Published, February 11,2022
RA05I003.001

Method of Test for Mammalian Nucleic Acids in Placental Samples

1. Scope

This method is applicable to the qualitative detection of mammalian nucleic acid components in placental samples.

2. Method

After DNA extraction, perform polymerase chain reaction (PCR) and confirm the PCR products by gel-electrophoresis.

2.1. Work environment

Procedure of sample pretreatment, DNA extraction, PCR reagent preparation, and PCR assay require separate compartments to avoid cross-contamination.

2.2. Equipment

2.2.1. Polymerase chain reaction machine: Veriti® Thermal Cycler, or an equivalent product.

2.2.2. Electrophoresis system: MJ-105A, or an equivalent product.

2.2.3. Evaporator: Genevac EZ-2, or an equivalent product.

2.2.4. Imaging system: ChemiDoc XRS$^+$, or an equivalent product.

2.2.5. Imaging system software: Image Lab Software, or an equivalent product.

2.2.6. Microvolume spectrophotometer: NanoDrop 2000, or an equivalent product.

2.2.7. Refrigerated microcentrifuge: centrifugal force ≥ 20,000 ×g, with temperature control at 4°C.

2.2.8. Water bath: capable of controlling water temperature at ± 1.0°C.

2.2.9. Laminar flow.

2.2.10. Balance: weighing up to 2,000 g with sensitivity of 0.1 g, weighing up to 100 g with sensitivity of 1 mg.

2.3. Chemicals

2.3.1. DNA extraction

Deionized water, PCR grade;
96-100% ethanol, chemical grade;
ammonium bicarbonate, BioUltra grade (≥ 99.5%);
commercial kits for animal DNA extraction.

2.3.2. PCR

2.3.2.1. Primer (Note1)

2.3.2.1.1. Mammal (12S mitochondrial DNA)

Primer F: 5’-CCCAAACTGGGATTAGATACCC-3’

Primer R: 5'-GTTTGCTGAAGATGGCGGTA-3'
PCR amplicon length: 215 bp

2.3.2.1.2. Mammal (16S mitochondrial DNA)
Primer F: 5'-GCCTGTTTACCAAAAACATCAC-3'
Primer R: 5'-CTCCATAGGGTCTTCTCGTCTT-3'
PCR amplicon length: 244 bp

Note1: After unpacking, dilute the synthesized primers with sterile deionized water to proper concentration, and store at -20°C until use.

2.3.2.2. PCR commercial kits (Containing DNA polymerase, reactions buffer, deoxy-ribonucleoside triphosphate (dNTP), diethyl pyrocarbonate- H_2O (DEPC-H_2O)).

2.3.3. Chemicals for gel-electrophoresis
Deionized water, PCR grade;
agarose, tris(hydroxymethyl)aminomethane (Tris), boric acid, ethylenediaminetetraacetic acid (EDTA), 6x loading dye, Safe view DNA stain and 100 bp DNA ladder marker, molecular bioanalytical grade.

2.3.4. Reference material

2.3.4.1. Negative reference material: deionized water.

2.3.4.2. Positive reference material: plasma or serum of mammal.

2.4. Apparatus (Note2)

2.4.1. Pipette: 10 µL, 20 µL, 100 µL, 200 µL, and 1,000 µL.

2.4.2. Pipette tips: 10 µL, 20 µL, 200 µL, and 1,000 µL.

2.4.3. Centrifuge tube: 200 µL, 1.5 mL, 2 mL, and 15 mL.

2.4.4. Glass or plastic bottle: 50 mL, 100 mL, 250 mL, 500 mL, 1,000 mL, and 2,000 mL.

Note 2: Plastic or glass containers must be DNase-Free.

2.5. Reagent preparation

2.5.1. 50 mM Ammonium bicarbonate solution
Dissolve 0.395 g of ammonium bicarbonate in 100 mL deionized water.

2.5.2. 0.5 M EDTA solution
Dissolve 14.612 g of EDTA in 100 mL deionized water.

2.5.3. PCR solution

PCR reaction buffer.....	5.0 µL
200 µM dNTP	1.0 µL

0.2 μM Primer F ..... 0.5 μL
0.2 μM Primer R..... 0.5 μL
2.5 U/μL Faststart Taq DNA polymerase..... 0.5 μL
Template DNA variable…… (Total amount 100 ng)
DEPC-H_2O..... to 50 μL

2.5.4. 0.5x TBE (Tris-borate-EDTA) buffer solution

Dissolve 54 g of Tris and 27.5 g of boric acid in appropriate amount of deionized water, add 20 mL of 0.5 M EDTA solution, adjust volume to 1,000 mL with deionized water, or dilute the 5x TBE buffer solution to 0.5x with deionized water.

2.5.5. 2% Agarose gel

Transfer about 2 g of agarose in glass bottle, add 100 mL of 0.5x TBE buffer solution, heat and swirl till the agarose is completely dissolved (avoid the splash of overboiling contents and affect the concentration of agarose gel), cool down to 50℃, add Safe view DNA stain 5 μL and mix properly, pour the molten agarose into the gel tray, use after solidification (Note 3).

Note 3: Pour slowly to avoid air bubbles in the gel.

2.6. Test procedure

2.6.1. Sample DNA isolation

2.6.1.1. Sample preparation

2.6.1.1.1. Powder/solid sample

Grind into powder, follow the protocol of commercial animal DNA extraction kit for sampling and pretreatment.

2.6.1.1.2. Capsule sample

Transfer 6 g of the contents of capsule into a 15 mL centrifuge tube, add 6 mL of ammonium bicarbonate solution, mix properly. Centrifuge at 20,000 ×g for 10 min. Take 3 mL of supernatant and 3 mL of lower dark and viscous layer separately into a 15-mL centrifuge tube, concentrate by the evaporator at 37℃ to 1/4 of the volume as sample for DNA extraction.

2.6.1.1.3. Liquid sample (such as positive reference materials, injection, etc.)

Transfer 1 mL of liquid sample into a 1.5-mL microcentrifuge tube, concentrate by the evaporator to 1/4 the of the volume at 37℃ as

sample for DNA extraction.

2.6.1.2. DNA extraction

Transfer proper amount of sample for DNA extraction from section 2.6.1.1. into a 1.5-mL microcentrifuge tube. Extract DNA by commercial DNA extraction kits, and then dispense DNA extract to a 1.5-mL sterile microcentrifuge tube, as DNA template. Measure the DNA concentration according to section 2.6.1.3., and store at 4°C.

2.6.1.3. Determination of concentration and purity of DNA template

Dilute DNA template with deionized water in a tube, determine the absorbance (O.D.) at wavelengths of 260 and 280 nm. Use the solution in which the sample was dissolved as blanks. Concentration of DNA template was calculated by the absorbance at OD 260 adjusted by the blanks and dilution factor. The purity of DNA solution was determined by the ratio of OD 260/280 and the ratio should be between 1.7~2.0.

Note4: It is recommended that each laboratory customize the absorbance value specification of the blank control group.

2.6.2. PCR protocol (Note 5)

Prepare the PCR solution according to the composition in section 2.5. Transfer the PCR solution into the PCR machine, and perform the reaction according to the following conditions. Transfer the PCR amplification products to perform agarose gel-electrophoresis.

Step	Temperature	Time
1. Initial Denaturation	95℃	3 min
2. Denaturation	95℃	30 sec
3. Annealing	58℃	30 sec
4. Extension	72℃	45 sec
Repeat step 2 to 4 for 40 cycles.		
5. Final extension	72℃	7 min
6. Cooling	4℃	45 sec

Note 5: The reaction conditions can be adjusted depending on the instruments used if the above reaction conditions are not applicable.

2.6.3. Agarose gel-electrophoresis

Add proper amount of 0.5x TBE buffer solution into gel box, place 2% agarose gel in the gel box. Mix 10 μL of PCR product with 2 μL of 6x loading dye properly, and load into gel. Load 8 μL of DNA Ladder Marker into gel for estimating the size of concurrently running samples. Perform electrophoresis at 100 voltage for 40 min (The running time depends on the actual situation). Remove the gel from gel box and place it in the imaging system to take pictures, and interpret the results. The positive and negative control should be tested simultaneously for each reaction.

2.6.4. Identification test

Compare the electrophoresis results of the PCR products with the positive control and the DNA Ladder Marker. If the sizes of the PCR products are 215 bp and 244 bp, it indicates that the sample contains mammalian nucleic acids.

Remarks

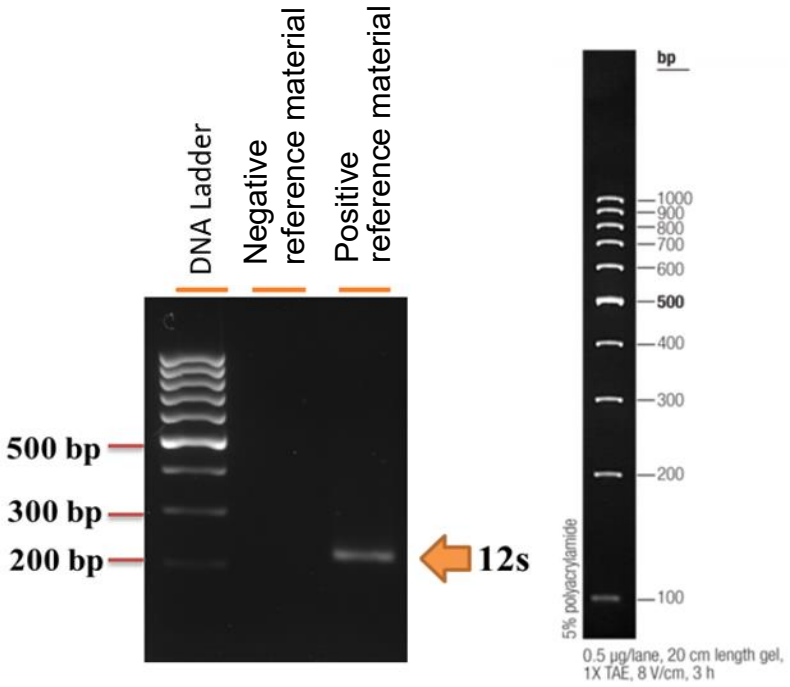
1. This method is applicable to the detection of DNA solutions with a content of more than 100 ng (inclusive).
2. If species identification is required, it is recommended that sequencing the two PCR amplification products (12S and 16S) of the sample DNA and determine the species by using NCBI BLAST comparison.
3. This method is applicable to products that can be used to extract DNA, but is not applicable to products that are highly processed to cause excessive DNA fragmentation.

References

1. Chang, T. L., Wu, S. F., Wang, D. Y. and Huang, C. H. 2020. A unified method for different placental products species identification. Pharmacogn. Mag. 16: S8-12.
2. U.S. National Library of Medicine. 2019. Nucleotide BLAST: Search nucleotide databases using a nucleotide query. [https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome]
3. Staats, M., Arulandhu, A. J., Gravendeel, B., Holst-Jensen, A., Scholtens, I., Peelen, T., Prins, T. W. and Kok, E. 2016. Advances in DNA metabarcoding for food and wildlife forensic species identification. Anal. Bioanal. Chem. 408: 4615-4630.

Reference gel-electrophoresis diagram

A



B

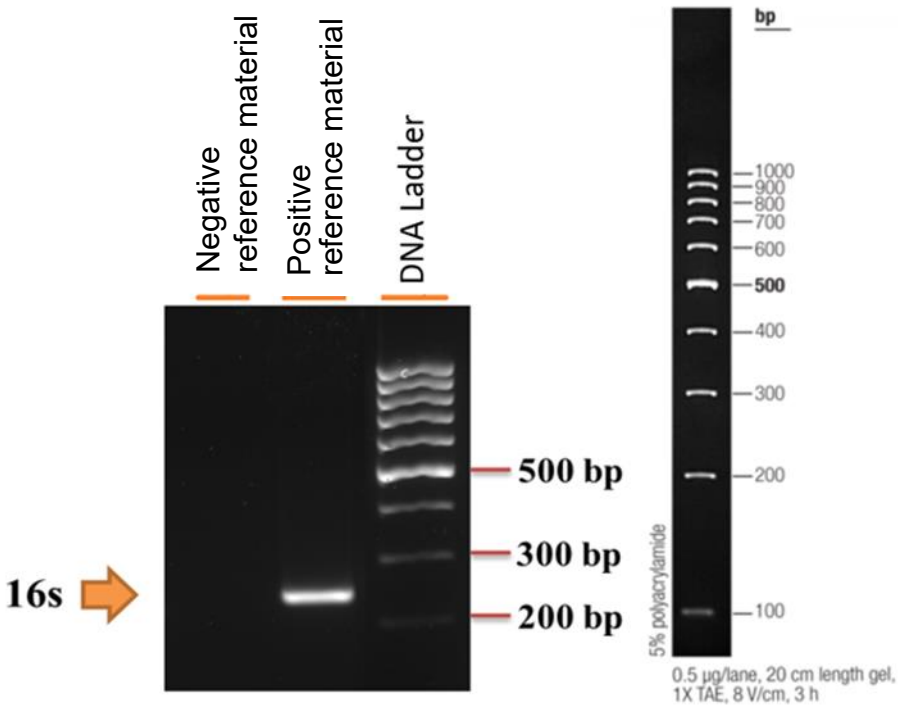


Figure. DNA ladder marker, negative, and positive control
(Figure A: 12S mitochondrial DNA; Figure B: 16S mitochondrial DNA).