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Textiles — Identification of some animal fibres by DNA analysis method — Cashmere, wool, yak and their blends

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National foreword

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Textiles — Identification of some animal fibres by DNA analysis method — Cashmere, wool, yak and their blends

Textiles — Identification de certaines fibres animales par la méthode d'analyse de l'ADN — Cachemire, laine, yack et leurs mélanges

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Foreword

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT), see the following URL: Foreword — Supplementary information.

The committee responsible for this document is ISO/TC 38, *Textiles*.

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Introduction

The composition of fibres in textile products is one of the most important properties. Labelling of composition of textile products is required globally by legislation or by voluntary regulation for fair trade.

The testing method to determine the composition of some animal fibres in the textile products has been developed as ISO 17751[3]. This is only one method to determine the animal fibre composition currently available. In this method, animal fibres are observed by microscope and identified from the shape of scales by experienced examiners. Many samples can be tested with a high degree of efficiency using this method. However, even experienced examiners have difficulties in identifying fibres, because textile products have a broad variety of colours and finishings, and there are many blends in animal fibres.

Given this situation, several testing methods to obtain the more accurate results have been investigated and developed. Among those methods, the DNA (deoxyribonucleic acid) analysis method has been found to be a practical and feasible method to identify the inherent type of animal fibres.

As it is well known, DNA is specific for animals. The DNA-PCR (polymerase chain reaction) method has recently been developed with high accuracy. A very trivial quantity of the DNA extracted from animal fibres is amplified by PCR to yield a huge quantity of copy DNA. Mitochondrial DNA is used for this analysis because it provides greater numbers than nuclear DNA.

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Textiles — Identification of some animal fibres by DNA analysis method — Cashmere, wool, yak and their blends

WARNING — The use of this International Standard can involve hazardous materials, operations, and equipment. This International Standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations and supplier's requirement for safety prior to use.

1 Scope

This International Standard specifies a testing method for DNA analysis of some animal fibres to identify cashmere, wool, yak, and their blends by using extraction, amplification by the polymerase chain reaction (PCR) method and DNA detection processes.

This International Standard is applicable to cashmere, yak, and wool and their blends as a qualitative method.

2 Caution

Test results for fibre identification by the DNA analysis method can be obtained with a high accuracy for the above-mentioned textile products which were processed at lower dye concentration levels or dyed in light colours.

However, when such textile products were processed under severe conditions or high temperatures, the mitochondrial DNA could have been damaged. In such cases, identification can be difficult because amplification of DNA by PCR cannot take place. If textile products were contaminated by using products from another species, such as cashmere grease on wool fibres, this situation may be solved by checking using microscopy techniques.

3 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 8655-2, *Piston-operated volumetric apparatus — Part 2: Piston pipettes*

4 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

4.1
DNA
deoxyribonucleic acid, that exists in nuclei and in mitochondria of animal fibre cells and is composed of a linear array of 4 bases (Adenine: A, Thymine: T, Guanine: G and Cytosine: C)

Note 1 to entry: The DNA sequence is identical and intrinsic for each animal fibre.

4.2
animal fibres
cashmere, wool, or yak fibres

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4.3
buffer solution
solution used to maintain pH of reaction solution at required value

4.4
reducing agent
agent that degrades animal fibres through reductive cleavage of S-S bonds in the fibres

4.5
DNA amplification
amplification of the specific fragment of DNA by the PCR method

4.6
PCR method
method of the polymerase chain reaction

Note 1 to entry: The amplification process of the DNA fragment with a constant length is explained in Annex A.

4.7
DNA polymerase for PCR method
heat-stable DNA polymerase that is used for PCR and has no proof reading activity

4.8
primer
short length fragment of a single strand DNA which is a reaction initiator and designed as the identical sequence of 18-30 bases to DNA of the animal fibre

4.9
primer set
set of primer with the reaction direction of forward and reverse

4.10
primer for cashmere
primer with an identical base sequence of mitochondrial DNA of cashmere

Note 1 to entry: The sequence of base for primers will be submitted to the public database.

4.11
primer for wool
primer with an identical base sequence to mitochondrial DNA of wool

4.12
primer for yak
primer DNA with an identical base sequence to mitochondrial DNA of yak

Note 1 to entry: Information concerning the primers may be obtained from ISO/TC 38 secretariat.

4.13
gel electrophoresis migration
method to detect the amplified constant length DNA fragments

5 Principle

Mitochondrial DNA is extracted from animal fibre samples by using a chemical and enzyme reaction. The extracted DNA is purified by using a precipitation method and centrifuge. The purified DNA is applied for the amplification reaction of PCR method. In the PCR method, primers for cashmere, yak and wool are respectively tested. If the sample is cashmere, only cashmere primer can amplify the constant length of DNA fragments. Then, the constant length of DNA fragments is detected by the electrophoretic migration method.

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The sample fibres are identified by knowing whether amplification was observed or not for the tests using all primers respectively.

6 Apparatus and equipment

6.1 Pipettes, capable of measuring and taking (0 to 20) µl (±0,20 µl), (20 to 200) µl (±1,60 µl), (200 to 1 000) µl (±8 µl) within systematic errors defined in ISO 8655-2.

6.2 Micro tube, capable of withstanding the centrifugation of 14 000*g* and autoclave.

The capacity is 2 ml for purification and 0,2 ml for PCR method. A tube of 0,2 ml for PCR method should follow the manufacturer's recommendation of the PCR instrument.

6.3 Cap lock, use for micro tube.

6.4 Heat block, with mounting holes for micro tubes and capable of heating up to about 80 °C (±1,0 °C).

6.5 Shaking agitator, capable of heating up to 50 °C and maintaining at the temperature of 50 °C and shaking micro tube at around 500 r/min or higher.

6.6 Shaking machine, capable of mounting micro tubes and shaking at around 500 times/min or higher.

This machine can be replaced by an equivalent instrument such as microtube rotator which is possible to rotate at 30 r/min or higher.

6.7 Centrifuge, capable of centrifuging of 14 000*g* or higher, setting up temperature from 0 °C to room temperature and mounting micro tubes or units of centrifugal ultrafiltration.

6.8 Unit of centrifugal ultrafiltration[1)], capable of capturing molecules with the molecular weight of 100 kDa (Dalton) or more.

6.9 PCR instrument[2)], capable of programing for temperature and time.

6.10 UV illuminator, UV irradiator.

6.11 Photo booth.

6.12 Generic plastic box with a resealable lid.

6.13 Comb, used for making wells in the agarose gel of the gel electrophoresis migration test.

6.14 Mixer mill, used for mixing and homogenizing animal fibres.

6.15 Erlenmeyer flask, with capacity 200 ml.

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1) Amicon Ultra is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

2) Life Technologies Corporation is a provider of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

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7 Reagents

7.1 Pure water.

Use pure water as defined in ISO 3696 with the purity of Grade 1. This water should not have DNase (DNA digesting) activity. It should not contain a significant amount of DNA which can be amplified by primers and should not show inhibitory effects for this testing method.

7.2 Chloroform/isoamyl alcohol reagent.

100 % concentration of the highest grade.

—	Chloroform	9,6 ml
—	Isoamyl alcohol	400 µl

7.3 Sodium perchlorate solution.

—	Sodium perchlorate	6,1 g

Make the solution up to 10 ml by adding pure water.

7.4 1 mol/l Tris – HCl [tris(hydroxymethyl)aminomethane].

Dissolve 12,1 g of tris(hydroxymethyl)aminomethane in 800 ml of pure water, then adjust the pH to 8,0 by adding HCl and using a pH-meter. Then, make it up to 1 000 ml by adding pure water.

7.5 500 mmol/l EDTA (ethylenediaminetetraacetic acid).

Dissolve 186,1 g of EDTA·Na_2·$2H_2O$ (disodium salt-dihydrate of EDTA) in 800 ml pure water, then adjust pH to 8,0 by adding NaOH and using a pH-meter. Then, make it up to 1 000 ml by adding pure water.

7.6 Buffer solution A.

—	1 mol/l Tris-HCL (7.4)	5 ml
—	500 mmol/l EDTA (7.5)	2 ml
—	Sodium lauryl sulfate (SLS) ($C_{12}H_{25}SO_4Na$)	0,2 g
—	Sucrose	1,2 g
—	Sodium chloride	170 mg
—	Dithiothreitol (DTT)	920 mg

Make it up to 10 ml by adding pure water. Prepare fresh before use.

Due to its volatile nature, DTT should be added after autoclaving, so that its effectiveness is not reduced.

7.7 Buffer solution B.

—	500 mmol/l EDTA (7.5)	0,2 ml

Make it up to 100 ml by adding pure water.

7.8 Protein resolving enzyme solution, the papain solution, with a papain of 10 units dissolved in pure water (e.g. 10 units/20 µl).

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7.9 Heat-stable DNA polymerase, without 3‘ to 5‘ exonuclease activity.

7.10 Animal fibre A primer 1, forward primers for cashmere, yak, and wool.

7.11 Animal fibre A primer 2, reverse primers for cashmere, yak, and wool.

7.12 DNA composition component, with a grade for the PCR method.

7.13 Buffer solution for polymerase, suitable for the polymerase of the PCR reaction.

This buffer solution may be designated by manufacturers of polymerase.

7.14 Salt, potassium chloride (KCl), used to stabilize the PCR reaction.

7.15 Magnesium chloride ($MgCl_2$), used to stabilize the PCR reaction.

7.16 Gel electrophoretic migration marker.

7.17 Agarose, a kind of agar with a grade for DNA electrophoretic migration.

7.18 Buffer solution for loading on the electrophoretic migration.

—	Glycerol	36 g
—	500 mmol/l EDTA (7.5)	6 ml
—	Bromophenol Blue	0,25 g
—	Xylene cyanol	0,25 g

Make it up to 100 ml by adding pure water. After adding the solution of more than 1/6 to the sample reaction solution, load it on the gel.

7.19 Buffer solution for electrophoretic migration.

—	tris(hydroxymethyl)aminomethane base (Trizma base)	242 g
—	Acetic acid (glacial acetic acid)	57,1 ml
—	EDTA·2Na	7,43 g

Dissolve using pure water and make the solution up to 1 l by adding pure water. Then, dilute it 50 times with pure water.

7.20 DNA dyeing colorant, ethidium bromide.

Ethidium bromide dissolves in the buffer solution for electrophoretic migration so as to be approximately 5 µg/ml.

NOTE Other DNA intercalating agents can be used as substitute for ethidium bromide.

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7.21 Common DNA fragment, primer.

Common DNA fragments are common base alignments existing in all the DNAs of cashmere, yak, and wool. Common primer 1 is a forward primer; common primer 2 is a reverse primer.

NOTE Information on primers can be obtained from ISO/TC 38 secretariat.

8 Sampling

Animal fibre samples shall represent the textile products for test. If the textile product is composed by several parts, separate them into identical parts and describe the details of the parts in the test report. Avoid contamination among the parts.

Two test specimens are selected from the sample. When the two results are not consistent, do not adopt the results and perform another test for two specimens again.

NOTE ISO 17751:2007, Annex B can be used as a reference for this procedure.

9 Test methods

9.1 General

The test should be performed in parallel for the primers for cashmere, yak, and wool.

9.2 Chipping sample

Chip the animal fibre sample of 100 mg with the length of less than 2 mm by scissors, mixer mill, or other instruments. Then, put the chipped sample of 50 mg into a micro tube[3)] with a 2,0 ml capacity.

9.3 DNA extraction

Follow the procedure described in 9.3.1 to 9.3.6.

9.3.1 Add 600 µl of buffer solution A (7.6) to the micro tube with the test sample (9.2).

9.3.2 Cap the micro tube and then tumble it by hand to immerse the sample in the buffer solution perfectly.

9.3.3 Lock the cap of the micro tube using a cap lock. Then, heat the micro tube with the test specimen at 60 °C for 20 min using the heat block. Take out the micro tube at 3 min, 6 min, 9 min, and 15 min from the heat block and tumble it for 10 s by hand. Then, put it back on the heat block again and continue heating.

9.3.4 After 20 min, take out the micro tube from the heat block and cool it down to room temperature.

9.3.5 Add 10 units of papain solution (7.8) to the micro tube with the test specimen. Then, heat up the micro tube to 50 °C. Maintain the temperature at 50 °C and shake it at 500 r/min for 1 h using the shaking agitator (6.5).

9.3.6 Add 10 units of papain solution (7.8) to the micro tube of 9.3.5. Maintain the temperature at 50 °C. Shake it at 500 r/min for over 12 h. Cool it down to room temperature.

3) Eppendorf tubes are an example of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

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9.4 DNA purification

Follow the procedure described in 9.4.1 to 9.4.13.

9.4.1 Add 300 µl of sodium perchlorate solution (7.3) to the micro tube of 9.3.6. Then, shake it for 10 min at a condition of 500 r/min using the shaking machine (6.6).

9.4.2 Add 300 µl of chloroform/isoamyl alcohol (7.2) to the micro tube of 9.4.1. Tumble it about for 10 s by hand. Then, shake it for 10 min using the shaking machine (6.6) (e.g. at 500 r/min for rotating shaker, 100 r/min for reciprocal shaker) or equivalent instrument (e.g. 30 r/min for vertical rotator) to mix well. The solution will become clear.

9.4.3 Centrifuge the micro tube of 9.4.2 at 3 300*g* for 1 min at room temperature. Then, take 500 µl of supernatant solution using a pipette (6.1) and put it in a new micro tube.

9.4.4 Add 500 µl of chloroform/isoamyl alcohol (7.2) to the micro tube of 9.4.3. Tumble it about for 10 s by hand. Then, shake it for 10 min using the shaking machine (6.6) or equivalent instrument to mix well. The solution will become clear.

9.4.5 Centrifuge the micro tube of 9.4.4 at 5 500*g* for 1 min at room temperature. Then, take 400 µl of supernatant solution by using a pipette (6.1) and put it in a new micro tube.

9.4.6 Centrifuge the micro tube of 9.4.5 at 13 000*g* for 7 min at 4 °C. Then, put 350 µl of supernatant solution in the unit of centrifuge type ultrafiltration. Attach another new micro tube under the unit.

9.4.7 Centrifuge the unit of centrifuge type ultrafiltration with the supernatant solution at a condition of 14 000*g* for 12 min at the temperature of 4 °C.

Filter the concentrated DNA solution on the filter membrane.

Discharge the filtrated solution as waste solution.

9.4.8 Attach the micro tube of 9.4.7 to the filtration unit again.

9.4.9 Then, add 450 µl of buffer solution B (7.7) to the unit of the centrifuge type ultrafiltration where the concentrated DNA solution is remained. Then, centrifuge the unit at 14 000*g* for 10 min at the temperature of 4 °C. Discharge the filtrated solution as the waste solution.

9.4.10 Repeat 9.4.8 and 9.4.9 procedures twice or more to purify the DNA.

9.4.11 Add 20 µl of buffer solution B (7.7) to the unit where the concentrated DNA solution remains.

9.4.12 Attach a new micro tube upside down to the upper part of the unit of centrifuge type ultrafiltration.

9.4.13 Reverse the unit and erect it and then, centrifuge it at 1 000*g* for 2 min at temperature of 4 °C. Then, collect the purified DNA solution finally.

This purified DNA solution is used for the DNA test hereafter and called it as the test specimen DNA.

If animal fibres coloured by dark pigments are being tested, it is possible to remain inhibitors for PCR. In that case, the additional purification method described in Annex B is recommended.

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9.5 DNA amplification

9.5.1 Composition of the reaction solution

The composition of the reaction solution for animal A is as follows:

— Test specimen DNA (9.4.12)	2 µl
— Heat stable DNA polymerase (7.9)	1–1,5 unit
— Animal A, Primer 1 (7.10)	1 µmol/l
— Animal A, Primer 2 (7.11)	1 µmol/l
— Common primer 1 for verification (7.21)	0,5 µmol/l
— Common primer 2 for verification (7.21)	0,5 µmol/l
— substrate nucleotide dATP, dTTP, dGTP, dCTP (7.12)	200 µmol/l each
— Buffer solution for polymerase (7.13)	designated amount by suppliers
— Salt, KCl	50 mmol/l
— $MgCl_2$	1,5 mmol

Make up the solution to 50 µl by adding pure water.

9.5.2 Condition of PCR amplification instrument for DNA amplification

The condition of PCR amplification instrument is shown in Table 1.

Table 1 — The program of PCR instrument

Item	Time/temperature	Number of cycles
Initiation of PCR	5 min to 10 min/95 °C	1
Amplification	30 s/95 °C 30 s/60 °C to 68 °C[a] 30 s/72 °C	35
Final extension	3 min to 7 min/72 °C	1
Preservation	No time limitation/4 °C	1

[a] Annealing temperature for the PCR amplification depends on the design of primers. This temperature should be adjusted to the primer to be used.

9.5.3 DNA Amplification test method

9.5.3.1 Put the reagents in a micro tube for PCR using micro pipettes according to 9.5.1 and make it up to 50 µl by adding pure water.

9.5.3.2 Mount the micro tube to the PCR amplification instrument and run the amplification procedure with the condition as shown in Table 1.

9.5.3.3 Run the amplification cycle up to 35 cycles.

9.5.3.4 When the reaction cycle has ended, take out the micro tube from the PCR amplification instrument and keep it in the refrigerator at 4 °C until running the following procedure.

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9.6 Detection and confirmation of DNA amplification

9.6.1 Preparation

9.6.1.1 Put the 0,9 g of agarose in the Erlenmeyer flask. Then, add 60 ml of electrophoretic migration buffer solution (7.19) to the flask. Melt the agarose completely by heating. Pour the melted agarose into a gel container and set a comb in the gel. Keep the gel container with the gel at room temperature for 30 min or more and materialize the gel.

9.6.1.2 Take out the comb carefully and create wells in the gel. Set the gel container in the electrophoretic migration unit. Pour the buffer solution (7.19) into the gel container to a depth of 3 mm to 5 mm from the surface of the gel to solution surface.

Take out the comb very carefully to avoid damaging the wells.

9.6.2 Electrophoretic migration test

9.6.2.1 Take 10 µl of PCR reaction solution (9.5.3.4) from the preserved micro tube using the micro pipette and put it into a new micro tube.

9.6.2.2 Add 2 µl of buffer solution (7.18) to the micro tube (9.6.2.1). Mix the contents of the micro tube pipetting.

9.6.2.3 Put the mixed solution (9.6.2.2) into the wells using a micro pipette.

9.6.2.4 Add 5 µl of gel electrophoretic migration marker (7.16) to one of the wells in the gel.

9.6.2.5 Apply a voltage of 100 V ± 50 V to the electrophoretic migration. Turn off the voltage when the dye in the electrophoresis loading buffer is migrated by 2/3 of whole migration distance.

9.6.2.6 Put the gel in a generic plastic box with a resealable lid (6.12). Then, add the ethidium bromide solution of 100 ml in it, and immerse the gel for 5 min and drain the solution. To increase the signal-to-noise ratio, immerse the gel in pure water for approximately 2 min to wash off the excess dye in the gel.

9.6.2.7 Take the gel out from the generic plastic box with a resealable lid (6.12) by a pallet and place it on the UV illuminator (6.10). Apply ultraviolet rays to the gel and observe the grow line due to the DNA, take a photograph of it in the photo booth (6.11).

10 Verification

10.1 General

The preparation of specimen for the verification is given in 10.2 to 10.6.

10.2 No amplification verification after extraction process (negative verification)

In the DNA extraction process described in 9.3, no animal fibre specimen is used. The DNA reaction solution for this purpose is produced by running through all the processes without a specimen. The frequency of the verification test is once per testing practice. There should be no DNA, so amplification shall not be observed for this verification test.

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10.3 Amplification verification after extraction process (positive verification)

The common DNA fragment for all animals, cashmere, yak and wool is applied to the extraction process (9.3). The DNA reaction solution is produced by running through all the processes. The frequency of the verification test is once per testing practice. Amplification shall be observed for this verification specimen.

10.4 Amplification verification after PCR process

The DNA fragment designated separately is applied to the PCR reaction solution. The PCR method is then carried out using this PCR reaction solution. Amplification shall be observed for this verification specimen. This verification is performed if the result of the test (10.3) is negative.

NOTE The DNA fragment for this purpose can be obtained in market. The information on the primer can be obtained from ISO/TC 38 secretariat.

10.5 No amplification verification after PCR process

The PCR amplification process is carried out by using the DNA reaction solution without a DNA fragment. Amplification shall not be observed. This verification is performed if the result of the test (10.2) is positive.

10.6 Amplification verification of PCR reaction solution

If the testing result for the animal fibre specimen is negative, the primers of any standard DNA primer and correspondent DNA is added to the PCR reaction solution. Then, PCR method is carried out by using the DNA solution with the primers. See Table 2.

NOTE Information on the DNA primer and correspondent DNA for this purpose can be obtained from ISO/TC 38 secretariat.

Table 2 — Process verification method

Verification method no.	10.2	10.3	10.4	10.5	10.6
Process	**DNA no extraction and process verification**	**DNA extraction and process verification**	**PCR process verification**	**PCR process verification**	**PCR reaction solution verification**
	No DNA added	**Standard animal fibre specimen**	**DNA added in reaction solution**	**No DNA added**	**DNA added in reaction solution**
Sampling of animal fibre	Not run	Run	Not run	Not run	Not run
DNA extraction	Run	Run	Not run	Not run	Not run
DNA purification	Run	Run	Not run	Not run	Not run
Amplification	Run	Run	Run	Run	Run
Detection	Run	Run	Run	Run	Run

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11 Assessment

Assessment is done as shown below and in Table 3 and includes the results of the verification specimen. If the test is not conclusive, repeat the test.

— The test for the animal fibre is not verified when amplification is observed even though it doesn't contain DNA (10.2 and 10.5) and when amplification is not observed even though DNA is contained in the verification tests (10.3, 10.4, and 10.6).

  The signal intensity of the amplified DNA fragment depends on the concentration of the dye, temperature and other factors. Thus, amplification of a specific DNA fragment should be judged by comparing the signal intensity with that of a reference marker, which can be universally usable.

— The animal fibre can be identified when the verification tests are entirely normal and the DNA amplification of the animal fibre is observed for the specific primer used for the test.

— The sample can be concluded that the animal fibre which primer used for the test is not included in the sample when the verification tests are entirely normal, and DNA amplification, using the primer, is not observed.

Table 3 — Assessment

Animal fibre specimen testing result	**Verification method No.**					**Assessment**
	10.2	10.3	10.4	10.5	10.6	
	DNA no extraction and process verification	**DNA extraction and process verification**	**PCR process verification**	**PCR process verification**	**PCR reaction solution verification**	
	No DNA	**Standard animal fibre specimen added**	**DNA added in reaction solution**	**No DNA**	**DNA added in reaction solution**	
Amplified	Not amplified	Amplified	Amplified	Not amplified	Amplified	Identified the animal fibre
Not amplified	Not amplified	Amplified	Amplified	Not amplified	Amplified	Confirmed no animal fibre
Any cases either amplified or not amplified	Amplified	—	—	—	—	Process problem The testing is not verified
	—	Not amplified	—	—	—	
	—	—	Not amplified	—	—	
	—	—	—	Amplified	—	
	—	—	—	—	Not amplified	

12 Precision

This test method is applied to actual samples. The repeatability and reproducibility are obtained as in Annex C. The tests for the samples which compositions are preknown are performed and the results are shown in Annex D.

13 Test report

The test report shall include the following information:

a) a reference to this International Standard, i.e. ISO 18074;

b) a description of the test sample;

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c) details of polymerase and PCR condition;

d) details of testing results;

e) details of any deviation from the specified procedure.

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Annex A
(informative)

Amplification of the constant length DNA fragment by PCR method

A.1 General

The DNA amplification runs through the following steps. The DNA fragment is amplified to double at each step using the PCR method, which is shown in Figure A.1 as a schematic diagram.

A.2 Amplification step by PCR method

A.2.1 0 step: The double helical strand becomes two single DNA strands by heating.

A.2.2 First step: Amplification is started by the PCR method. The two different primers for one animal fibre which have the different growth directions of forward and reverse are prepared. The primers act as an initiator and the substrate nucleotides are added selectively on the strands bases so as to make a copy. The length of the DNA fragment is not determined on this step yet.

Before the second step, DNA is heated and again the double helical strand becomes two single DNA strands. There are two lengths of single strand DNA which is a long DNA originated from animal fibre and the middle length DNA with one starting point by initiators.

A.2.3 Second step: Two kinds of amplification occur in this step: 1) amplification of the first step; and 2) another amplification. Then another amplification takes place on the short DNA fragment obtained from the first step amplification. At this point, the length is not determined yet, but it has the initiated point by the forward primer or reversed primer. For this DNA fragment, the amplification started by the primers with the opposite directions is terminated at the starting position of the first step.

So, amplified DNAs are heated again and the double helical strands become two single DNA strands. There are 3 kinds of the single strand DNA which is a long DNA originated from animal fibre and the middle length DNA with one starting point by initiators and the short length DNA with both ends created by the primers as a starting point of amplification.

A.2.4 Third step: Amplification of the first and second steps continues. Amplification of the short length DNA with both ends created by the primers begins, creating a copy with the same short length.

Again, the amplified DNAs are heated and made single strand of DNA.

A.2.5 Fourth step: Amplification of the first, second and third steps continues. The major parts of the amplification are taken place for the short length DNA with a constant length.

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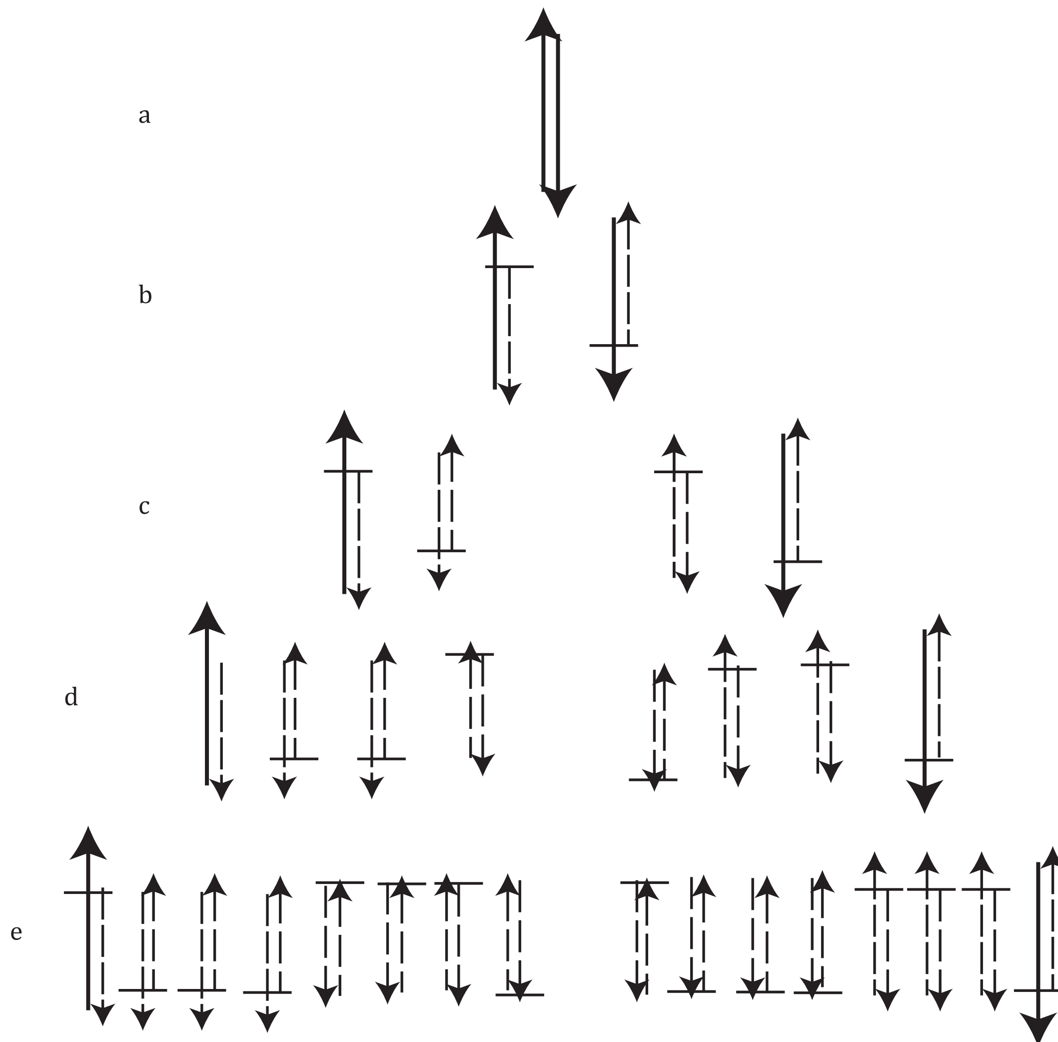

Key

a Former animal fibre DNA, the arrow are directions of growth. Double DNA chain becomes single chain by heating.

b Amplification of the 1st step. 2 strands start to amplify, then heated.

c Amplification of the 2nd step. 4 strands start to amplify, then heated.

d Amplification of the 3rd step. 8 strands start to amplify, then heated.

e Amplification of the 4th step. 12 strands start to amplify, then heated.

Figure A.1 — Amplification process of DNA fragment by PCR method

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Annex B
(informative)

DNA additional purification

B.1 General

When the animal fibres contain a dark colour pigment, there is a possibility that the pigment acts as a PCR inhibitor. In this annex, a testing example is described for an additional purification method with the sample containing approximately one fifth of yak fibre to eliminate the pigment.

B.2 Reagent

B.2.1 10 % CTAB solution with sodium chloride.

— *Cetyltrimethylammonium bromid (CTAB)* $(C_{16}H_{33})N(CH_3)_3Br$ 10 g

— Sodium chloride 4,1 g

Make up the solution to 100 ml by adding pure water. Heat the solution at 55 °C for dissolving CTAB powder completely. After the dissolution, the solution should be kept at a temperature higher than 15 °C to avoid the precipitation of the powder.

B.2.2 5 mol/l sodium chloride solution.

— Sodium chloride 29,2 g

Make up the solution to 100 ml by adding pure water.

B.3 Purification procedure

The procedure for additional DNA purification using the CTAB solution is given in B.3.1 to B.3.15.

B.3.1 Add 300 µl of buffer solution B (7.7) to the micro tube with the purified sample (9.4.13).

B.3.2 Add 50 µl of 5 mol/l sodium chloride solution (B.2.2) to the micro tube.

B.3.3 Add 40 µl of 10 % CTAB solution containing 0,7 mol sodium chloride (B.2.1) the micro tube.

B.3.4 Then, heat the micro tube with the test specimen at 68 °C for 10 min by using the heat block. During heating, take out the micro tube temporarily from the heat block and tumble it by hand, then return it to the heat block to continue heating until 10 min has passed.

B.3.5 After 10 min, take the micro tube out from the heat block and let it cool down to room temperature.

B.3.6 Add 300 µl of chloroform/isoamyl alcohol (7.2) to the micro tube of B.3.5. Then, shake it for 10 min by the shaking machine (6.6) (e.g. at 500 r/min for rotating shaker, 100 r/min for reciprocal shaker or equivalent instrument e.g. 30 r/min for vertical rotator) to mix well. The solution will become clear.

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B.3.7 Centrifuge the micro tube of 9.4.6 by 3 300*g* for 2 min at room temperature. Then, take 250 µl of supernatant solution by using a pipette (6.1).

B.3.8 Place it in the unit of the centrifuge type ultrafiltration. Attach another micro tube under the unit to receive the discharged solution.

B.3.9 Centrifuge the micro tube of B.3.8 by 14 000*g* for 15 min at 4 °C. The condensed DNA solution remains on the filter membrane. Discharge the filtrated solution as waste solution.

B.3.10 Re-attach the micro tube to the filtration unit.

B.3.11 Add 300 µl of buffer solution B (7.7) to the unit of the centrifuge-type ultrafiltration where the condensed DNA solution remains. Then, centrifuge it by 14 000*g* for 7 min at the temperature of 4 °C. Discharge the filtrated solution as waste solution.

B.3.12 To purify the DNA, repeat once more the procedures in B.3.10 and B.3.11.

B.3.13 Add 30 µl of buffer solution B (7.7) to the unit of the centrifuge-type ultrafiltration where the condensed DNA solution remains.

B.3.14 Attach a new micro tube upside down to the upper part of the unit of the centrifuge type ultrafiltration to recover the purified DNA solution.

B.3.15 Reverse the unit and erect it. Then, centrifuge it at a condition of 1 000*g* for 2 min at temperature of 4 °C. Finally, collect the purified DNA solution.

This purified DNA solution is used for the DNA test and is called the test specimen DNA.

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Annex C
(informative)

Repeatability and reproducibility

C.1 Repeatability

C.1.1 Cashmere

C.1.1.1 Sample

The pure cashmere was collected from cashmere goats in Mongolia.

C.1.1.2 Polymerase and PCR condition

The electrophoresis image for cashmere is shown in Figure C.1. A test for a single specimen consists in 3 lanes which is for the either primer set of cashmere, wool, and yak (labelled as C, W, and Y in Figure C.1 respectively). Each lane contains the test by positive control primers and there is a glow spot for the DNA fragment amplified by the positive control primers. It should be noted that there is a competition of amplification by specific identification primers and positive control primers, thus the lane for cashmere sample amplified by primers for cashmere can contain 2 glow spots in a single lane. The right-hand 3 lanes are the negative control for the test of no contamination in the reagent. 5 sets of 3 glow spots are test results for 6 cashmere specimens.

— Positive control: Clear images are obtained.

— Negative control: The right-hand 3 lanes containing no gloss spots, indicating no contamination.

The test is judged as effective.

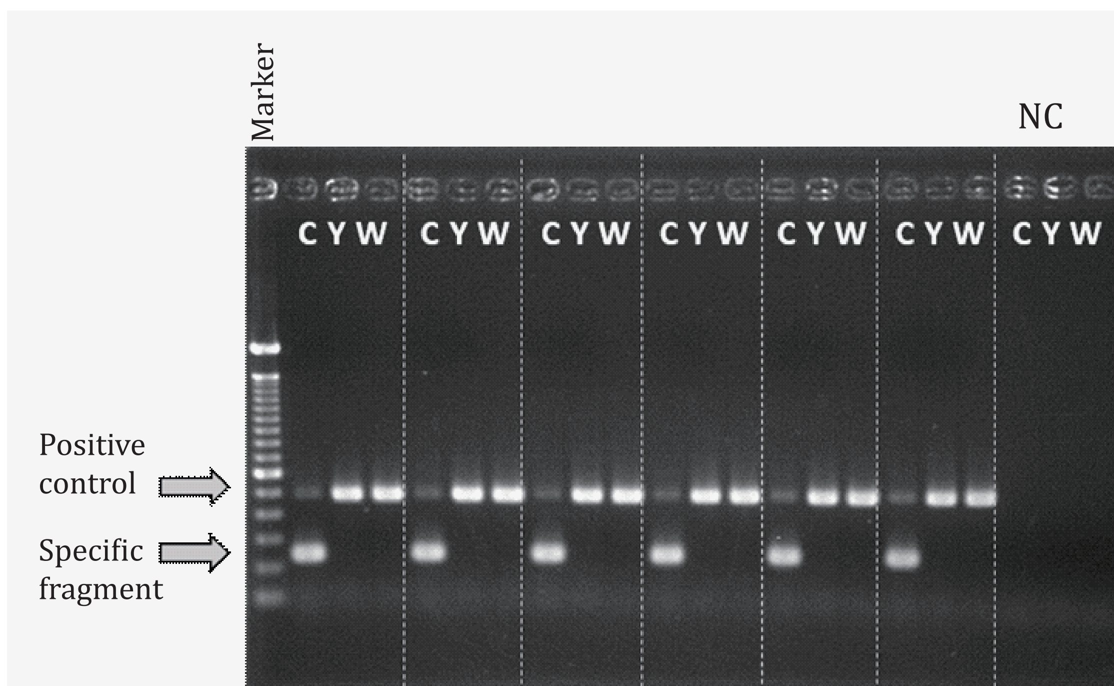

Figure C.1 — Test result for six specimens of cashmere

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C.1.1.3 Test result

Very clear images are obtained for the tests for six cashmere specimens repeatedly.

C.1.2 Wool

C.1.2.1 Sample

The pure wool was collected in Australia from sheep.

C.1.2.2 Polymerase and PCR condition

The electrophoresis image for wool specimens is shown in Figure C.2. The array of the glow spots is same as cashmere, but the test results are for seven wool specimens. There is no lane for negative control in Figure C.2 for the electrophoresis.

— Positive control: Clear images are obtained.

The test is judged as effective.

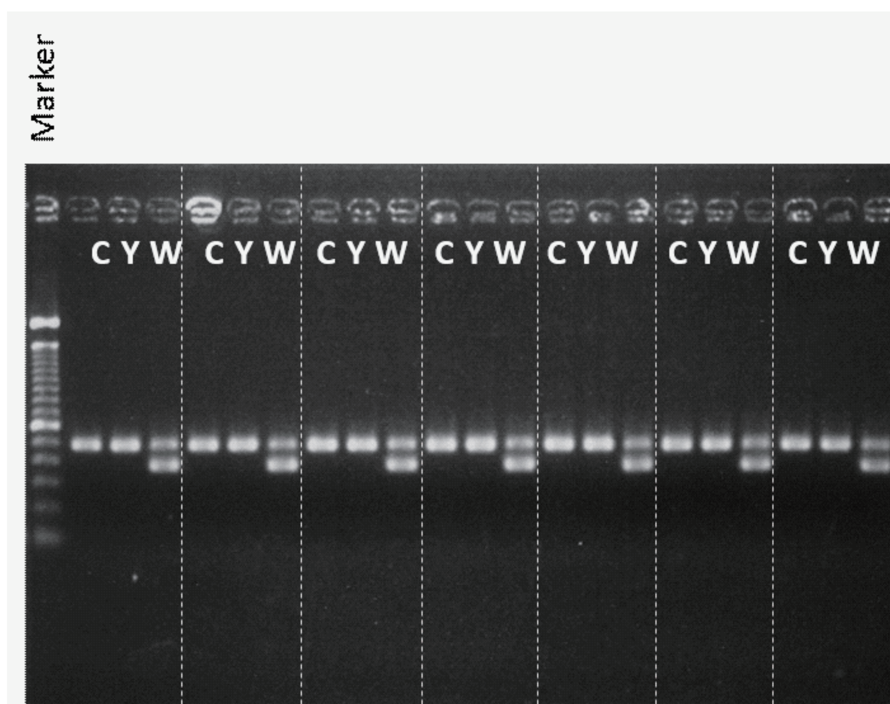

Figure C.2 — Test results for seven specimens of wool

C.1.2.3 Test result

The seven test results for wool specimens are shown in Figure C.2 and very clear images are obtained repeatedly.

C.1.3 Yak

C.1.3.1 Sample

The pure yak was collected in Mongolia from yak.

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C.1.3.2 Polymerase and PCR condition

The electrophoresis image for yak specimens is shown in Figure C.3. The array of the glow spots is same as cashmere, but the test results are for seven yak specimens.

— Positive control: Clear images are obtained.

— Negative control: The right-hand three glow spots are clear, indicating no contamination.

The test is judged as effective.

Figure C.3 — Test results for seven specimens of yak

C.1.3.3 Test result

The seven test results for yak specimens are shown in Figure C.3 and very clear images are obtained repeatedly.

C.2 Limit of detection

Using a mixture of animal fibres, the test is executed for determination of the limit of detection experimentally. 0,5 % mixed in cashmere fibre were able to be detected by this test method. 0,5 % cashmere fibre mixed in wool hair was able to be detected as well.

C.3 Reproducibility

C.3.1 Test samples

The same samples used in C.1 are tested. The specimens of animal fibres are prepared as 100 %, two specimens for each animal, total 6 specimens.

C.3.2 Test house

Three testing houses or laboratories in Japan participated for this test.

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C.3.3 Test result

The tests are executed by using primers of each animal species for four specimens and for one negative control sample. The testing results in Table C.1 show how many testing houses gave the results in line with the expectation.

Table C.1 — Reproducibility tests by 3 testing houses

Primer	Samples				
	Cashmere C.1.1	Wool C.1.2	Yak C.1.3	Fabric[a]	Negative control
Cashmere	3/3	3/3	3/3	3/3	3/3
Wool	3/3	3/3	3/3	3/3	3/3
Yak	3/3	3/3	3/3	3/3	3/3

[a] A dark blue cashmere fabric was used for the test.

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Annex D
(informative)

Practical application to various textile products

The coloured textile samples with cashmere label are obtained from the market. These samples are observed by microscopy and tested by DNA analysis. The results are shown in Table D.1.

Table D.1 — Result of DNA analysis for the practical samples

No	Sample colour	Microscope observation				DNA analysis		
		Cashmere	Wool	Yak	Other	Cashmere	Wool	Yak
1	Brown knit	O				O		
2	Grey knit	O	trace			O	trace	
3	Purple knit	O				O		
4	Beige knit	O				O		
5	Brown knit	O	O		O	O	O	
6	Orange knit	O	O	O		O	O	O
7	Black knit	O	trace		O	O	trace	
8	Beige knit	O			O	O		
9	**Beige felt**	O	O			No signal		
10	Brown knit	O				O		
11	Beige knit	O	O	O		O	O	trace
12	**Black felt**	O	O			No signal		
13	Grey knit	O	O		O	O	O	
14	Blue knit	O				O		
15	Woven fabric	O	O			O	O	
16	Grey knit	O				O		
17	Black knit	O				O (weak)		
18	Woven fabric	O				O (weak)		
19	Pink knit	O	O		O	O	O	
20	Black knit	O				O		
21	Black knit	O	trace		O	O	trace	
22	Beige knit	O	O	O		O		O
23	Black and Grey knit	O	O			O	O	

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