	mtDNA
H ₂ O sterile	8 µL	8 µL
Pre-Mastermix	10 µL	10 µL
Pooled and purified SOE1 amplification products	2 µL	2 µL
	20 µL	20 µL

8. Amplify using the denaturation, annealing, and extension times and temperatures listed in the table below:

Cycle number	Denaturation	Annealing	Extension
Initial hold	2 min at 95°C		
5 cycles	15 s at 95°C	45 s at 72°C	
28 cycles	15 s at 95°C	30 s at 60°C	45 s at 72°C
Final hold	30 min at 72°C		

9. Check amplicons on a gel and clone into TAvector according to the manufacturers instructions.
10. Verify the induced mutations and the correct sequences of the IPCs by sequencing.

3.2.2. *rtPCR for Quantitation of nuclear DNA (+ mtIPC)*

1. Set up PCR for quantitation of nuclear DNA (+ mtIPC) by mixing the following reagents:

rtPCR Pre-Mastermix

10X BSA (2.5 mg/mL)	2 µL
pRB1 F (10 µM)	0.6 µL
RB1-2750 R (10 µM)	0.6 µL
RB1-2727 MGB (10 µM)	0.4 µL
mt-8294 IPC F (10 µM)	0.28 µL
mt-8436 R (10 µM)	0.28 µL
mt-8345 MGB (10 µM)	0.4 µL
plasmid mt-IPC (40,000 copies/µL)	0.44 µL

2. rtPCR Mastermix for quantitation of nuclear DNA (+ mtIPC); 10 µL of TaqMan® Universal PCR Master Mix, 5 µL of rt-PCR Pre-Mastermix for quantitation of nuclear DNA (+mt-IPC); and up to 20 µL of H₂O and/or sample (*see Note 15*).
3. When the setup is complete, seal reactions, spin briefly (1 min at 1000g), place plate into the thermal cycler block, and start the run (*see Note 16*).
4. Interpretation guidelines for possible results, which can be obtained with the real time PCR-setup: DNA quantitation and test for PCR inhibition.

Target DNA	IPC	
+	+	Detectable signals (i.e. Ct values smaller than the number of cycles performed) both for the target DNA and the IPC means that the extract contains no inhibitors. To exclude partial inhibition, the Ct-values of the IPC obtained for the unknown samples have to be in the range of those observed in the no template controls (NTCs).
+	–	A high copy number of target DNA can lead to elevated Ct values or even a complete drop-out of the IPC resulting from the using up of the reaction components by the more abundant target.

- | | | |
|---|---|--|
| – | + | No detectable signal for the target DNA but a detectable signal for the IPC indicates that the DNA content of the extract is below the detection limit of the rt-PCR method. |
| – | – | No detectable signal for the target DNA and IPC in the unknown sample, but detectable IPC signal in the no template controls, indicates the presence of PCR-inhibitor(s) in the extract. The copy number of the target can't be assessed at this stage |

4. Notes

1. When plasmids (containing the nuclear or mitochondrial target) are used for the generation of standard curves, rtPCR amplification efficiencies have to be compared to genomic DNA (by comparing the slopes of the standard curves) as the circular form of plasmids may negatively affect PCR efficiency. If this is the case, the plasmids have to be linearized.
2. Besides increased sequence specificity and lower fluorescent background of MGB probes in comparison to unmodified probes, MGB probes increase the apparent PCR-efficiency for longer amplicons as displayed in **Table 1**.
3. To include an internal positive control into the real time PCR setup following rules have to be followed: 1) the IPC must not influence the PCR efficiency for the target sequence, especially at low DNA concentrations of the target sequence; and 2) Ct-values for the IPC should be stable over a wide range of copies of target DNA (The Ct-value of the IPC can be adjusted either by limiting primer concentrations or the amount of the IPC-template).
4. To demonstrate that the rtPCR setup with the IPC included is appropriate to reveal the presence of PCR inhibitors in a specific DNA extract, we performed mock DNA extractions on materials known to contain PCR inhibitors and spiked our standard curve with constant volumes of the extracts. The effect of PCR-inhibitor(s) on the rtPCR reaction parameters are shown in **Fig. 4**, considering blue denim as example.
5. The region of overlap between the two SOE primers should contain a number of bases corresponding to a T_m of approx 68–72°C.
6. The 3' ends of both SOE-primers should be at least 10 bases in length without mismatch to the original target sequence.
7. For the addition of phenol and chloroform/isoamylalcohol we use a dispenser. Before use the dispenser pipe is cleaned with 10% hydrogen peroxide solution and the first two volumes are discarded from the dispenser.
8. Phenol is a hazardous waste material that needs to be disposed properly. Phenol is highly corrosive and can cause severe burns to skin and damage clothing. Gloves, safety glasses, and a lab coat are to be worn whenever working with phenol, and all manipulations should be carried out in a fume hood.

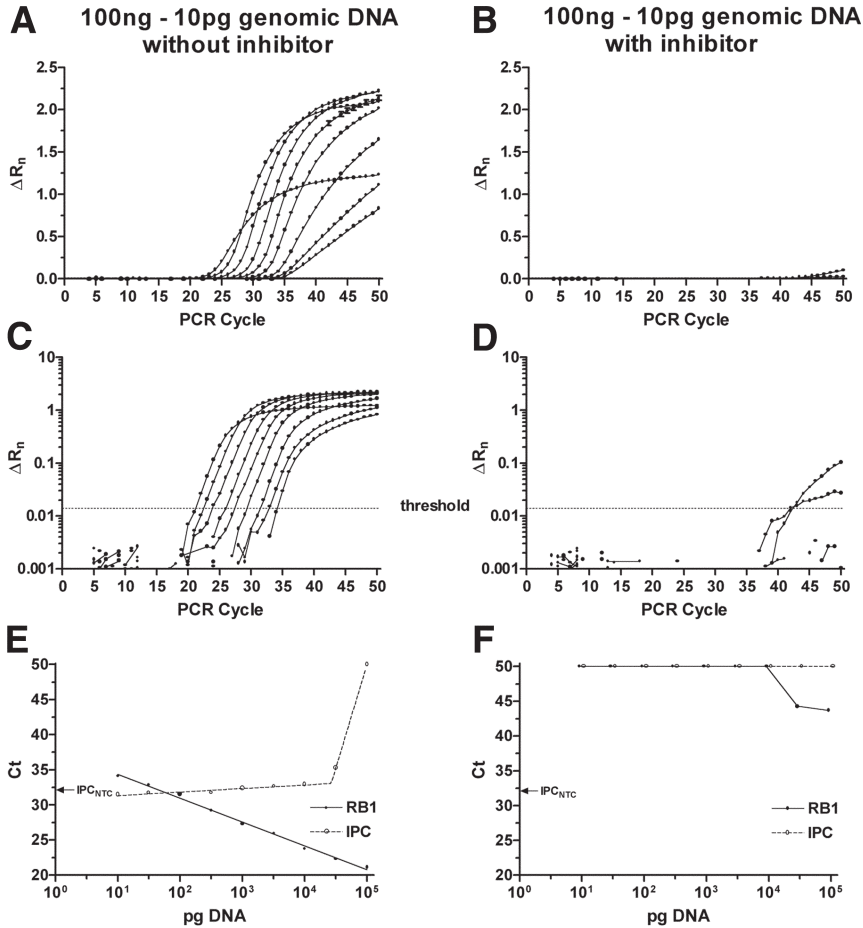


Fig. 4. (A) Amplification plots of a dilution series (10 pg to 100 ng) of the DNA standard—linear view. (B) Amplification plots of the same dilution series of the DNA standard spiked with constant amounts of the inhibitor—linear view. The addition of extract from blue denim led to complete inhibition of rtPCR at any concentration of the standard curve up to 10 ng input DNA (C_t -values equal to that of the no template controls and the total amount of cycles). (C) is the same as (A) but displayed in log view. (D) is the same as (B) but displayed in log view. (E) Standard curve derived from the data shown in (A) and (C); C_t values are plotted against the amount of template DNA. Also shown are C_t values of the IPC (note the elevated C_t values and the complete drop-out of the IPC at high amounts of input target DNA as a result of the consumption of the reaction components by the more abundant target). (F) Standard curve derived from the data shown in (B) and (D).

9. The two phases (organic phase and aqueous layer) need to be carefully separated in that the nucleic acids and proteins tend to be at the interface. Leaving too much of the aqueous layer behind will cause loss of material and aspirating too close to the interface can include protein.
10. Precipitation of the DNA at -20°C should be performed at least for 1.5 h. It is possible to place the samples at -20°C overnight at this stage.
11. For extractions from stains with small amounts of DNA, use LPA as a coprecipitant. LPA offers the advantage that it is chemically synthesized and is not derived from biological sources. Other carriers may contain small amounts of contaminating nucleic acids, which may cause problems, especially when typing mtDNA as seen in our laboratory when working with glycogen as carrier.
12. While drying the DNA pellet in the vacuum centrifuge, the screw caps of the tubes are kept in sterile Petri dishes.
13. Concerning DNA extraction from sperm-cell/nonsperm-cell mixtures, when removing the stain material with tweezers, it is important to squeeze the stain firmly to prevent loss of liquid and DNA.
14. Depending on the target sequence, the addition of nontemplate 3' overhanging residues by *Taq* DNA polymerase can lead to undesired mutations when using the SOE technique. To remove 3' overhanging residues and nonincorporated primers, purify the PCR products using the ExoSapIT Kit from Amersham/Pharmacia.
15. It is recommended to include at least three no template controls in each reaction plate in addition to extraction blocks.
16. rtPCR cocktails in a plate can be stored in the dark at room temperature for a couple of hours.

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Forensic DNA Typing Protocols

Carracedo, A. (Ed.)

2005, X, 280 p. 81 illus., Hardcover

ISBN: 978-1-58829-264-3

A product of Humana Press