File No. 11014/07/2021-QA

Food Safety and Standards Authority of India

(A statutory Authority established under the Food Safety and Standards Act, 2006)

(Quality Assurance Division)

FDA Bhawan, Kotla Road, New Delhi – 110002

Dated, the 9th October, 2024

Order

Subject: FSSAI Manual of Methods of Analysis of Foods- Meat and Meat products - reg.

The FSSAI Manual of Methods of Analysis of Foods- Meat and Meat products which has been approved by the Food Authority in its 44th meeting held on 19.06.2024 is enclosed herewith.

2. This manual supersedes the test methods for Meat and Meat Products specified under the Manual of Methods of Analysis of Foods- Meat and Meat Products & Fish and Fish Products issued vide Office Order No. 1-90/FSSAI/SP (MS&A)/2009 dated 09.01.2017.

3. The approved methods shall be implemented with immediate effect. The notified laboratories shall include the new methods in their respective scope of accreditation within six months from the date of issue of this order.

4. Since the process of updation of test methods is dynamic, any changes happening from time to time will be notified separately. Queries/concerns, if any, may be forwarded to email: sp-sampling@fssai.gov.in.

Encl: as above

Dr. SATYEN KUMAR PANDA

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(Dr. Satyen Kumar Panda)
Advisor (QA)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories
3. CEO, National Accreditation Board for Testing and Calibration Laboratories (NABL)

फा. सं. 11014/07/2021 – **क्यूए**

भारतीय खाद्य सुरक्षा और मानक प्राधिकरण

(खाद्य सुरक्षा और मानक अधिनियम, **2006** के अंतर्गत स्थापित एक वैधानिक प्राधिकरण**)**

(**गुणवत्ता आश्वासन विभाग**)

एफडीए भवन, कोटला रोड, नई दिल्ली-110002

दिनांक: 09 अक्टूबर, 2024

आदेश

विषय: खाद्य पदार्थों के विश्लेषण के तरीकों की एफएसएसएआई मैनुअल – मांस और मांस उत्पाद - के संबंध में।

खाद्य पदार्थों के विश्लेषण के तरीकों की एफएसएसएआई मैनुअल - मांस और मांस उत्पाद, जिसे खाद्य प्राधिकरण ने 19.06.2024 को आयोजित अपनी 44वीं बैठक में अनुमोदित किया है, इसके साथ संलग्न है।

2. यह मैनुअल कार्यालय आदेश संख्या 1-90/एफएसएसएआई/एसपी (एमएस&ए)/ 2009 दिनांक 09.01.2017 के तहत जारी किए गए खाद्य पदार्थों-मांस और मांस उत्पादों और मछली और मछली उत्पादों के विश्लेषण के तरीकों के मैनुअल के तहत निर्दिष्ट मांस और मांस उत्पादों के लिए परीक्षण विधियों का स्थान लेता है।.

3. अनुमोदित विधियां तत्काल प्रभाव से लागू किये जायेंगे। अधिसूचित प्रयोगशाला इस आदेश के जारी होने की तारीख से छह महीने के भीतर मान्यता के अपने संबंधित दायरे में नई विधियों को शामिल करेगी।

4. चूंकि परीक्षण विधियों के अद्यतन की प्रक्रिया गत्यात्मक है, समय-समय पर होने वाले किसी भी परिवर्तन को अलग से अधिसूचित किया जाएगा। प्रश्न/चिंताएं, यदि कोई हों, ईमेल: sp-sampling@fssai.gov.in, पर अग्रेषित की जा सकती हैं ।

संलग्नक: उपरोक्त अनुसार

Dr. SATYEN KUMAR PANDA

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(डॉ. सत्येन कुमार पंडा)
सलाहकार (गुणवत्ता आश्वासन)

प्रति:

1. सभी एफएसएसएआई अधिसूचित प्रयोगशालाएं
2. सभी राज्य खाद्य परीक्षण प्रयोगशालाएं
3. सीईओ, राष्ट्रीय परीक्षण और अंशशोधन प्रयोगशाला प्रत्यायन बोर्ड

एफएसएसएआई
fssai
भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India

सत्यमेव जयते

स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare
GOVERNMENT OF INDIA

MANUAL OF METHODS OF ANALYSIS OF FOODS

MEAT AND MEAT PRODUCTS

OCTOBER 2024

जी. कमलावर्धन राव
G. Kamala Vardhana Rao
मुख्य कार्यकारी अधिकारी
Chief Executive Officer

सत्यमेव जयते

एफएसएसएआई
fssai
भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare

FOREWORD

We are delighted to present the **FSSAI Manual of Methods of Analysis of Foods- Meat & Meat Products**, a comprehensive guide that serves as an invaluable resource for food testing laboratories, researchers & quality control professionals, food technologists, and anyone involved in the analysis of Meat & Meat Products.

This manual has been meticulously crafted to offer a wide range of analytical methods specifically tailored for Meat & Meat products. It encompasses various aspects of analysis as per FSSR. In an ever-evolving scientific landscape, it is essential to stay abreast of emerging technologies and methodologies. Therefore, we encourage users of this manual to actively contribute their experiences and expertise. By fostering a collaborative environment, we can continuously refine and expand our understanding of Meat & Meat Products, driving innovation and improvement in the field.

It gives us immense pleasure to release this **FSSAI Manual of Methods of Analysis of Foods-Meat & Meat Products**. The FSSAI notified laboratories shall use these testing methods only for analyzing samples under the Food Safety and Standards Act, 2006 and Regulations made thereunder. This Manual may serve as a catalyst for scientific advancements, quality assurance, and consumer safety, ultimately contributing to the overall well-being and satisfaction of individuals worldwide.

October 2024

Shri G. Kamala Vardhana Rao,
Chief Executive Officer

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Eat Right India
सही भोजन. बेहतर जीवन.

डॉ. सत्येन कुमार पंडा, एआरएस
Dr. Satyen Kumar Panda, ARS
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सत्यमेव जयते

आज़ादी का अमृत महोत्सव

एफएसएसएआई
fssai
भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare

PREFACE

Food safety is assurance that food is acceptable for human consumption according to its intended use. Testing of food to instil confidence amongst consumers that food is safe to eat is important part of the food safety ecosystem. Food testing ecosystem is complex in India and challenges start from sample preparation to final result generation.

Each method in the **FSSAI Manual of Methods of Analysis of Foods- Meat & Meat Products** has been carefully selected based on its scientific rigor, applicability, and relevance to the food testing laboratories, QA/QC Professionals of industry. The procedures are meticulously detailed, providing step-by-step instructions, necessary reagents, and equipment requirements.

We express our sincere gratitude to the numerous experts who have contributed their knowledge, expertise, and insights to the development of this manual especially Dr. Vishnuraj M.R, Scientist, ICAR- National Meat Research Institute, Hyderabad, for valuable insight. I am thankful to the Chairperson, FSSAI and CEO, FSSAI for their support and constant encouragement without which the work would not have seen the light of day.

Any suggestions/feedback from the stakeholders, which will contribute towards updating the manual from time to time are welcome.

October 2024

Dr. Satyen Kumar Panda
Advisor (QA)

G20 भारत 2023 INDIA

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Eat Right India
सही भोजन, बेहतर जीवन.

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Table of Contents

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for determination of Nitrite**		
Method No.	**FSSAI 05.001:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document is to provide a procedure for the determination of the nitrite content in meat and meat products using spectrophotometric method.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	The present method describes a spectrophotometric method for the determination of nitrite based on the reaction of nitrite with sulfanilamide to form a diazonium salt, then coupling the diazotized sulfanilamide with N- (1-Napthyl) ethylenediamine dihydrochloride to form an intensely purple coloured azo-dye which is measured spectrophotometrically.		
Apparatus/Instruments	1. Meat mincer - fitted with a perforated plate with holes not greater than 4 mm in diameter 2. Analytical Balance 3. Volumetric flasks - 100 mL, 250 and 1000 mL 4. Pipette 10 mL 5. Conical flask 6. Boiling water bath 7. Filter paper (Fluted) 8. Photoelectric colorimeter or spectrophotometer.		
Materials and Reagents	1. NED reagent (1-Napthyl ethylenediamine dihydrochloride) 2. Sulphanilamide reagent 3. Nitrite standard solution 4. Filter paper - Test for nitrite contamination by analyzing 3-4 sheets at random. Filter approx. 40 mL water through each sheet. Add 4 mL of sulphanilamide reagent, mix, let stand 5 minutes, add 4 mL of NED reagent, mix and wait for 15 minutes. If any sheets are positive do not use them.		
Preparation of Reagents	1. NED reagent - Dissolve 0.2 gm N- (1-Napthyl) ethylenediamine dihydrochloride in 150 mL, 15% (v/v) acetic acid. Filter if necessary and store in a glass stoppered brown glass bottle 2. Sulphanilamide reagent- Dissolve 0.5 gm sulphanilamide in 150 mL 15% acetic acid (v/v). Filter, if necessary and store in a glass stoppered brown bottle. 3. Nitrite standard solution- (i) Stock solution - 1000 ppm $NaNO_2$ - Dissolve l.000 gm pure $NaNO_2$ in water and make up to 1 liter. (ii) Intermediate solution - 100 ppm - Dilute 100 mL of stock solution to 1 liter with water. (iii) Working solution- l ppm - Dilute 10 mL of intermediate sol to 1 liter with water.		
Sample Preparation	To prevent loss of moisture during preparation and subsequent handling, do not use small test samples. Keep ground material in glass or similar containers with air and water-tight covers. **(a) Fresh and frozen meat, cured meats, smoked meats etc.** Separate as completely as possible from any bone, pass rapidly three times through food chopper with plate opening equal to 1/8th inch (3 mm), mixing thoroughly after each grinding and begin all determinations promptly. If any delay occurs, chill the sample to		

	inhibit decomposition. In case of cured meats, mix thoroughly with a spatula or pass through a food chopper or mix in a homogenizer/blender to a uniform mass as appropriate. Transfer to a wide-mouth glass or other suitable container with an airtight stopper. Carry out the analysis as soon as possible. **(b) Canned Meat:** Pass the entire contents of the can through the food chopper or blender to obtain a uniform mass. Dry portions of samples are not needed for immediate analysis either in a vacuum at less than 60°C or by evaporating on a steam bath 2 -3 times with alcohol. Extract fat from dried product with petroleum ether (b. p. less than 60°C) and let petroleum ether evaporate spontaneously, finally expelling the last traces by heating for a short time on a steam bath. Do not heat test the sample or separate fat longer than necessary because of a tendency to decompose.
Method of analysis	1. Weigh a 5 gm prepared sample in a 50 mL beaker. Add about 40 mL of water heated to 80°C. 2. Mix thoroughly with a glass rod taking care to break all lumps and transfer to a 500 mL volumetric flask. 3. Thoroughly wash the beaker and glass rod with successive portions of hot water adding all washings to the flask. Add enough hot water to bring the volume to about 300 mL. 4. Transfer the flask to the steam bath and let stand for 2 hours shaking occasionally. Cool to room temperature, dilute to volume with water and remix and Filter. 5. If turbidity remains after filtration, centrifuging will usually clear the solution. 6. Add 2.5 mL of sulphanilamide sol. to an aliquot containing 5-50 µg $NaNO_2$ in a 50 mL vol. flask and mix. 7. After 5 minutes add 2.5 mL NED reagent, mix dilute to vol, mix and let colour develop for 15 minutes. 8. Transfer a portion of the solution to the photometer cell and determine the absorbance at 540 nm against a blank of 45 mL water and 2.5 mL of sulphanilamide reagent and 2.5 mL of NED reagent.
Calculation with units of expression	Nitrite content is expressed as $NaNO_2 = c \times 2000 / M \times V$ Where, V = volume in mL of an aliquot portion of filtrate taken for the test M =mass in gm of the sample taken c = concentration of sodium nitrite in µg/mL read from the calibration curve that corresponds with the absorbance of the solution prepared from the sample
Reference	1. AOAC 22nd edition, 2024, 983.18 Meat and Meat Products, Preparation of test sample (a) and (b) 2. AOAC 22nd edition, 2024, 973.31 Nitrites in cured meats - Colorimetric method, Adopted as Codex Reference method (Type II)
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Alternate Method for determination of Nitrite**		
Method No.	**FSSAI 05.002:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document is to provide a procedure for the determination of the nitrite content in meat and meat products using Photoelectric colorimeter or spectrophotometer.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	Extraction of a test portion in hot water, precipitation of the proteins and filtration by addition of Carrez solution. In the presence of nitrite development of red colour by the addition of sulphanilamide and N – naphthyl ethylenediamine dihydrochloride to the filtrate and photometric measurement at 538 nm.		
Apparatus/Instruments	1. Meat mincer - fitted with a perforated plate with holes not greater than 4 mm in diameter 2. Analytical Balance 3. Volumetric flasks - 100 mL, 250 and 1000 mL 4. Pipette 10 mL 5. Conical flask 6. Boiling water bath 7. Fluted filter paper 8. Photoelectric colorimeter or spectrophotometer.		
Materials and Reagents	**A. Solutions for precipitation of proteins** a) Potassium ferrocyanide solution b) Zinc acetate solution c) Borax solution **B. Standard Sodium nitrite solution** **C. Solution for colour development**		
Preparation of Reagents	**1. Potassium ferrocyanide solution:** Dissolve 106gm of Potassium ferrocyanide trihydrate in water and dilute to 1000mL **2. Zinc acetate solution:** Dissolve 220gm of Zinc acetate dehydrate and 30mL glacial acetic acid in water and dilute to 1000mL **3. Borax solution:** Dissolve 50gm of disodium tetra borate dehydrate in 1000mL of tepid water and cool to room temperature **4. Standard Sodium nitrite solution** - Dissolve 1.000 gm pure sodium nitrite in water and dilute to 100 mL in a volumetric flask. Pipette 5 mL of the solution into a 1000 mL volumetric flask and make upto volume. Prepare a series of standard solutions by pipetting 5 mL, 10 and 20 mL of the solution into 100 mL volumetric flasks and diluting to mark with water. These standard solutions contain 2.5 µg, 5.0 µg, and 10 µg sodium nitrite respectively. The standard solutions and the 0.05 gm/L solution from which they are prepared shall be made on the day of the use. **5. Solution for colour development** (i) Dissolve by heating on a water bath, 2 gm of sulphanilamide in 800 mL water. Cool, filter if necessary and add 100 mL of cone HC1 while stirring. Dilute to 1000 mL with water. (ii) Dissolve 0.25 gm of N – napthyl ethylenediamine dihydrochloride in water. Dilute to 250 mL with water Store in a stoppered brown bottle in a refrigerator for not more than one week (iii) Dilute 445 mL of Concentrated HCl (sp.gr 1.19) to 1000mL with water.		

Sample Preparation	To prevent loss of moisture during preparation and subsequent handling, do not use small test samples. Keep ground material in glass or similar containers with air and water-tight covers. **(a) Fresh and frozen meat, cured meats, smoked meats etc.** Separate as completely as possible from any bone, pass rapidly three times through a food chopper with a plate opening equal to 1/8th inch (3 mm), mixing thoroughly after each grinding and begin all determinations promptly. If any delay occurs, chill the sample to inhibit decomposition. In the case of cured meats, mix thoroughly with a spatula or pass through a food chopper or mix in a homogenizer/blender to a uniform mass as appropriate. Transfer to a wide-mouth glass or other suitable container with an airtight stopper. Carry out the analysis as soon as possible. **(b) Canned Meat:** Pass the entire contents of the can through the food chopper or blender to obtain a uniform mass. Dry portions of samples are not needed for immediate analysis either in a vacuum at less than 60°C or by evaporating on a steam bath 2-3 times with alcohol. Extract fat from dried product with petroleum ether (b. p. less than 60°C) and let petroleum ether evaporate spontaneously, finally expelling the last traces by heating for a short time on a steam bath. Do not heat test the sample or separate fat longer than necessary because of a tendency to decompose.
Method of analysis	1. Weigh to the nearest 0.001 gm, about 10 gm of the test sample, transfer quantitatively to a 300 mL conical flask and add successively 5 mL of borax solution and 100 mL water at a temperature, not below 70°C 2. Heat the flask for 15 minutes in the boiling water bath and shake repeatedly. Allow the flask and its contents to cool to room temperature and add successively 2 mL of potassium ferrocyanide followed by 2 mL of zinc acetate. 3. Mix thoroughly after each addition. Transfer the contents to a 200 mL volumetric flask. Dilute to mark with water and mix. Allow the flask to stand for 30 minutes at room temperature. 4. Carefully decant the supernatant liquid and filter it through fluted filter paper to obtain clear solution. **Colour Development:** 5. Pipette an aliquot of the filtrate (v mL) not more than 25 mL into a 100 mL volumetric flask and add water to make upto 60 mL. 6. Add 10 mL of sulphanilamide solution followed by 6 mL of conc. HCl and leave the solution in the dark for 5 minutes. 7. Add 2 mL of N-Napthylethylenediamine solution and leave for 5-10 minutes in the dark. Dilute to mark with water. 8. Measure the absorbance of the solution in a 1 cm cell using a photoelectric colorimeter or spectrophotometer at a wave length of about 538 nm Prepare a calibration curve by taking 10 mL water in 4 separate volumetric flasks, adding 10 mL each of the standard sodium nitrite solution containing 2.5, 5.0 and 10 µg of nitrite/mL, developing the colour and measuring as above. **Preparation of calibration curve:** 9. Prepare a calibration curve by taking 10 mL water in 4 separate volumetric flasks, adding 10 mL each of the standard sodium nitrite solution containing 2.5, 5.0 and 10 µg of nitrite/mL, developing the colour and measuring as above.

Calculation with units of expression	Nitrite content is expressed as $NaNO_2$= c x 2000/M x V Where, V = volume in mL of an aliquot portion of filtrate taken for the test M =mass in gm of the sample taken c = concentration of sodium nitrite in µg/mL read from the calibration curve that corresponds with the absorbance of the solution prepared from the sample
Reference	1. AOAC 22nd edition, 2024, 983.18 Meat and Meat Products, Preparation of test sample (a) and (b) 2. IS 5960 (Part VII): 1996 / ISO 2918: 1975 Meat and Meat Products Methods of Test - Determination of Nitrite content
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for determination of Nitrite in Processed meat and meat products like Ready to eat / ready to cooked products**		
Method No.	**FSSAI 05.003:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document is to provide a procedure for the determination of the nitrite content using Ion exchange chromatography Method in Processed meat and meat products like Ready to eat / ready to cooked products.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	The sample is extracted and purified using the relevant method after the protein precipitates and the fat is skimmed before being separated by an anion exchange column with KOH solution as an eluate and detected with a conductivity detector. It is then determined with an external standard method by taking retention time as for quantitative analysis.		
Apparatus/Instruments	1. Ion chromatograph: including a conductivity detector, suppressor, high-capacity anion exchange column, measuring ring in 25μL. 2. Food disintegrator. 3. Supersonic cleaner. 4. Analytical balance: readability 0.1mg and 1mg. 5. Centrifuge: rotational speed no less than 10000rpm with 5mL or 10mL centrifugal tubes. 6. 0.22 μm syringe filters with hydrophilic filterable membrane. 7. Decontaminating column: including C18 column, Ag column and Na column or its equivalent. 8. Syringe: 1.0 mL and 2.5 mL. All glassware should be soaked in 2mol/L of NaOH solution and water for 4h, respectively, followed by rinsing with water for 3-5 times before ready for use later.		
Materials and Reagents & Preparation of Reagents	1. Ultrapure water: with its conductivity of 18.2MΩ.cm. 2. CH3COOH: analytically pure 3. KOH: analytically pure 4. CH3COOH solution (3%): 3 mL CH3COOH (3.2) into 100mL volumetric flask, diluted to a mark with water and fully homogenized. 5. Nitrite ion ($NO2^-$) stock solution (100 mg/L, aqueous solution). 6. Nitrate ion ($NO3^-$) stock solution (1000 mg/L, aqueous solution). 7. Mixed standard solution of nitrate (counted on $NO3^-$ ion, the same herein below) and nitrite (counted on $NO2^-$ ion, the same herein below): accurately pipette 1.0 mL of nitrite ion ($NO2^-$) stock solution and nitrate ion ($NO3^-$) stock solution to 100 mL volumetric flask, diluted to a mark with water, which 1mL of this solution contains 1.0 μg of nitrite ion and 10.0 μg of nitrate ion.		
Sample Preparation	Meat, egg, aquatic products and their processed products: an adequate amount or full of materials is taken with quartering, and then prepared into a slurry with a stamp mill for use later.		
Method of analysis	**A. Extraction** a) Meat and their processed products: 5 gm (accurately weighed to 0.001gm) of sample in a homogeneous slurry form are taken and washed into a 100 mL volumetric flask with 80 mL water, extracted for 30min with an ultrasonic generator, shaken once every 5min to make sure that the solid phase is fully distributed.		

	Leave it on a water bath at 75°C for 5min before making volume with water. A portion of the solution after filtering is then subjected to centrifuge at 10000 rpm for 15 min; the supernatant is ready for use later. b) Salted meat, and other processed products: 2 gm (accurately weighed to 0.001gm) of sample in a homogeneous slurry form are taken and washed into an 100 mL volumetric flask with 80 mL water, extracted for 30 min with an ultrasonic generator, shaken once every 5 min to make sure that the solid phase is fully distributed. Leave it on a water bath at 75°C for 5 min before making volume with water. A portion of solution after filtered is then subjected to centrifuge in 10000 rpm for 15 min; the supernatant is ready for use later. c) 15 mL of supernatant are taken to run through a 0.22 μm syringe filters with hydrophilic filterable membrane and C18 column, the front segment in 3 mL is discarded (if Cl^- ion is over 100 mg/L, the supernatant should be successively run through syringe filters, C18 column, Ag column and Na column, the front segment in 7 mL shall be discarded), the eluate collected is then determined. The solid phase extraction column should be activated before applied. The activation is carried out as follows: if C18 column (1.0 mL), Ag column (1.0 mL) and Na column (1.0 mL) are engaged in the application: C18 column is run through with 10 mL of methanol, 15 mL of water before use, and then activated by resting for 30 min. Ag column (1.0 mL) and Na column (1.0 mL) are run through with water before activated with resting for 30 min. **B. Chromatographic conditions for reference:** Chromatographic conditions for reference Chromatographic column: selectivity of hydroxide, high-capacity anionic exchange column compatible to gradient elution **(a) Elution solution** i) General samples: KOH solution with its concentration of 6 mmol/L- 70 mmol/L, elution gradient is 6 mmol/L for 30 min, 70 mmol/l for 5 min and 6 mmol/l for 5 min. Flow rate is 1.0 mL/min. ii) Powder infant formula foods: KOH solution with its concentration of 5 mmol/L - 50 mmol/L, elution gradient is 5 mmol/L for 33 min, 50 mmol/L for 5 min and 5 mmol/L for 5 min. Flow rate is 1.3 mL/min. **(b) Inhibitor:** Anion inhibitor with regenerated membrane in automatic and continuous mode, or its equivalent **(c) Detector:** Conductivity detector with its temperature of detector cell at 35°C **(d) Sample volume:** 25 μL (enabled to be modified according to the content of ion to be measured). **C. Determination:** **(a) Standard curve:** The mixed standard solution of nitrite and nitrate pipetted is diluted with water to prepare a series of standard solutions with nitrite ion concentration of 0.00 mg/L, 0.02 mg/L, 0.04 mg/L, 0.06 mg/L, 0.08 mg/L, 0.10 mg/L, 0.15 mg/L, 0.20 mg/L, and with nitrate ion concentration of 0.0 mg/L, 0.2 mg/L, 0.4 mg/L, 0.6 mg/L, 0.8 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L. The chromatographic diagram of standard solution above with each concentration is obtained by successive injection of

	samples one by one from the lowest concentration. The calibration curve is plotted using concentration (mg/L) of nitrite and nitrate ions as abscissa and peak height (μS) and peak area as ordinate to calculate the linear regression equation  Fig.1 Chromatographic diagram of mixed standard solution of nitrite and nitrate **(b) Determination of samples:** 50 μl blank solution and 50 μl sample solution are injected into ion chromatograph one by one at the same working condition, respectively, chromatographic diagrams are then recorded. The peak height (μS) and peak area are individually measured using retention time for qualitative analysis.
Calculation with units of expression	**Formulation of analytical results:** The contents of nitrite (counted on $NO2^-$ ion) and nitrate (counted on $NO3^-$ ion) in samples are calculated in accordance with formula (1): $$X = \frac{(c - c_0) \times V \times f \times 1\,000}{m \times 1\,000} \quad \text{.....(1)}$$ Where, X = The content of nitrite or nitrate in samples, mg/kg; C = The content of nitrite or nitrate in samples for measurement, mg/L; C_0 = The content of nitrite or nitrate in blank solution, mg/L; V = The volume of sample solution, mL; f = dilution factor of sample solution; m = sample taken, gm **Note:** The content of $NO2^-$ in the sample multiplies by 1.5, to represent the nitrite content (calculation per sodium nitrite). The content of $NO3^-$ in the sample multiplies by 1.37, to represent the nitrate content (calculation per sodium nitrate). The result is represented by the mean arithmetical value from two independent determination results under the same condition, and keeps two digits. **Precision:** The absolute difference between two independently measured results under the same condition will not be over 10% of the arithmetic mean.
Reference	Chinese standard method for determination of Nitrate and Nitrite in Foods; GB5009.33-2010
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.004:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide the procedure for the determination of Ascorbic acid in meat and meat products using an Indophenol reagent (oxidation-reduction indicator dye).		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	Ascorbic acid reduces oxidation-reduction indicator dye 2, 6 dichlorophenol Indophenol to a colourless solution. At the endpoint excess unreacted dye is rose pink in the acid solution. The vitamin is extracted and the titration is performed in the presence of metaphosphoric acid acetic acid solution to maintain proper acidity and avoid auto-oxidation of ascorbic acid at high pH.		
Apparatus/Instruments	1. Titration apparatus 2. Centrifuge		
Materials and Reagents	1. Extracting solution 2. Ascorbic acid standard solution (1 mg/mL) 3. Indophenol standard solution		
Preparation of Reagents	**(a) Extracting solution** Metaphosphoric acid-acetic acid solution-Dissolve with shaking 15 gm HPO3 pellets or freshly pulverized sticks in 40 mL acetic acid and 200 mL water. Dilute to 500 mL. Filter rapidly through fluted filter paper into a glass stoppered bottle. Store in a refrigerator. The solution remains satisfactory for 7-10 days. **(b) Ascorbic acid standard solution (1 mg/mL)**: Accurately weighs 50 mg USP Ascorbic acid reference standard that has been stored in a desiccator away from sunlight. Transfer to 50 mL vol flask. Dilute to vol with metaphosphoric-acetic acid extracting solution before use. **(c) Indophenol standard solution:** Dissolve 50 mg 2,6 dichlorophenol indophenols sodium salt in 50 mL of water to which have been added 42 mg of NaHCO3. Shake vigorously and when the dye dissolves dilute to 200 mL with water. Filter through the fluted filter in an amber-coloured glass bottle. Keep stoppered and store in a refrigerator. (**Note:** Decomposition products that make endpoint indistinct occur in some batches of dry indophenol and also develop with time in stock solution. Add 5 mL of extracting solution with excess ascorbic acid to 15 mL of dye solution. If the reduced solution is not practically colourless discard it and prepare a new stock solution.) **Standardization of Indophenol solution:** Transfer 3 aliquots of 2.0 mL Ascorbic acid standard sol. to 3 conical flasks containing 5 mL of metaphosphoric-acetic acid extracting solution. Titrate rapidly with indophenol dye from 50 mL burette until a light distinct rose pink remains for 5 seconds. Each titration should require about 15 mL of indophenol solution and differ from each other by 0.1 mL. Similarly, titrate 3 blanks composed of 7 mL of metaphosphoric-acetic acid water equal to the vol. of indophenol sol used in the earlier titration. Titrate		

	with indophenol. Titre for the blank should be approx 0.1 mL. Subtract the blank from earlier titration and calculate the concentration of indophenol solution as mg ascorbic acid equivalent to 1 mL of solution. Standardize indophenol solution daily with the freshly prepared ascorbic acid standard solution
Sample Preparation	Make the sample homogeneous by passing it at least twice through the meat mincer and mixing. Keep the homogenized sample in a completely filled airtight closed container and store it in such a way that deterioration and change in composition is prevented.
Method of analysis	1. Take about 25-50 gm of prepared sample (containing 100 to 200 mg ascorbic acid). 2. Dilute with 100 mL of metaphosphoric-acetic acid solution and mix thoroughly in a laboratory homogeniser. Centrifuge and decant the supernatant liquid through an acid-washed filter paper. 3. Take 3 sample aliquots containing about 2.0 mg of ascorbic acid, and add 5 mL of metaphosphoric-acetic acid solution. Make a blank using 7 mL of extracting solution. 4. Titrate with indophenol solution.Titrate the blank also by diluting it with water to the extent of the indophenol sol used in the sample. **Alternate Method:** 1. Extract 50 gm prepared sample with 100 mL meta phosphoric acid - acetic acid mixture prepared by dissolving 30 gm metaphosphoric acid in 1000 mL water containing 80 mL glacial acetic acid. Mix thoroughly in a laboratory homogenizer. 2. Centrifuge and decant the supernatant through an acid-washed filter paper. Titrate 2 mL of the extract with a solution of 2,6 - dichloro - N - p -hydroxy phenyl- p - benzoquinone monoamine (30 gm in 200 mL water) until a permanent pink colour persists for at least 1 minute. 3. 0.1 mL = 7 mg of total ascorbate
Calculation with units of expression	Ascorbic acid mg/100 gm $= \frac{\text{(Sample titre–blank) x mg ascorbic acid/mL x Vol. made x 100}}{\text{Aliquot taken x wt of sample}}$
Reference	1. AOAC 22nd edition, 2024, Official method 967.21 Ascorbic acid in vitamin preparation and juices 2. Pearsons Composition and Analysis of Foods 9th edn1991, page 500
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.005:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a procedure for the determination of ascorbic acid using High-Performance Liquid Chromatography (HPLC) with UV detection.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	Vitamin C is extracted from products with a combination of ethylenediamine tetra acetic acid (EDTA), tris (2-carboxy-ethyl) phosphine (TCEP) hydrochloride, and metaphosphoric acid. These reagents precipitate proteins and stabilize vitamin C. There are two chromatographic options available for use with this method. **Option 1 —** A portion of prepared sample is injected onto a 4.6 × 75 mm, 4 µm, 80 Å, reversed-phase phenyl column that is compatible with 100% aqueous mobile phases where vitamin C is separated from other early- and late-eluting compounds present in the sample with a sodium acetate/EDTA mobile phase containing the ion pairing agent, dodecyl trimethylammonium bromide. The fraction of eluant from the 4.6 × 75 mm phenyl column containing vitamin C is collected on a 4.6 × 150 mm, 4 µm, 80 Å, reversed- phase phenyl column where vitamin C is further separated from compounds present in the sample. After vitamin C elutes from the 4.6 × 150 mm column, it is detected and quantitated by UV at 254 nm. **Option 2 —** A portion of prepared sample is injected onto a 3 × 100 mm, 2.5 µm, 100 Å, reversed-phase phenyl column where vitamin C is separated from other compounds present in the sample with a sodium acetate/EDTA mobile phase containing the ion pairing agent, dodecyl trimethylammonium bromide. After vitamin C elutes from the column, it then passes through the UV detector cell. Option 1 is ideal for laboratories with high sample throughput analyzing multiple samples daily every week. With option 1, thousands of samples can be analyzed before the columns need to be cleaned. Option 2 is ideal for laboratories with low sample throughput analyzing a few samples every week. With option 2, the column will typically need to be cleaned after approximately 200 injections. Also, with option 2 it may be necessary to clean and store the column in 50% acetonitrile if the system is not used daily or weekly.		
Apparatus/Instruments	1. HPLC instrument — Autosampler, vacuum degasser, pump, UV detector, and column switching valve, or equivalent. 2. HPLC autosampler vials and caps 3. HPLC columns — **Option 1 —**Reverse phase phenyl column size: 4 µm, 80 Å, 4.6 × 75 mm or reverse phase phenyl column, size: 4 µm, 80 Å, 4.6 × 150 mm each with an appropriate guard column, or equivalent. **Option 2 —** Reverse phase phenyl column, size: 2.5 µm, 100 Å, 3 × 100 mm with an appropriate guard column, or equivalent. 4. Analytical and top-loading balances — Capable of weighing 0.0001 gm and up to 1000gm. 5. Filtering apparatus —Vacuum filter apparatus for membrane		

	filters, including 2 L flask (Millipore, or equivalent). 6. Filter membrane —Nylon, 0.45 µm. 7. Filter paper — Medium flow and nominal particle retention rating of 8 µm. 8. Vacuum flasks —2000 mL. 9. Homogenizer or blender. 10. Light shields —Yellow or clear shields with a cutoff of 385 nm. 11. pH meter. 12. Pipets — Volumetric, Class A, assorted sizes. 13. Pipettors —Mechanical, 1–5 mL, 100–1000 µL, and 10–100 µL with tips, repipet heads, or equivalent. 14. Syringe —Disposable, 1 mL. 15. Syringe filters —Nylon, 13 mm, 0.45 µm . 16. Ultrasonic bath. 17. Vials - Glass with screw cap, to hold approximately 5.5 mL vitamin C stock standard.
Materials and Reagents	Storage of chemicals or reagents at any temperature between 2 and 30°C in an airtight, inert container is appropriate unless otherwise stated. Also, unless otherwise stated, the re-evaluation date for all opened reagents is 2 years from the date received. Regardless of expiration or re-evaluation dates, discontinue the use of any chemicals or solutions whenever 0indications of contamination, chemical degradation, or changes in concentration are evident. 1. Acetonitrile — HPLC grade 2. Control sample — A representative sample is analyzed with each batch of samples to monitor method performance. 3. Dodecyl trimethylammonium bromide—Reagent grade 4. Drierite (desiccant)—Anhydrous calcium sulfate, 8 mesh, or equivalent. 5. EDTA, disodium salt— Analytical Grade 6. Laboratory water—Distilled or deionized water 7. Meta-phosphoric acid— Analytical Grade 8. Phosphoric acid—85%, Analytical Grade 9. Sodium acetate—Anhydrous; Analytical Grade 10. TCEP hydrochloride—Analytical Grade 11. Takadiastase 12. Ascorbic acid —USP reference standard for instrument calibration. 13. NIST SRM 1849—NIST infant/adult nutritional formula with certified values for 14. evaluation of method performance
Preparation of Reagents	**Solutions Preparation:** Solutions can be stored at 2–30°C in tight inert containers unless otherwise noted. Preparation should be performed under shielded fluorescent lighting with a minimum UV cutoff of 385 nm. **(a) 6% Metaphosphoric acid—**Weigh 30.0gm (±10%) metaphosphoric acid into a 500 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store refrigerated. Expiration: 1 week. **(b) 3% Metaphosphoric acid—**Weigh 15.0gm (±10%) metaphosphoric acid into a 500 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store refrigerated. Expiration: 1 week.

	(c) 0.2% EDTA—Weigh 2.0 gm (±10%) EDTA into a 1000 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store at room temperature. Expiration: 6 months. **(d) 1% TCEP—**Weigh 0.1 gm (±10%) TCEP hydrochloride into a 10 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Expiration: 2 months. **(e) Ascorbic acid stock standard (2000 mg/L)—**Weigh 0.2000 gm (±2%) ascorbic acid into a 100 mL volumetric flask. Dissolve and dilute to volume with 3% metaphosphoric acid. Mix well. Store approximately 5.5 mL aliquots frozen in individual vials. Expiration: 2 years. **(f) Ascorbic acid intermediate standard (100 mg/L) —** Quantitatively transfer 5.0 mL ascorbic acid stock standard into a 100 mL volumetric flask with a volumetric pipet. Dilute to volume with 3% metaphosphoric acid. Mix well. Expiration: 1 day. Discard after use. **(g) Ascorbic acid routine working standards (15, 7, and 2 mg/L)-** Quantitatively transfer 15.0, 7.0, and 2.0-mL ascorbic acid intermediate standard into separate 100 mL volumetric flasks with volumetric pipets. Add 5 mL (±10%) 0.2% EDTA, 100 µL (±10%) 1% TCEP and 5mL (±10%) 6% metaphosphoric acid and dilute to volume with laboratory water. Mix well. Filter the working standards through 0.45 µm syringe filters. Discard the first millilitre and collect the second millilitre in HPLC autosampler vials. Store refrigerated. Expiration: 14 days. **(h) Ascorbic acid working standards for low sample concentrations (1, 0.5, 0.25, and 0.125 mg/L)—**To make the 1.0 and 0.5 mg/mL working standards, quantitatively transfer 1 and 0.5-mL ascorbic acid intermediate standard into separate 100 mL volumetric flasks with volumetric pipets. Add 5 mL (±10%) 0.2% EDTA, 100 µL (±10%) 1% TCEP, and 5 mL (±10%) 6% metaphosphoric acid and dilute to volume with water. Mix well. To make the 0.25 and 0.125 mg/mL working standards quantitatively transfer 5 mL of the 0.5 mg/mL and 5 mL of the 0.25 mg/mL working standard into a 10 mL volumetric flask. Add 0.5 mL (±10%) 0.2% EDTA, 10 µL (±10%) 1% TCEP, and 0.5 mL (±10%) 6% metaphosphoric acid and dilute to volume with laboratory water. Mix well. Filter the working standards through 0.45 µm syringe filters. Discard the first milliliter and collect the second milliliter in HPLC autosampler vials. Expiration: 1 day. **(i) 0.25 M sodium acetate buffer—**Weigh 41.0 gm (±10%) sodium acetate anhydrous into a weighing dish. Quantitatively transfer to a 2000 mL beaker containing approximately 1800mL laboratory water and dissolve. Adjust pH to 3.0 (±0.1) with concentrated phosphoric acid. Quantitatively transfer solution to a 2000 mL volumetric flask and dilute to volume with laboratory water. Expiration: 1 month. **(j) Mobile phase —** Quantitatively transfer 200 mL (±10%) 0.25 M sodium acetate buffer and 100 mL (±10%) 0.2% EDTA to a beaker

	containing approximately 1650 mL laboratory water. Weigh 1.0 gm (±10%) dodecyl trimethylammonium bromide and quantitatively transfer it to the beaker. After all of the dodecyl trimethylammonium bromide has dissolved, adjust the pH of the solution to 3.15 (±0.05) with phosphoric acid. Quantitatively transfer the solution to a 2000 mL volumetric flask and dilute to volume with laboratory water. Filter the solution through a 0.45 µm filter and degas. Expiration: 1 month. **(k) Precipitant—**Dilute 1 part 6% metaphosphoric acid with 1 part 0.2% EDTA. Mix well. Expiration: 16 h. **(l) 6% Takadiastase solution—**Weigh 0.6 gm (±10%) into a 50 mL beaker. Add approximately 10 mL of laboratory water. Stir until dissolved. Expiration: 8 h.
Sample Preparation	**Sample preparation—**Liquid samples must be freshly opened and sample containers tightly closed between samplings. Sample preparations must be completed within 20 min after the container is opened. Sample preparations are good for up to 18 h. After 18 h sample preparations cannot be injected and must be prepared again. All samples, liquids, semisolids, and powders should be as uniform and representative of the product as possible. This should be accomplished by thoroughly mixing or stirring the product prior to sampling. Mixing of liquid products should be performed to minimize the production of foam. Powdered products may require a preliminary reconstitution. Sampling of liquid products should be performed immediately after a final, gentle mixing or stirring to prevent inaccurate sampling due to stratification induced by foaming or creaming. Powdered products, which are not homogeneous at the subgram level, should first be reconstituted. Some products may require homogenization in order to assure accurate sampling. For powder products which are not homogeneous at the subgram level, reconstitute the samples by dissolving appropriate amounts of the powders in water or precipitant. If necessary, homogenize or blend the reconstitutions to breakup any large clumps. Appropriate sample sizes can be calculated from the following equation: Sample size = 400/E Where, sample size is the theoretical sample size, in grams; E is the expected ascorbic acid concentration in mg/L or mg/kg of the liquid or reconstituted sample; and 400 is the desired amount, in micrograms (µg), of ascorbic acid in the sample preparation. The net conversion factor for µg to mg and kg to grams is unity. Weight and volume sample sizes are interchangeable where feasible provided appropriate corrections are made to the calculations using the product density. All sample weights should be recorded to at least three significant figures. • Immediately after preparing sample reconstitutions or after opening samples, weigh the sample into a 100 mL volumetric flask and record the weight to the nearest 0.0001 gm. If the sample contains a significant amount of starch, add 0.5 mL 6% takadiastase and allow it to react for 1 min before continuing. • Prepare a control sample with each sample set. • Immediately after weighing the sample into a 100 mL volumetric flask, add 5 mL
Method of analysis	**Instrument Operating Conditions:**

A. Instrument conditions—Option 1 —Note: Option 1 is ideal for laboratories with high sample throughput analyzing multiple samples daily. With option 1 thousands of samples can be analyzed before the columns need to be cleaned.

1. Mobile phase flow rates (both pumps)—1.0 mL/min.
2. Columns— Reverse phase phenyl columns of size 4.6 × 75 mm, 4 µm, 80 Å and reverse phase phenyl column of size, 4.6 × 150 mm, 4 µm, 80 Å.
3. Injection volume—20 µL.
4. Detector wavelength—254 nm.
5. Run time—11 min.
6. System configuration—See Figure 2012.21A.

A. Configuration 1—0–2 min.
B. Configuration 2—2–3 min.
C. Configuration 1—3–11 min.

B. Instrument conditions— Option 2

Option 2 is ideal for laboratories with low sample throughput analyzing

A few samples every week. With option 2 the column will typically need to be cleaned after approximately 200 injections. Also, with option 2 it may be necessary to clean and store the column in 50% acetonitrile if the system is not used daily or weekly.

1. Mobile phase flow rate—0.4 mL/min.
2. Column —Reverse phase phenyl column of size 3 × 100 mm, 2.5 µm, 100 Å.
3. Injection volume—20 µL.
4. HPLC detector wavelength—254 nm.
5. Run time —15 min

C. Instrument start-up — Equilibrate the instrument by pumping the mobile phase through the columns for at least 0.5 h before injecting standards and samples onto the column. When new columns are installed, the mobile phase must pass through the columns for at least 3 h to equilibrate the columns with ion pairing agent.

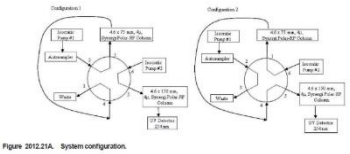
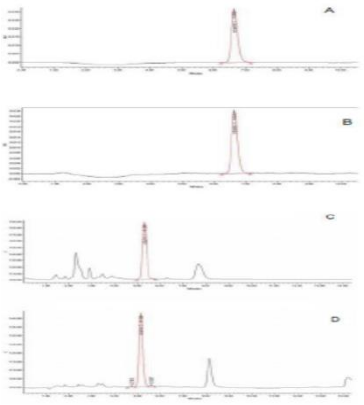


Figure 2012.21A. System configuration.

D. HPLC analysis of standards and samples —Inject the most concentrated standard 3–4 times and note the peak areas. Confirm that the precision of the peak areas is ≤2% RSD and the peak areas are not steadily increasing or decreasing by more than 4% from the first injection to the third or fourth injection. If the RSD >2%, locate the source of the imprecision and correct it before beginning the sample analysis. Once the system has been calibrated, inject the control and sample preparations. The control sample preparation should be the first injection after the system has been calibrated. For batch analyses, all working standards should be injected every 8 h which will allow analysis of the control sample and up to 44 samples before another set of working standards must be analyzed.

	E. HPLC column and system maintenance— **a. Option 1 —**If the vitamin C peak responses drop and curves have acceptable linearity, clean off the columns using 50% water– acetonitrile solution at 0.6–0.8 mL/min for 1 h. After cleaning the columns, reequilibrate the columns with mobile phase for at least 3 h. **b. Option 2 —** After approximately 200 sample injections, the vitamin C retention time will decrease and the vitamin C peak will begin to merge with other peaks present in the chromatograms. When this happens, clean off the columns using 50% water–acetonitrile solution at 0.6–0.8 mL/min for 1 h. After cleaning the columns, re-equilibrate the columns with mobile phase for at least 3 h.
Calculation with units of expression	The ascorbic acid concentrations of samples analyzed with the HPLC system are determined by comparison of the ascorbic acid peak areas from samples of known weight with the peak areas of standards of known concentration. **A.** Calculation of the standard concentrations. 1. Concentration of stock standard 2. Concentration of intermediate standard $C_i = \frac{C_s \times 5.0}{100} = C_s \times 0.05 = W \times P \times 500$ Where, Ci is the concentration of the intermediate standard solution in mg/L; 5.0 is the volume of the stock standard solution aliquot in mL; 100 is the dilution volume in mL. 3. Concentration of working standards: $Cw = (Ci \times A)/100 = W \times P \times A \times 5$ Where, Cw is the concentration of the working standard in mg/L; A is the volume of the intermediate standard in mL; 100 is the dilution volume in mL **B.** Measurement of peak areas—Peak areas are measured with a data system. Before calculating concentrations, compare the ascorbic acid standard peaks with the ascorbic acid sample peaks to make sure that there are not any interfering compounds and that ascorbic acid is separated from all other components in the sample. The concentration of ascorbic acid cannot be calculated if there are interferences or if there is poor separation. Also check to see that the ascorbic acid peak areas of the samples fall within the range of the standards. Peak areas of the same standards injected before and after a set of samples should not change by more than 6%. If they do, the system was not equilibrated or the columns need to cleaned or replaced and the data are not acceptable. **C. Preparation of the standard curve—**Average the standard peak responses from the standards injected before and after a set of samples. Prepare a standard curve by performing a linear least squares routine (regression) on the concentration of the working standards versus their corresponding averaged peak areas. The linear correlation coefficient (r) of the curve should be >0.999.

	D. Calculation of ascorbic acid concentration in a sample— $$C_{(AA)} = \frac{C_{(1)} \times 100}{S} \times R \times D$$  **Figure 2012.21B. HPLC analysis of (A) vitamin C standard, option 1; (B) infant nutritional product, option 1; (C) infant nutritional product, option 2; and (D) adult nutritional product, option 2.** Where, C(AA) is the concentration of ascorbic acid in the original sample in mg/kg or mg/L; 100 is the dilution volume of the sample in mL; S is the sample size in grams or mL; C(I) is the concentration of ascorbic acid in the injected sample determined from the standard curve in mg/L; R is the reconstitution rate (total weight of sample reconstitution in grams divided by the weight of powder or solid used in grams), if no sample reconstitution is required R = 1; and D is the density of the product in g/mL (if results for weighed samples 29 need to be converted from mg/kg to mg/L). **E.** Calculation of ascorbic acid concentrations in control samples — Calculate the concentration of ascorbic acid in the control sample. The control result must be within ±3 standard deviations of the control mean. If the control result is outside 3 standard deviations of the control mean, the samples must be prepared and analyzed again.
Reference	1. AOAC SMPR 2012.012. 2. AOAC SMPR 2012.012: Standard Method Performance Requirements for Vitamin C in Infant Formula and Adult/Pediatric Nutritional Formula, Journal of AOAC INTERNATIONAL, Volume 96, Issue 3, 1 May 2013, Page 491
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.006:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a procedure for the determination of the total phosphorous content in meat and meat products using acid digestion method.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	Mineralization of a test portion (wet digestion) with sulphuric and nitric acid, precipitation of phosphorous as quinoline phosphomolybdate and drying and weighing of the precipitate. Alternatively, the sample can be ashed and ash taken up in 15 mL conc. nitric acid in a conical flask adding water to make up to 75 mL, heating on a boiling water bath for 30 minutes, cooling and making up to a known volume.		
Apparatus/Instruments	1. Mechanical meat mincer - fitted with a plate with holes of dia not exceeding 4 mm. 2. Analytical balance 3. Kjeldahl flask 4. Heating device on which the flask can be heated in an inclined position in such a way that the source of heat only touches the wall of the flask which is below the level of the liquid. 5. Suction device to remove the acid fumes formed during the digestion. 6. Fritted glass filter - pore diameter 5-15 mm. 7. Drying oven capable of being adjusted to 260 — 20°C 8. Conical suction flask 9. Desiccator		
Materials and Reagents	1. Conc. Sulphuric acid - 1.84 gm/mL 2. Conc. Nitric acid - 1.40 gm/mL 3. Precipitating reagent		
Preparation of Reagents	**Precipitating reagent -** Dissolve 70 gm of Sod. Molybdate dehydrate in 150 mL water. Dissolve 60 gm of Citric acid monohydrate in 150 mL water and add 85 mL of cone nitric acid. Mix the two solutions and stir slowly. To another 100 mL water add 25 mL nitric acid and 5 mL of distilled quinoline. Gradually add this solution to the first solution while stirring. Leave for 24 hrs at room temperature. Store the reagent in a stoppered plastic bottle in the dark		
Sample Preparation	Make the sample homogeneous by passing it at least twice through the meat mincer and mixing. Keep the homogenized sample in a completely filled airtight closed container and store it in such a way that deterioration and change in composition is prevented. Analyse the sample as soon as possible, but in any case, within 24 hrs. If the sample is not immediately analysed after passage through the mincer, liquid separation may occur. Therefore, homogenize the sample thoroughly immediately before testing		
Method of analysis	1. Weigh to the nearest 0.001 gm about 3 gm of sample into the flask, add 20 mL nitric acid and some glass beads. 2. Place the flask in an inclined position on the heating device and heat for 5 minutes. Cool and then add 5 mL of sulphuric acid. 3. Heat the flask gently until the foaming has ceased, then heat more strongly. Add more nitric acid and continue heating. 4. Repeat the operation until evolution of brown fumes has ceased.		

	Finally, when the liquid has become colourless heat until white fumes appear. Cool add 15 mL water and boil gently. 5. Transfer the liquid to a 250 mL beaker or conical flask rinsing the flask with water. 6. Add 10 mL nitric acid. The total volume should then be 50mL. **Determination:** 1. Add 50 mL of the precipitating reagent to the liquid in the beaker or conical flask. Cover with a watch glass and boil for 1 minute on a hot plate. 2. Allow to cool to room temperature, during cooling swirl the contents three or four times. 3. Filter under the suction through the fritted glass filter which has been previously heated for 30 minutes at a temperature of 260 ± 2°C, cooled in a desiccator and weighed to the nearest 1 mg. 4. Wash the ppt on the filter five times with 25 mL water using the same water to wash away any remaining ppt from the conical flask onto the filter. 5. Dry in the oven at 260 ± 2°C for 1 hr. Cool in a desiccator and weigh to the nearest 1 mg. 6. Carry out a blank test using same procedure and the same quantity of reagents but omitting the test portion.
Calculation with units of expression	Phosphorous (%) as P2O5 = 0.03207 x m1/ m0 x 100 or = 3.207 x m1/ m0 Where, m0 = mass in gm of the test portion m1 = mass in gm of the quinoline phosphomolybdate precipitate. Report the result to the nearest 0.01 gm of phosphorous pentoxide/100 gm
Reference	IS 5960 (Part 9): 1988 / ISO 2294:1974 - Meat and Meat Products-Methods of Test - Determination of Total Phosphorous Content
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.007:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a process for the determination of the presence of polyphosphates in meat and meat products using Thin Layer Chromatography		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	Extraction of meat or meat product with trichloro acetic acid, cleaning of the serum obtained with ethanol/diethyl ether mixture, separation of the phosphates by thin layer chromatography and detection of polyphosphates by spraying with reagents for colour development.		
Apparatus/Instruments	1. Glass plates - 10 cm x 20 cm 2. Spreading device for preparing layers of 0.25 mm thickness 3. Laboratory mixer 4. Dessicator 5. Mechanical meat mincer fitted with a plate with holes of diameter not more than 4 mm. 6. Fluted filter paper, 15 cm dia 7. Micropipette - 1 µL or micrometer syringe 8. Paper lined glass tank. 9. Hair dryer 10. Sprayer 11. Oven capable of being maintained at 60°C		
Materials and Reagents	1. Trichloro acetic acid 2. Diethyl ether 3. Ethanol 95% (v/v) 4. Cellulose powder for TLC 5. Soluble starch		
Preparation of Reagents	**Reference mixture:** Dissolve in 100 mL water 200 mg of Sodium dihydrogen phosphate monohydrate (NaH2PO4.H2O), 300 mg of tetra sodium diphosphate decahydrate (Na4P2O7.10H2O), 200mg of penta sodium triphosphate (Na5P3O10) and 200mg of sodium hexametaphosphate (NaPO3)2 [lx >10]. The reference mixture is stable at 4 °C for at least 4 weeks. **Developing Solvent:** Mix 140 mL isopropyl alcohol, 40 mL of a 135 gm/liter solution of trichloro acetic acid and 0.6 mL of ammonium hydroxide 0.9 gm /ml, about 25 % (m/m) solution. **Spray Reagent I:** Mix equal volume of a 75 gm/liter solution of ammonium molybdate tetrahydrate [(NH4)6Mo2)24-4H2O] and conc. nitric acid (1.4 gm/mL) and dissolve 10 gm tartaric acid in 100 mL of this mixture **Spray reagent II:** Dissolve 0.5 gm of 1 amino 2 naphthol- 4 sulphonic acid in a mixture of 195 mL of a l50 gm/L solution of sodium disulphite (Sodium metabisulphite) and 5 mL of a 200 gm/L solution of sodium sulphite (Na2S2O3). Dissolve 40 gm of sod acetate trihydrate in the mixture. Store the reagent in a tightly closed brown bottle in the refrigerator, Discard the solution after 1 week.		
Sample Preparation	Proceed from a laboratory sample of at least 200 g. See IS0 3100.		

	Prepare the test sample on the day of its receipt in the laboratory. Homogenize the sample by passing it at least twice through the meat mincer and by mixing. Keep it in a completely filled, air-tight, closed container and store it, if necessary, in a refrigerator. Analyze the sample as soon as possible, but in any case, within 5 h.
Method of analysis	**Preparation of TLC plates:** Dissolve 0.3 gm starch in 90 mL boiling water, add 15 gm of cellulose powder and homogenize in the laboratory mixer for 1 minute. Apply the slurry onto glass plates with the spreading device adjusted to obtain a layer of 0.25 mm. Air dry the plates at room temperature and heat them finally for 10 minutes at 100°C. Store the plates in a desiccator. Alternatively, ready-to-use plates may be used. **Preparation of serum:** Macerate 50 gm of test sample with 15 mL water at 40 - 60°C in a beaker with a spatula or flattened stirring rod until a homogeneous mass is obtained taking no more than 5 minutes. Add 10 gm of trichloracetic acid and mix again. Immediately place in a refrigerator for 1 hr and then collect the separated serum by decanting through the fluted filter paper. If the filtrate is turbid shake once with an equal volume of diethyl ether. Remove the ether layer with a small pipette and add an equal volume of ethanol to the aqueous phase. Shake for 1 minute. Allow the mixture to stand for a few minutes and filter through a fluted filter paper. **Chromatographic separation:** Pour the developing solvent into the developing tank to a depth of 5 - 10 mm and close the tank with its lid. Allow to stand for at least 30 minutes at ambient temperature. Apply 3 µL of serum or 6 µL if the clearing procedure was carried out to the cellulose layer on a pencil line drawn at about 2 cm from the bottom. Keep the spots small by applying 1 µL at a time. Use a warm air stream from the hair dryer for drying. In the same way, apply 3 µL of reference mixture to the plate at a distance of 1 - 1.5 cm from the sample spot. Remove the lid from the tank and quickly place the cellulose plate in the tank. Develop the plate until the solvent front has ascended to approx 10 cm from the pencil line. Remove the plate, dry for 10 minutes in the oven at 60°C or for 30 minutes at room temperature. Spray the plates lightly but uniformly with spray reagent No 1. Yellow spots appear immediately. Dry the plate in a stream of warm air from a hair dryer and heat it in the oven for 1 hr at 100°C to remove the last traces of nitric acid. Check that the plate is free from the pungent smell of nitric acid. Allow the plate to cool and spray lightly with spray reagent no 2. Blue spots appear immediately. Spraying with reagent 2 is not an absolute necessity but the intense blue spots produced improve the detection considerably.
Inference (Qualitative Analysis)	Compare the migration distance of the phosphate spots from the sample and the reference mixture. An orthophosphate spot is always present. If the sample contains condensed phosphates, a diphosphate spot and/or spots of more highly polymerized phosphates are visible.
Reference	IS 5960 (Part 13): 1988 / ISO 5553: 1980 Meat and Meat Products – Methods of Test - Detection of Polyphosphates
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.008:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a procedure for the determination of Glucono- Delta- Lactone in meat and meat products		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	This is an enzyme ultraviolet procedure recommended by ISO and BSI (ISO 4133 and BS 4401, part 13). A test combination kit is available.		
Apparatus/Instruments	1. Centrifuge 2. Homogenizer 3. Pipettes 4. Volumetric Flask (250mL) 5. Fluted filter paper 6. Spectrophotometer or Colorimeter		
Materials and Reagents	1. Perchloric acid 2. Potassium hydroxide 3. Buffer Solution - pH 8.0 4. Nicotinamide adenine dinucleotide phosphate (NADP 5. Adenosine -5- triphosphate (ATP) 6. 6 - Phosphogluconate dehydrogenase (6 PGDH)- Commercial suspension containing 2mg 6- PGDH / mL from yeast 7. Gluconate kinase (GK)- Suspension containing mg/mL from E. coli.		
Preparation of Reagents	1. Perchloric acid - 0.4 M - Dilute 17.3 mL Perchloric acid (70 % m /m) to 500 mL with water 2. Potassium hydroxide - 2 M - Dissolve 56.l gm Pot. Hydroxide in water- Dilute to 500 mL 3. Buffer Solution - pH 8.0 - Dissolve 2.64 gm glycylglycine and 0.284 gm magnesium chloride hexahydrate in 150 mL water. Adjust to pH 8 with potassium hydroxide. Dilute to 200 mL with water 4. Nicotinamide adenine dinucleotide phosphate (NADP) - Dissolve 50 mg of NADP disodium salt in 5 mL water. 5. Adenosine -5- triphosphate (ATP) - Dissolve250 mg ATP disodium salt and 250 mg sodium hydrogen carbonate in 5 mL water.		
Sample Preparation	Proceed from a representative sample of at least 200g. Store the sample in such a way that deterioration and change in composition are prevented.		
Method of analysis	1. Weigh 50 gm of prepared sample into a homogenizer. Add 100 mL of cold (0°C) 0.4M perchloric acid and homogenize. Transfer the slurry to a 100 mL centrifuge tube. 2. Centrifuge at 3000 rpm for 10 minutes. Move the fat layer. Decant the supernatant through a fluted filter paper into a 200 mL conical flask and discard the first 10 mL. 3. Transfer 50 mL of the filterate to a 100 mL beaker. Adjust to pH 10 with Pot. Hydroxide and make up to 100 mL in a volumetric flask with water. Cool in ice for 20 minutes. 4. Filter through a fluted filter paper. Discard the first 10 mL. Pipette 25 mL of the filtrate (V mL) into a 250 mL volumetric flask. Dilute to mark with water (maximum concentration of D (+) gluconate is 400 mg/L). 5. This is the prepared extract. Pipette into each of 2 photometric cells - 2.5 mL of pH 8 buffer, 0.1 mL NADP, 0.1 mL ATP. 6. Into one of the cells pipette 0.2 mL extract, into the other 0.20 mL		

	water. Pipette 0.05 mL of 6 - PGDH suspension on to a plastic spatula, mix with the contents of one of the cells. 7. Repeat the operation with the second cell. Read the absorbance of each cell against air at 365 nm after 5 minutes. Retain the cells for reaction. 8. Al = absorbance of test solution A AIB = absorbance of blank 9. Pipette 0.01 mL of GK suspension on to the plastic spatula. Mix with the contents of one of the cells. Repeat the operation with the other cell. Read the absorbance of each cell at 365 nm after 10 minutes and again after 2 minutes until a constant rate of absorbance is obtained. Plot the absorbance against time and extrapolate the linear part of the curve back to zero time. 10. A2 = Absorbance (T = 0) of the test solution 11. A2 B = Absorbance (T = 0) of the blank solution
Calculation with units of expression	A = (A2 - Al) - (A2B - A1B) Glucona - delta lactone % by mass = $\frac{15058 \times A}{V \times m}\left(100 + \frac{M \times m}{(100)}\right)$ Where V = volume in mL of the filtrate to make prepared extract M = moisture content of prepared sample per cent m/m m = mass in gm of test sample
Reference	1. Pearsons Composition and Analysis of Foods 9th edn1991, page 502 2. Methods of test for meat and meat products: Part 11 – IS 5960: Part 11:1998, Determination of glucone- delta-lactone content
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.009:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a procedure for the determination of total fat content in meat and meat products using the Soxhlet apparatus.		
Caution	In order for the solvent to thoroughly penetrate the sample, it is necessary for the sample to be as finely comminuted as possible. Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	Total fat content is determined by extracting the fat from the sample using a solvent, and then determining the weight of the fat recovered. The sample is contained in a porous thimble that allows the solvent to completely cover the sample. The thimble is contained in an extraction apparatus that enables the solvent to be recycled over and over again. This extends the contact time between the solvent and the sample and allows it time to dissolve all of the fat contained in the sample.		
Apparatus/Instruments	1. Soxhlet apparatus 2. Desiccator 3. Analytical balance		
Materials and Reagents	1. Ammonium hydroxide (NH4OH) 2. Conc. HCl 3. Ethyl ether/Petroleum ether 4. Filter Paper		
Preparation of Reagents	1. Ammonium hydroxide (NH4OH) 2. Conc. HCl 3. Ethyl ether/Petroleum ether 4. Filter Paper		
Sample Preparation	Proceed from a representative sample of at least 200 g. Render the sample uniform by passing it at least twice through the meat mincer and mixing. Keep it in a completely filled airtight container and store it in such a way that deterioration and change in composition are prevented. Analyze the sample as soon as possible, but in any case, within 24 hours.		
Method of analysis	1. Weigh accurately 3-4 gm of well-mixed sample in a 100 mL beaker. Add a few drops of NH4OH and warm on a steam bath. Add 10mL of conc. HCl and boil for approx. 30 minutes. Cool, filter through a wetted filter paper. Wash filter paper with hot water. 2. Dry the filter paper containing the residue of the sample, roll and insert in an extraction thimble and extract fat in a Soxhlet apparatus using ethyl ether or petroleum ether, and transfer to another flask. Remove solvent. Keep the flask in an air oven maintained at 100°C for 30 minutes to remove residual solvent if any. Transfer the flask to a desiccator to allow it to cool. Weigh the residue and calculate the total fat.		
Calculation with units of expression	The total fat content of the sample, percent by weight, is equal to $100 \times \frac{W_2 - W_1}{W_0}$ where W_2 - weight, in g, of the flask with the dried fat; W_1 = weight, in g, of the empty extraction flask with boiling		

	W_0 = weight, in g, of the test portion. Take the result as the average of the two determinations.
Inference (Qualitative Analysis)	-
Reference	1. ISO 1443- 1973 Codex approved method - Extraction / gravimetric Type I method 2. IS: 5960 (Part 3) 1970 Methods of test for meat and meat products - Determination of total fat content
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.010:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide the procedure for the determination of total protein content in meat and meat products using Kjeldahl method.		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	Method consists in a procedure of catalytically supported mineralization of organic material in a boiling mixture of sulfuric acid and sulfate salt at digestion temperatures higher than 400 °C. During the process, the organically bonded nitrogen is converted into ammonium sulfate. Alkalizing the digested solution liberates ammonia which is quantitatively steam distilled and determined by titration.		
Apparatus/Instruments	1. Distillation apparatus 2. Pipettes 3. Titration apparatus		
Materials and Reagents	1. Kjeldahl catalyst 2. Sulphuric Acid - Concentrated 3. NaOH solution- 50% (1+1). Let stand until clear 4. Standard NaOH solution 5. Standard acid solution 6. Methyl Red Indicator		
Preparation of Reagents	1. Kjeldahl catalyst: - 15 gm Potassium Sulphate + 0.5 gm Copper sulphate 2. Standard NaOH solution-0.1 N=0.1 M (4.00 gm/liter) 3. Standard acid solution- Prepare either HCl or H2SO4 solution HCl sol-0.1 N= 0.1 M (3.646 gm/liter); H2SO4 sol - 0.1N=0.05 M (4.9 gm/liter) 4. Methyl Red Indicator - 0.5 gm in 100 mL ethanol		
Sample Preparation	Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1. It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Proceed from a representative sample of at least 200 g.		
Method of analysis	1. Weigh 1-1.5 gm of prepared sample and transfer to a kjeldahl digestion flask. Add 15 gm of Potassium sulphate, 0.5 gm of copper sulphate and 25-40 mL of Sulphuric acid. 2. Heat the flask gently in an inclined position until frothing ceases then boil briskly for 2 hours. Allow to cool. Add approx. 200mL of water and 25ml of Sodium thiosulphate solution (80 gm/L) and mix. 3. Add a piece of granulated Zinc or anti-bump granules and carefully pour down the side of the flask sufficient Sodium Hydroxide sol (1+1) to make the contents strongly alkaline (about 110 mL). 4. Before mixing the acid and alkaline layers connect the flask to a distillation apparatus incorporating an efficient splash head and condenser. 5. To the condenser fit a delivery tube which dips just below the surface of a pipetted vol. of the digestion flask and boil until about 150 mL of the distillate has been collected. 6. Add 5 drops of methyl red indicator and titrate with 0.1N NaOH. Carry out a blank, l mL of 0.1 HCl or H2SO4 is equivalent to 0.0014 of N.		
Calculation with units of	Total protein is equal to N X 6.25		

expression	
Reference	1. AOAC 22nd edition, 2024, Official Method 928.08 Nitrogen in Meat (Alternative II) 2. IS- 5960 (Part 1) 1996/ISO 937-1978 Meat and Meat Products - Determination of Nitrogen Content
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Method for determination of pH</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.011:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">The scope of this document is to provide the procedure for the determination of the pH of meat and meat products using a digital pH meter and also the Nitrazine – Yellow Test.</td></tr>
<tr><td>Caution</td><td colspan="3">Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.</td></tr>
<tr><td>Principle</td><td colspan="3">The potential difference is measured between a glass electrode and a reference electrode, which are placed in a sample or a sample extract of the meat or meat product.
The pH is a measure of the acidity or alkalinity in solutions or water-containing substances. pH values lower than 7 are considered acidic, while pH values higher than 7 are considered alkaline. A pH of 7 indicates neutrality. pH values are related to the concentration of hydrogen ions (H+) in the substance.

Typical pH values for meat and meat products are:
<table><tr><th>Product</th><th>Product pH value (range)</th></tr><tr><td>Meat mixes in jelly + vinegar added</td><td>4.5 to 5.2</td></tr><tr><td>Raw fermented sausage</td><td>4.8 to 6.0</td></tr><tr><td>Beef</td><td>5.4 to 6.0</td></tr><tr><td>Pork</td><td>5.5 to 6.2</td></tr><tr><td>Canned meats</td><td>5.8 to 6.2</td></tr><tr><td>Curing brines</td><td>6.2 to 6.4</td></tr><tr><td>Blood sausages</td><td>6.5 to 6.8</td></tr><tr><td>Muscle tissues, immediately after slaughter</td><td>7.0 to 7.2</td></tr><tr><td>Blood</td><td>7.3 to 7.6</td></tr></table>
pH measurement is useful for:
▪ Evaluation of meat quality for further processing, in particular the water binding capacity
▪ Control of ripening of raw fermented products, which is connected with drop in pH
▪ Control of acidity of ingredients such as brines, marinades etc.

The pH can be measured by following methods –
1. Digital pH meter
2. Chemical indicator method (Nitrazine yellow)</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">1. Digital pH meter
2. Stirring rod</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">1. Nitrazine yellow indicator (1:10000)
2. Distilled water</td></tr>
<tr><td>Preparation of Reagents</td><td colspan="3">-</td></tr>
<tr><td>Sample Preparation</td><td colspan="3">Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1. It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Proceed from a representative sample of at least 200 g.</td></tr>
</table>

<table>
<tr><td>Method of analysis</td><td>
A. Digital pH meter:
Portable instruments are battery driven and have glass electrodes. The pH–value in meat and meat products can be measured by direct contact between the sensitive diaphragm of the electrode and the meat tissue. Through the diaphragm differences in electrical load between the meat and electrolyte solution (e.g., Potassium chloride KCl) inside the glass electrode are measured and directly indicated as the pH-reading. In raw fresh meat, it is recommended to spray small amounts of distilled water onto the tissue at
the point of measurement (prior to inserting the electrode), because the operation requires some fluidity in the sample and the glass electrode should be thoroughly wet. The amount of water necessary will not appreciably alter the pH. For accurate pH readings, the pH meter should be calibrated before use and adjusted to the temperature of the tissues to be measured. The electrode must be rinsed with distilled water after each measurement.
Procedure:
i. Blend 15 gm of meat with 30 mL distilled water at 27-300C.
ii. Note the pH with a glass electrode pH meter.
B. Nitrazine- Yellow Test: This test determines the acidity of meat.
Procedure:
i. Take a piece of meat free of blood, fat, and connective tissue in a petri dish.
ii. Add Nitrazine yellow indicator (1:10000) sufficient to cover the meat piece
iii. Mix with stirring rod
iv. Note the colour change with the standard chart provided.
</td></tr>
<tr><td>Inference
(Qualitative Analysis)</td><td>
<table>
<tr><th>pH</th><th>Colour</th><th>Inference</th></tr>
<tr><td>6.0</td><td>Yellow</td><td>Good keeping quality</td></tr>
<tr><td>6.4</td><td>Olive Green</td><td>Not having same good keeping quality</td></tr>
<tr><td>6.8</td><td>Bluish violet</td><td>Suspect on signs of incipient spoilage</td></tr>
</table>
</td></tr>
<tr><td>Reference</td><td>Chicken broth flavor and pH by Pippen et al. (1965), Poultry Sci. 44: 816-823</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

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Method No.	**FSSAI 05.012:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document pertains to providing the Estimation of moisture content in meat & meat products using Rapid Microwave Drying Method. It has been established for meat & meat products		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	Moisture is removed (evaporated) from the sample by using microwave energy. Weight loss is determined by electrical balance readings before and after drying and is converted to moisture content by the microprocessor with a digital per cent readout.		
Apparatus/Instruments	Microwave moisture analyzer — 0.2 mg H20 sensitivity, moisture/solids range of 0.1-99.9%, 0.01% resolution. Includes automatic tare electronic balance, microwave drying system, and microprocessor digital computer control. The electronic balance pan is located inside the drying chamber. (Balance sensitivity: 0.2 mg at 15 g capacity or 1.0 mg at 40 g capacity. (CEM Corp., PO Box 200, Matthews, NC, 28106), or equivalent.)		
Materials and Reagents	Glass fiber pads. — 9.8 x 10.2 cm rectangular glass fiber pads (CEM Corp.), or equivalent.		
Sample Preparation	Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1. It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Proceed from a representative sample of at least 200 g.		
Method of analysis	1. Prepare test samples as mentioned in the sample preparation document. 2. Place 2 rectangular glass fiber pads on the balance pan in the microwave moisture analyzer drying chamber, and tare. 3. Remove pads from the chamber, rapidly and evenly deposit ca 4 g well-mixed test portion on the rough side of one pad. 4. Place the second pad over the test portion and replace the pads and test portion on the balance pan. 5. Dry sample with pads 3-5 min at 80- 100% power, depending on product type. 6. At the completion of the microwave drying cycle, read per cent moisture which is displayed on the digital readout panel. 7. Certain product classes require the addition of an adjustment factor to readout for accurate results, as follows: cooked sausage, preblends/emulsions, cured/cooked meats, factor = 0.55.		
Reference	1. JAOAC 68, 876 (1985) 2. Journal of AOAC INTERNATIONAL		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

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Method No.	**FSSAI 05.013:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document is to provide the procedure for the determination of Extract Release Volume (ERV) for meat and meat products.		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	The technique was first described in 1964 and has been shown to be a value in determining incipient spoilage in meat as well as in predicting refrigerator shelf life. The technique is based on the volume of aqueous extract released by homogenate of meat when allowed to pass through the filter paper for a given period of time, by this meat of good organoleptic and microbial quality release large volume of extract, whereas meat of poor-quality releases smaller volume or none.		
Apparatus/Instruments	1. Beaker 2. Distilled water 3. Cellulose-based qualitative filter paper 4. Pestle and mortal 5. Graduated cylinder		
Materials and Reagents	No reagents are used in this method		
Sample Preparation	Homogenize 20 gm of meat with 100 mL distilled water for 2 minutes. Pour the homogenate directly into the funnel lined with cellulose-based qualitative filter paper, folded thrice so as to make eight sections. Allow the homogenate to seep between the folds and collect the extract in a 100 mL graduated cylinder for 15 min.		
Method of analysis	**Procedure:** 1. Take a 25 gm meat sample in 100 mL distilled water 2. Bend it within pestle and mortal 3. Filter through cellulose-based qualitative filter paper, folded thrice so as to make eight sections. 4. Allow the homogenate to seep between the folds 5. Collect the extract in a 100 mL graduated cylinder for 15 min. 6. Record extract release volume and interpret results		
Inference (Qualitative Analysis)	ERV (ml) | ERV (ml) > 25 mL | Good quality > 20 mL | Incipient spoilage < 20 mL | Spoiled meat		
Reference	1. Release of aqueous extracts by beef homogenates and factors by Jay (1964), Food Technol. 18: 129-132. 2. Beef microbial quality determined by extract release volume (ERV) by Jay (1964), Food Technol. 18: 132-137. 3. Alternatively, BIS standard method IS 5960: part 10: 2011 (methods of test for meat and meat products: Part 10 Measurement of pH – Reference) may be used		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

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Method No.	**FSSAI 05.014:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document is to provide the procedure for the determination of meat swelling capacity in meat and meat products.		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	This test determines the freshness of meat. Swelling capacity of meat increases during spoilage due to protein degradation and penetration of more amounts of water in the protein matrix. A method of measuring the water-binding capacity of muscle proteins with low water-holding forces is known as meat swelling (SW).		
Apparatus/Instruments	1. Centrifuge 2. Blender 3. Graduated cylinder		
Materials and Reagents	1. Distilled water		
Preparation of Reagents	No reagents are used in this method		
Sample Preparation	Since no reagents, no preparation is required.		
Method of analysis	**Procedure:** 1. Take 25 gm of meat in 100 mL of distilled water 2. Blend it for 2 min 3. Centrifuge 35 mL of homogenate at 2000 rpm for 15 min 4. Measure the volume of supernatant (S) 5. Record the volume and denote it as “S”.		
Calculation with units of expression	The percentage of meat swelling can be determined as % Meat Swelling = (35-S-7)/7 X 100		
Reference	Determination of meat swelling capacity as a method for investigating the water binding capacity of muscle proteins with low water holding forces. II Application of the swelling methodology by Wierbicky et al (1963) Fleischwirtschaft 15: 404.		
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Method No.	**FSSAI 05.015:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide the procedure for the determination of the Total Volatile Basic Nitrogen (TVBN) in meat and meat products using the micro diffusion technique.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	The volatile bases in most species of fish consist of ammonia together with appreciable quantities of amines. In meat, trimethylamine is only present in significant quantities and total volatile nitrogen consists almost entirely as ammonia. As ammonia production due to the deamination of protein increases during spoilage, its determination represents a simple method of following the course of determination of the quality of lean meat. Meat extract is treated with relatively weak alkali and the volatile base is distilled or diffused over into standard acid or boric acid.		
Apparatus/Instruments	1. Conway Unit for micro diffusion 2. Titration apparatus		
Materials and Reagents	1. Boric acid reagent 2. Trichloroacetic acid (TCA) extract 3. TVBN reagent 4. Potassium carbonate (K2CO3) 5. 0.02 N sulphuric acid (H2SO4)		
Preparation of Reagents	**1. Boric acid reagent:** Dissolve 5 gm of boric acid in 100 mL of 95% Alcohol and add 350 mL of water. After the acid had dissolved add 5mL of indicator (0.066% methyl red and 0.33% bromocresol green in alcohol). Add alkali (40% sodium hydroxide) until a faint Reddish colour is produced. Make the volume up to 500 mL with alcohol. **2. Trichloroacetic acid (TCA) extract:** Take 10 gm of meat sample and blend with 90 mL of distilled water for 2 min. To 5 mL of this homogenate add an equal volume of 10% TCA (w/v in distilled water), allowed to stand for 15 min. Filtered through cellulose-based qualitative filter paper. The clear TCA extract thus obtained is used to determine the TVBN value following the technique of Conway (1947) and Pearson (1968 b). **3. Preparation of TVBN reagent:** Take 92 mL of 2% boric acid, 4 mL of 0.1% of alcoholic solution of bromocresol green and 4 mL of 0.1% of alcoholic methyl red are mixed to make 100 mL TVBN reagent. **4. 0.02 N sulphuric acid (H2SO4)** - 0.02N=0.01 M (0.98 gm/liter)		
Sample Preparation	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Method of analysis	**Determination of TVBN by Micro diffusion technique** **Procedure:** 1. Add One mL of TVBN reagent in to the inner well of the Conway unit. 2. Deposit one mL of TCA extract in outer wall followed by the addition of one mL of saturated potassium carbonate (K2CO3). 3. Close the Conway unit immediately with an airtight ground glass plate 4. Rotate clockwise and antilock wise and incubate at room temperature for 3 hours.		

	5. The TVBN reagent in the inner well is back-titrated with 0.02 N sulphuric acid (H2SO4) till the blue color changes to pink.
Calculation with units of expression	14 X a X b = 'N' mg/mL of extract C= 100 x N Where, 14 = Molecular weight of Nitrogen a = Normality of H2SO4 b = volume of H2SO4 (Titration value) c = mg% of TVBN value TVBN values are expressed as mg %.
Reference	1. Micro-diffusion analysis and volumetric error by Conway (1947), D.Van Nostrand Co. Inc., New York 2. Application of chemical methods for the assessment of beef quality II Methods related to protein breakdown by Pearson (1968), J. Sci. Fd. Agric. 19: 366-369. 3. Alternatively, BIS reference method IS 5960: Part 3: 1970 may be used.
Approved by	Scientific Panel on Methods of Sampling and Analysis

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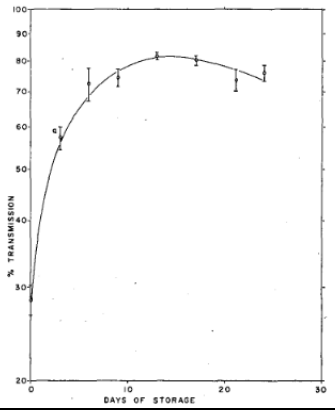
Method No.	**FSSAI 05.016:2024**	**Revision No. & Date**	**0.0**
Scope	The Scope of this document is to provide a procedure for the determination of the Picric Acid Turbidity test in meat and meat products.		
Caution	Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.		
Principle	Picric acid turbidity test has been adapted to assay the skin of ready-to-cook broilers i-n order to measure objectively the relative freshness of a group of broilers during refrigerator storage.		
Apparatus/Instruments	1. Meat mincer 2. spectrophotometer		
Materials and Reagents	1. 70% ethanol 2. Saturated aqueous picric acid 3. Ashless quantitative filter 4. Paper (20 µm nominal particle retention rating) 5. spectrophotometer		
Method of analysis	1. Blend 5 gm of meat with 20 mL of 70 % ethanol for 30 sec 2. Add 5 mL of saturated aqueous picric acid solution and re-blend the slurry for about 20 sec. 3. Filter the slurry through ashless quantitative filter paper. 4. Measure the optical density of the filtrate at 540 nm wavelength with visible spectrophotometer as a turbidity of the solution.		
Calculation with units of expression			
Inference (Qualitative Analysis)	It is possible practical freshness test of meat. It is to detect presence of reducing sugar.		
Reference	The picric acid turbidity: A possible practical freshness test for ice shrimps by Kurtzman and Synder (1960), Food Technol. 14(7): 337.		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Method for determination of Dye reduction Capacity</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.017:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">The scope of this document is to provide the procedure for the determination of dye reduction capacity in meat and meat products using the Resazurin dye reduction test (RDRT)</td></tr>
<tr><td>Caution</td><td colspan="3">Use only reagents of recognized analytical quality, distilled or demineralized water or water of equivalent purity.</td></tr>
<tr><td>Principle</td><td colspan="3">A dye reduction method aims for estimation of total aerobic and/or psychrotrophic bacterial (bacteria that are capable of surviving or even thriving in extremely cold environment) counts in ground pork. The method is based on color changes in indicator disks placed on the meat surface.
This test estimates bacterial population in meat sample indirectly.
1. Resazurin dye reduction test (RDRT)
2. Methylene blue reduction test (MBRT)</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">1. Resazurin dye/tablet
2. Filter paper strips
3. Polythene bag
4. Nutrient</td></tr>
<tr><td>Method of analysis</td><td colspan="3">Procedure- A:
1. Four Resazurin tablets dissolved in 100 mL of the water sample.
2. Filter paper strips are dipped in the above solution and dried in a dark and cool room.
3. For testing, the strip is moistened and a drop of meat juice to be tested is placed on it for 1 minute.
4. The strip is then placed in a polythene bag and kept in a dark room (22-23 ^{0}C).
5. Time taken for the blue colour of the paper to change to red is noted.

Procedure- B:
1. Soak the swab in nutrient broth
2. Sample 1 cm2 area of meat
3. Break the swab in 10 mL nutrient broth yeast extract medium
4. Collect the washings in a sterile glass beaker containing 0.3 mL of freshly prepared 0.05% aqueous Resazurin dye
5. Incubate the beaker at 30oC in a dark room
6. Note the time taken for the change in colour from violet/ blue to colourless</td></tr>
<tr><td>Inference
(Qualitative Analysis)</td><td colspan="3"><table><tr><th>Reduction time</th><th>Meat quality</th></tr><tr><td>10 min</td><td>Meat not acceptable</td></tr><tr><td>10-30 min</td><td>Doubtful</td></tr><tr><td>30-60 min</td><td>Good quality</td></tr><tr><td>> 60 min</td><td>Very good quality</td></tr></table></td></tr>
<tr><td>Reference</td><td colspan="3">1. Textbook of “Methods in Meat Science”.
2. Resazurin dye reduction tests for shelf-life estimation of poultry meats by Wells (1959), Food Technol. 13: 584-586.
3. Rapid procedure for approximation of bacterial counts in shrimps and oysters by Novak et al (1955), Food Technol. 10: 66 – 67.</td></tr>
<tr><td>Approved by</td><td colspan="3">Scientific Panel on Methods of Sampling and Analysis</td></tr>
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Method No.	**FSSAI 05.018:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a procedure for the determination of chloride content in meat and meat products using Volhard Method.		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	Extraction of a test portion with hot water and precipitation of the proteins. After filtration and acidification, addition of an excess of silver nitrate solution to the extract, and titration of this excess with potassium thiocyanate solution.		
Apparatus/Instruments	1. Homogenizing equipment 2. One-mark volumetric flasks 3. Conical flasks 4. Burette 5. One-mark pipettes 6. Boiling water bath 7. Analytical balance		
Materials and Reagents	1. Water, distilled and halogen-free 2. Nitrobenzene or nonan-1-ol. 3. Solutions for precipitation of proteins. 4. Silver nitrate, standard volumetric solution, c(AgNO3) = 0.1 mol/l 5. Potassium thiocyanate, standard volumetric solution, c(KSCN) = 0.1 mol/l 6. Ammonium iron (III) sulfate		
Preparation of Reagents	**Water, distilled and halogen-free:** Halogen-free test: Add 1 ml of silver nitrate (c(AgNO3) = 0.1 mol/l] and 5 ml of nitric acid (c(HNO3) L- 4 mol/l) to 100 ml of water. No more than a slight turbidity shall be produced. **Solutions for precipitation of proteins:** **Reagent A** Dissolve in water 106 g of potassium hexacyanoferrate(ll) trihydrate [K4Fe (CN)6.3H2O. Transfer quantitatively to a 1000 ml one-mark volumetric flask and dilute to the mark with water. **Reagent B** Dissolve in water 220 g of zinc acetate dihydrate [Zn (CH3COO)2.2H2O) and add 30 ml of glacial acetic acid. Transfer quantitatively to a 1 000 ml one-mark volumetric flask and dilute to the mark with water. **Silver nitrate, standard volumetric solution, c(AgNO3) = 0.1 mol/l.** Dissolve in water 16.989 g of silver nitrate, previously dried for 2 h at 150 ^{0}C ± 2 ^{0}C and allowed to cool in a desiccator. Transfer quantitatively to a 1000 ml one-mark volumetric flask and dilute to the mark with water. Store this solution in a dark glass container out of direct sunlight. **Potassium thiocyanate, standard volumetric solution, c(KSCN) = 0.1 mol/l.** Dissolve in water about 9.7 g of potassium thiocyanate. Transfer quantitatively to a 1000 ml one-mark volumetric flask and dilute to the mark with water. Standardize the solution to the nearest 0.000 1 mol/l against the silver nitrate solution using the ammonium iron(lll) sulfate solution as an indicator.		

	Ammonium iron (III) sulfate Prepare a saturated aqueous solution at room temperature from the dodecahydrate [NH4Fe(SO4)2H2O).
Sample Preparation	1. A recommended sampling method is given in IS0 3100-I. Proceed from a representative sample of at least 200 g. 2. Homogenize the laboratory sample with the appropriate equipment (5.1). Take care that the temperature of the sample material does not rise above 25 ^{0}C. If a mincer is used, pass the sample at least twice through the equipment. 3. Fill a suitable airtight container with the prepared sample. Close the container and store in such way that deterioration and change in composition are prevented. Analyse the sample as soon as practicable, but always within 24 h of homogenization.
Method of analysis	**NOTE 1** If it is required to check whether the repeatability requirement is met, carry out two single determinations in accordance with 8.1 to 8.4 under repeatability conditions. Weigh, to the nearest 0.001 g, about 10 g of the test sample and transfer it quantitatively to a conical flask. **Deproteination:** 1. Add 100 ml of hot water to the test portion. Heat the flask and its contents for 15 min in the boiling water bath. Periodically shake the contents of the flask. 2. Allow the flask and its contents to cool to room temperature, then add successively 2 ml of reagent A and 2 ml of reagent B. Mix thoroughly after each addition. 3. Allow the flask to stand for 30 min at room temperature. Transfer the contents quantitatively to a 200 ml volumetric flask and dilute to the mark with water. Mix the contents thoroughly and filter through a fluted filter paper. **NOTE 2** If this method is used for the determination of the nitrate and nitrite content or if ascorbic acid is present in the sample in concentrations higher than 0.1 %, it is necessary to add also 0.5 g of activated charcoal to the test portion. After mixing reagents A and 8, adjust the pH to between 7.5 and 8.3 by means of a sodium hydroxide solution. **Determination:** 1. Transfer 20 ml of the filtrate to a conical flask by means of a pipette and add, from a graduated measuring cylinder, 5 ml of the dilute nitric acid and 1 ml of the ammonium iron(lll) sulfate solution as indicator. 2. Transfer 20 ml of the silver nitrate solution to the conical flask by means of a pipette. Add 3 ml of the nitrobenzene or nonan-1-ol from a graduated measuring cylinder and mix thoroughly. Shake vigorously to coagulate the precipitate. Titrate the contents of the conical flask with the potassium thiocyanate until the appearance of a persistent pink coloration. Record the volume of the potassium thiocyanate solution required, to the nearest 0.05 ml. **Blank test:** Carry out a blank test using the same volume of silver nitrate solution.
Calculation with units of expression	Calculate the chloride content of the sample from the following equation:

	$$w_{Cl} = 0{,}058\ 44(V_2 - V_1) \times \frac{200}{20} \times \frac{100}{m} \times c$$ $$= 58{,}44 \times \frac{V_2 - V_1}{m} \times c$$ where W_{Cl} is the chloride content of the sample, expressed as sodium chloride as a percentage by mass; V_1 is the volume, in millilitres, of the potassium thiocyanate solution (4.6) used in the determination V_2 is the volume, in millilitres, of the potassium thiocyanate solution (4.6) used in the blank test C is the concentration of the potassium thiocyanate solution in moles per litre m is the mass, in grams, of the test portion. Report the result to the nearest 0,05 % (m/m)
Reference	IS 5960 (Part 6/Sec 1): 1997 Indian Standard Meat and Meat Products - Methods of Test Part 6, Determination of Chloride Content - Section 1 Volhard Method
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.019:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide the procedure for the determination of L (-) hydroxyproline Content in meat and meat products. It is applicable to meat and meat products containing less than 0.5 % (m/m) hydroxyproline.		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Principle	Hydrolysis of a test portion in sulfuric acid at 105 “C. Filtration and dilution of the hydrolysate. Oxidation of the hydroxyproline by chloramine-T, followed by the formation of a red compound with p-dimethyl-amino benzaldehyde. Photometric measurement at a wavelength of 558 nm.		
Apparatus/Instruments	1. Electric meat mincer 2. Round- or flat-bottomed hydrolysis flasks 3. Drying oven 4. Filter paper discs 5. pH-meter 6. Aluminium or opaque plastic foil 7. Water bath 8. Spectrometer or a photoelectric calorimeter 9. Glass cells 10. Analytical balance 11. Volumetric flasks 12. Watch glasses		
Materials and Reagents	1. Sulfuric acid solution, c(HzS0,) ~ 3 mol/l 2. Buffer solution, pH = 6.8 3. Chloramine-T reagent 4. Colour reagent 5. Hydroxyproline, standard solutions.		
Preparation of Reagents	**Sulfuric acid solution, c(HzS0,) ~ 3 mol/l:** Add 750 ml of water to a 2-litre one-mark volumetric flask. Add slowly, with agitation, 320 ml of concentrated sulfuric acid (ρ_{20} = 1.84 g/ml). Cool to room temperature and makeup to the mark with water. **Buffer solution, pH = 6.8:** 260 g of citric acid monohydrate (C6H8O7.H2O); 14.0 g of sodium hydroxide; 78.0 g of anhydrous sodium acetate (Na (CH3CO2)). Dissolve the reagents in 500 ml of water and transfer quantitatively into a 1-litre one-mark volumetric flask. Add 250 ml of propane-1-ol and makeup to the mark with water. When stored at 4 “C in the dark, this solution is stable for several weeks. **Chloramine-T reagent:** Dissolve 1,41 g of sodium N-chloro-ρ-toluene-sulfonamide trihydrate (chloramine-T) in 100 ml of the buffer solution. Prepare this solution immediately before use. **Colour reagent:** Dissolve 10.0 g of ρ -dimethyl amino benzaldehyde in 35 ml of perchloric acid solution [60 % (m/m)] and then slowly add 65 ml of propan-2-ol. Prepare this solution on the day of use. If the purification of ρ -dimethyl amino benzaldehyde is necessary proceed as follows. Prepare a saturated solution G: ρ -dimethyl amino benzaldehyde in hot		

	70 % (V/V) ethanol. Cool, first at room temperature and finally in a refrigerator. After about 12 h, filter on a Buchner funnel. Wash with a little 70 % (V/V) ethanol. Again, dissolve in hot 70 % (V/V) ethanol. Add cold water and agitate thoroughly. Repeat this procedure until a sufficient quantity of milk-white crystals has been formed. Place in the refrigerator overnight. Filter on the Buchner funnel. Wash with 50 % (VA4 ethanol and vacuum dry over phosphorus(V) oxide. **Hydroxyproline-standard solutions:** Prepare a stock solution by dissolving 50 mg of 4-hydroxy pyrrolidine-a-carbonic acid (hydroxyproline) in water in a 100 ml one-mark volumetric flask. Add 1 drop of the sulfuric acid solution and makeup to the mark with water. This solution is stable for at least 1 month at 4 ^{0}C. On the day of use, transfer 5 ml of the stock solution to a 500 ml one-mark volumetric flask and makeup to the mark with water. Then prepare four standard solutions by diluting 10 ml, 20 ml, 30 ml and 40 ml of this solution to 100 ml with water to obtain hydroxyproline concentrations of 0.5 µg/ml, 1 µg/ml, I.5 µg/ml and 2 µg/ml respectively.
Sample Preparation	It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in IS0 3100-I. Proceed from a representative sample of at least 200 g. Store the sample in such a way that deterioration and change in composition are -prevented. **Raw meat and meat products** Reduce intact meat into small cubes (approx. 0.5 cm3, i.e., sides of length approx. 8 mm) by cutting it while it is cold (just below 0^{0}C), using a sharp knife. Either place the sample in a container and seal the latter hermetically, or vacuum-pack the sample in heat-resistant plastic film. Then heat the container and sample so as to maintain a temperature of at least 70 ^{0}C for at least 30 min. Cool and proceed as below section. During the remaining stages of preparation of the test sample and the weighing out of the test portions, ensure that the sample is kept well mixed and, in particular, that any fat or fluid is kept evenly distributed. **NOTE 1** The heat treatment softens the raw connective tissue and makes it less resistant to homogenization by mincing. However, it may also lead to the separation of a fluid containing gelatine. The presence of fat may also demand special attention for the production of a homogeneous test sample. **Cooked meat and cooked meat products:** Homogenize the sample in the meat mincer. Keep the homogenized sample in a completely filled, 1 air-tight, closed container and store it in such a way that deterioration and change in composition are prevented. Analyse the test sample as soon as possible, but always within 24 h.
Method of analysis	**Test portion:** Weigh, to the nearest 0.001 g, about 4 g of the test sample into a hydrolysis flask. Ensure that no sample material adheres to the side walls of the flask. **Hydrolysis:** 1. Add 30 ml + 1 ml of sulfuric acid solution to the flask. Cover the flask with a watch glass and place in the oven for 16 h (conveniently,

	overnight) at 105 ^{0}C. **2.** Filter the hot hydrolysate through filter paper into a 250 ml one-mark volumetric flask. Wash the flask and filter paper three times with 10 ml portions of hot sulfuric acid solution and add the washings to the hydrolysate. Makeup to the mark with water and mix. **Colour development and measurement of absorbance:** 1. Using a pipette, transfer to a one-mark 250 ml volumetric flask a volume (V) of the hydrolysate so that, after dilution to 250 ml, the hydroxyproline concentration will be within the range 0.5 µg/ml to 2 µg/ml. Makeup to the mark with water. **NOTE 2** In most cases, V will be in the order of 5 ml to 25 ml depending on the amount of connective tissue present in the sample. 2. Transfer 4,00 ml of this solution to a test tube and add 2.00 ml of chloramine-T reagent. Mix and leave at room temperature for 20 mint ± 1 min. 3. Add -2.00 ml of the colour reagent (4.41, mix thoroughly and cap the tube with aluminium or plastic foil 4. Transfer the tube quickly into the water bath set at 60 ^{0}C, and heat for exactly 20 min. 5. Cool the tube under running tap water for at least 3 min and leave at room temperature for 30 min 6. Measure the absorbance at 558 nm ± 2 nm in a glass cell against water, using the spectrometer or the photoelectric calorimeter equipped with an interference filter 7. Subtract the absorbance measured for the blank solution and read the hydroxyproline concentration of the diluted hydrolysate from the calibration graph obtained as described in upcoming sessions. **Blank test** Carry out in duplicate the above-described procedure in 2 to 7 points inclusive, substituting water for the diluted hydrolysate. **NOTE 3** If the absorbance of the blank exceeds 0.040, a fresh colour reagent should be prepared and, if necessary, the ρ -dimethyl amino benzaldehyde should be purified. **Calibration Graph** 1. Carry out the above-described procedure in 2 to 7 points inclusive, substituting in turn 4.00 ml of each of the four diluted standard hydroxyproline solutions for the diluted hydrolysate. 2. Plot the measured absorbance values, corrected for the blank value, against the concentrations of the standard hydroxyproline solutions. Construct the best-fitting straight line through the plotted points and the origin. Prepare a new calibration graph for each series of analyses.
Calculation with units of expression	For each test portion, calculate the hydroxyproline content, as a percentage by mass, from the formula $$w_h = \frac{6{,}25c}{m \times V}$$ where W_h is the hydroxyproline content, expressed as a percentage by mass,

	obtained from the formula: c is the hydroxyproline concentration, in micrograms per millilitre, of the diluted hydrolysate as read from the calibration graph; m is the mass, in grams, of the test portion V is the volume, in millilitres, of the aliquot portion of the hydrolysate taken for dilution to 250 ml Report the result to the nearest 0.01 %.
Reference	IS 5960(Part15): 2000. Indian Standard: Meat and Meat Products - Methods of Test - Part 15 Determination of L (-) Hydroxyproline Content
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.020:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document is to provide a procedure for the determination of starch content in meat and meat products.		
Caution	All reagents shall be of recognized analytical quality. The water used shall be distilled water or water of at least equivalent purity.		
Principle	Heating of a test portion with ethanolic potassium hydroxide solution until the meat components are totally dissolved. Decantation, washing of the remaining residue with hot ethanol, filtering, dissolution in hydrochloric acid, and hydrolysis. Titrimetric determination of the glucose formed.		
Apparatus/Instruments	1. Mechanical Meat Mincer 2. Boiling Water bath 3. Fluted filter paper 4. Asbestos plate 5. Conical flask 6. Condenser 7. Boiling aids (for example pumice stone or glass beads) 8. Burette, capacity 50 ml, complying with class A of ISOIR 385 9. pH meter		
Materials and Reagents	1. Potassium hydroxide, ethanolic solution 2. Ethanol, 80 % (V/V). 3. Hydrochloric acid, 1.0 M solution (chlorine-free) 4. Bromothymol blue 5. Sodium hydroxide 6. Solutions for the precipitation of proteins 7. Copper reagent 8. Starch Indicator solution 9. Hydrochloric acid, 25 % (m/m) solution (chlorine-free) 10. Sodium thiosulphate 0.1N Standard solution 11. Potassium iodide Solution		
Preparation of Reagents	1. **Potassium hydroxide, ethanolic solution** - Dissolve 50 g of potassium hydroxide in 800 ml of 95 % (V/V) ethanol and dilute to 1 000 ml with the same ethanol. 2. **Hydrochloric acid, 1.0 M solution (chlorine-free)** 3. **Bromothymol blue, 10 g/l solutions in 95 % (V/V) ethanol**. 4. **Sodium hydroxide, 300 g/l solution.** 5. **Solutions for precipitation of proteins.** **Solution-I** - Dissolve 106 g of potassium hexacyanoferrate (II) trihydrate [K4Fe(CN)6.3H20] in water in a 1000 ml one-mark volumetric flask and dilute to the mark. **Solution-II** - Dissolve 220 g of zinc acetate dihydrate [Zn $(CH3COO)_2.2H_2O$] in water in a 1 000 ml one-mark volumetric flask. Add 30 ml of glacial acetic acid, and dilute to the mark with water. 6. **Copper reagent** - Prepare the following solutions: a) 25 g of copper sulphate pentahydrate (CuSO4.5H20) in 100 ml of water; b) 144 g of sodium carbonate (Na2CO3) in 300 to 400 ml of water at 50 ^{o}C; c) 50 g of citric acid monohydrate ($C_6H_8O_7.H_2O$) 50 ml of water Add solution c) slowly and carefully, stirring continuously to		

	solution b). Continue stirring until the evolution of carbon dioxide ceases. Add solution a) to this mixture, stirring continuously. Allow to cool to room temperature, transfer quantitatively to a 1 000 ml one-mark volumetric flask, dilute to the mark and filter after 24 h. The pH of the solution, after 1 + 49 dilution with freshly boiled and cooled water, should be 10.0 + 0.1. 7. **Starch indicator solution** Add a mixture of 10 g of soluble starch, 10 mg of mercury(II) iodide (as a preservative) and 30 ml of water to 1 liter of boiling water. Continue boiling for 3 min and cool, 8. **Sodium thiosulphate, approximately 0.1 N standard volumetric solution.** **Preparation:** Dissolve, in 1 000 ml of freshly boiled and cooled water, 25 g of sodium thiosulphate pentahydrate (Na&03.5H20) and add 0,2 g of sodium carbonate decahydrate (Na2CO3.10H20). Allow the solution to stand for one day before standardizing. **Standardization:** Weigh 150.0 mg of dried potassium iodate, dissolve it in 25 ml of water and add 2 g of potassium iodide and 10 ml of the hydrochloric acid solution. Titrate with the solution thiosulphate solution while stirring continuously. Add 1 ml of the starch indicator solution when the solution has become pale yellow, and continue the titration until the blue colour disappears. The normality T of the sodium thiosulphate solution is then calculated from the formula: $$\frac{T = 6m}{214.0\ V}$$ Where, m is the mass, in milligrams, of the potassium iodate; V is the volume, in millilitres of the sodium thiosulphate solution added to the potassium iodate solution $$\frac{214.0}{6}$$ is the relative molecular mass of potassium iodate 9. **Potassium iodide, 100 g/l solution** Dissolve 10 g of potassium iodide in water, and dilute to 100 ml. Store the solution in a dark brown bottle. 10. **Hydrochloric acid, 25 % (m/m) solution (chlorine-free)** Dilute 100 ml of concentrated, chlorine-free hydrochloric acid (ρ_{20} 1.19 g/ml) with 60 ml of water.
Sample Preparation	Proceed from a representative sample of at least 200 g. See IS0 3100. Store the sample, if necessary, in such a way deterioration and change in composition are prevented.
Method of analysis	**Preparation of the Test Sample:** Homogenize the sample by passing it at least twice through the meat mincer and mixing. Keep it in a completely filled, air-tight, closed container and store it. Analyze the sample as soon as possible after homogenization, but always within 24h. **Test Portion:** Weigh into a 500 or 600 ml beaker, to the nearest 0.1g, about 25 g of the test sample. If the mass of starch in this test portion is expected to be more than 1 g, reduce the mass of the test portion accordingly.

	Isolation of starch: Add to the test portion, while stirring with a glass rod, 300 ml of hot ethanolic potassium hydroxide solution and cover the beaker with a watch glass. Heat on the boiling water bath for 1 h, stirring occasionally. Decant the solution through a filter paper and then wash the starch quantitatively on the filter paper using hot ethanol and with the aid of a rubber-tipped glass rod. Keep the filter moist. NOTE: In some cases, centrifuging may be more advantageous than filtration. **Hydrolysis:** means of a glass rod. Pierce a hole in the filter paper and wash the starch through it into a 250 ml beaker, using 100 ml of hot hydrochloric acid solution. Cover the beaker with a watch glass and immerse it in the boiling water bath for 2,5 h, stirring the solution occasionally with a glass rod. Cool the solution and neutralize it by adding the sodium hydroxide solution drop by drop, taking care that the pH does not exceed 6,5; check this with the pH meter. Transfer the mixture quantitatively into a 200 ml volumetric flask, washing with water, add 3 ml of Solution I and, after mixing, 3 ml of Solution II and dilute to the mark. Mix and filter through a fluted filter paper Immediately before pipetting an aliquot portion for the next stage, make the filtrate alkaline to bromothymol blue by adding 1 or 2 drops of the sodium hydroxide solution. **Determination of glucose:** If the approximate starch content of the sample is unknown, carry out a preliminary trial analysis to estimate it. Dilute an aliquot portion (V_2) of the filtrate with water to a known volume (V_3) so that 25 ml of the diluted solution contains preferably 40 to 50 mg of glucose and in no circumstances more than 60 mg of glucose. Mix and pipette 25.0 ml of the diluted solution into the conical flask. Pipette 25.0 ml of the copper reagent into the flask and add some boiling aids. NOTE - It is essential that the total volume of liquid at this stage is always 50.0 ml. Fit the condenser to the flask. Place the flask and condenser on a metal wire gauze surmounted by the asbestos plate. Bring the liquid to the boil over a gas flame in about 2 min and continue to boil gently for exactly 10 min. Then cool quickly to room temperature. Remove the condenser and add 30 ml of the potassium iodide solution and next, carefully but as quickly as possible, 25 ml of the hydrochloric acid solution. Stopper the flask until titration. Titrate the liberated iodine with the standard volumetric 100 g of sample. sodium thiosulphate solution (6.9). When the solution has become pale yellow, add about 1 ml of the starch indicator solution (6.8) and continue the titration until the blue colour disappears. **Blank determination:** Carry out a blank determination, following the same procedure as in 9.5, taking 25.0 ml of water instead of 25.0 ml of the diluted filtrate.
Calculation with units of expression	Calculate the difference between the volumes noted in the two titrations, expressed in millilitres of exactly 0.1 N sodium thiosulphate solution, from the formula $10\ T \times (V_0 - V_1)$ Where, T is the normality of the standard volumetric sodium thiosulphate solution

V_0 is the volume, in millilitres, of the standard volumetric sodium thiosulphate solution needed for the blank determination
V_1 is the volume, in millilitres, of the standard volumetric sodium thiosulphate solution needed for the diluted filtrate
Calculate the starch content, as a percentage by mass, from the formula

$$\frac{m_1}{1\,000} \times 0{,}9 \times \frac{V_3}{25} \times \frac{200}{V_2} \times \frac{100}{m_0} = 0{,}72 \times \frac{V_3}{V_2} \times \frac{m_1}{m_0}$$

Where,
V_2 is the volume, in millilitres, of the undiluted aliquot portion.
V_3 is the volume, in millilitres, of the diluted aliquot portion.
m_0 is the mass, in grams, of the test portion
m_1 is the mass, in milligrams, of glucose as determined from the expression 10 T x (V_0 – V_1) by reference to the table or the graph
0.9 is the factor for the conversion of the mass of glucose m_1 to the corresponding mass of starch.
Report the result to the nearest 0.1 %

Table: Conversion of millilitres of 0.1 N sodium thiosulphate solution to milligrams of glucose

10 T x (V0 – V1)	Corresponding mass of glucose	
ml of 0.1 N sodium thiosulphate solution	m_1 mg	Δm_1 mg
1	2.4	2.4
2	4.8	2.4
3	7.2	2.6
4	9.1	2.5
5	12.2	2.5
6	14.7	2.5
7	17.2	2.6
8	19.8	2.6
9	22.4	2.6
10	25.0	2.6
11	27.6	2.7
12	30.3	2.7
13	33.0	2.7
14	35.7	2.8
15	38.5	2.8
16	41.3	2.9
17	44.2	2.9
18	47.1	2.9
19	50.0	2.9
20	53.0	3.0
21	56.0	3.0
22	59.1	3.1
23	62.2	3.1

Reference	IS0 554 -1978 (E) Meat products - Determination of starch content
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.021:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document to provide the procedure for the determination of agar in meat & meat products. It has been established for meat & meat products.		
Caution	Use only reagents of recognized analytical quality and distilled or demineralized water or water of equivalent purity.		
Apparatus/Instruments	1. Centrifuge 2. Water bath		
Materials and Reagents	1. Trichloroacetic acid solution 2. Iodine solution— Approx. 0.033N. 3. Benedict's qualitative solution 4. Na citrate 5. anhydrous Na2CO3 6. CuSO4.5H20 7. 10% NaOH solution 8. litmus paper		
Preparation of Reagents	**1. Trichloroacetic acid solution** — 25 g acid in 50 mL H2O. **2. Benedict qualitative solution**—Dissolve 17.3 g Na citrate and 10 g anhydrous Na2CO3 in ca 80 mL hot H2O; dissolve 1.73 g CuSO4.5H20 in 10 mL H2O. Filter alkaline citrate solution, add CuSO4 solution slowly with constant stirring, and dil. with H2O to 100 mL.		
Sample Preparation	Boned chicken or meat. — Refrigerate overnight to gel broth. With a thin-blade spatula, separate as much gel as possible, and warm it on a steam bath until completely liquefied. Consommé or broth. — No preparation necessary		
Method of analysis	**Detection of Gum** 1. Transfer up to 40 mL liquefied gel from meat, or 40 mL consommé, to 100 mL beaker. 2. Add 5 mL trichloroacetic acid solution, stir, and let stand 15-30 min. 3. Transfer to 50 mL conical centrifuge tube and centrifuge 15-20 min at ca 1200 rpm. 4. Decant clear supernatant into 250 mL (8 oz) centrifuge bottle or nursing bottle, add 4-5 vols alcohol and let stand until precipitate coagulates, or overnight. (No precipitation indicates the absence of gums.) 5. Centrifuge at 1200 rpm 15-30 min until precipitate packs to the bottom of centrifuge bottle. 6. Carefully decant alcohol, taking care not to disturb packed gum precipitate. 7. Remove the few remaining drops of alcohol by spontaneous drying or by gentle air current. 8. Add 1 drop of 0.033A I solution Evanescent violet or black colour indicates the presence of agar. (Negative the test does not necessarily mean agar is absent.) 9. Add 3 mL hot H2O and warm on a steam bath until the gum precipitate dissolves. 10. Chill gum solution in ice and H2O mixture Thickening, or stiff jell, indicates agar. warm the cooled mixture on a steam bath, transfer to a 50 mL beaker, and rinse the centrifuge bottle with 3-4 mL H2O, and add rinsings to the gel solution.		

	11. Add 1 mL HC1 and boil for 30 sec. Transfer 1 mL hydrolysed gum solution to the test tube, and neutralize with 10% NaOH solution, using litmus paper as an indicator (ca 2 mL required). 12. Remove litmus paper, add 5 mL Benedict solution, and boil cautiously over the free flame for 30-60 sec. Green, yellow, or brick-coloured precipitate after spontaneous cooling indicates agar (or other hydrolysable gum).
Calculation with units of expression	Since it is a qualitative method, no calculation is needed.
Inference (Qualitative Analysis)	Green, yellow, or brick-coloured precipitate after spontaneous cooling indicates agar (or other hydrolysable gum).
Reference	AOAC 22nd edition, 2024, 945.57: Agar in Meat
Approved by	Scientific Panel on Methods of Sampling and Analysis

Part-B:
Meat Speciation

(Inauthenticity and mislabeling in foods of animal origin)

Chapter 1

General requirements and sample preparation – Nucleic acid-based methods

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Method No.	**Chapter 1.1**	**Revision No. & Date**	0.0
Scope	This document specifies the general requirements for DNA extraction/purification from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed, including stages of sample preparation.		
Caution	The objective of nucleic acid extraction methods is to provide nucleic acids suitable for subsequent analysis. The "quality" of DNA depends on the average length of the extracted DNA molecules, the chemical purity and the structural integrity of the DNA sequence and of the double helix.		
Principle	The basic principle of DNA extraction consists of releasing the DNA present in the matrix and further, concurrently or subsequently, purifying the DNA from polymerase chain reaction (PCR) inhibitors. Method-selection is an experience- based choice of the user, taking into account the scope and type of matrices to be tested. Alternative protocols are suitable provided that the method has been validated on the respective matrix under investigation.		
Apparatus/Instruments	Refer methods FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements. A fume hood is necessary for handling organic chemicals.		
Materials and Reagents	Refer methods FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements.		
Preparation of Reagents	Refer methods FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements.		
Sample Preparation - General	**Laboratory requirements - General** Accidental contamination of DNA can originate from dust and spreading aerosols. As a consequence, the organization of the work area in the laboratory is logically based on; • The systematic containment of the methodological steps involved in the production of the results, and • A "forward flow" principle for sample handling. The latter ensures that the DNA to be analyzed and the amplified DNA generated byPCR remain physically segregated. **Preparation of test portion - General** • Commodity-specific variables (e.g., humidity) and processing can impact the amount and quality of DNA extracted from the material under investigation. Therefore, the performance characteristics of a given DNA extraction method depend on the nature of the matrix. • Take appropriate measures to ensure that the test portion is representative of the laboratory sample. • The test portion shall be of sufficient size and shall contain a sufficient number of particles to be representative of the laboratory sample (e.g., 3000 particles at an LOD of 0.1 %) to allow a statistically valid conclusion to be made (see ISO 21568). For practical/technical reasons, it is not recommended to exceed a size of 2 g.		

	• Any restrictions that arise from the size of the test portion which prevent it from being representative shall be reported and taken into consideration inthe interpretation of the analytical results.
	• The methods for DNA extraction describe test portions from 50 mg to 500 mg, which are usually adequate for DNA-rich raw materials. However, for certain matrices containing very low amounts or degraded DNA, insufficient DNA suitable for analysis can be extracted. In these cases, the size of test portion may be increased.
	• DNA extractions shall be carried out at least on two test portions.
	• Storage of standards, samples and test portions shall comply with ISO 20813 and shall be organized in such a way as to preserve the biochemical parameters to be analyzed (for details, see ISO/IEC 17025).
	Samples - General
	• All operations for the preparation of test samples (e.g., grinding, homogenization, division, drying) shall be carried out in accordance with the procedures described in ISO 20813, taking care to prevent all contamination of the sample or modification of its composition.
	• Laboratory samples shall be sufficiently homogeneous before reducing the laboratory sample and taking the test portion.
	• For liquid samples, shake the vessel containing the sample to improve the homogenization of the product.
	• For solid matrices that cannot easily be suspended, the matrix shall be ground to reduce the particle size and/or facilitate the extractability of DNA. In such a case, attention shall be paid to the particle size. The test portion subjected to extraction shall contain a minimum number of particles as specified in ISO 21568. Milling/grinding devices should be capable of being thoroughly cleaned and shall be selected in order to achieve the expected particle number and particle size distribution within the test portion as defined in ISO 21568.
	• If components of the laboratory sample have been removed prior to extraction, then such procedures shall be reported.
	• Final food products that are solid or paste and have high lipid contents are often not easy to grind to the desired particle size in a single step. Several procedures may therefore be added, such as lipid removal using hexane after intermediate grinding, freezing or freeze-drying before grinding.
	• In order to facilitate the grinding of paste or viscous products, it is possible to apply one of the following treatments to certain matrices:
	  • Heating to a maximum temperature of 40 °C;
	  • Dissolving in an appropriate liquid such as water;
	  • Freezing at a temperature below or equal to −20 °C.
	• Homogenize the whole laboratory sample. Sample the two test portions, taking into account possible dilutions or concentrations.
	• During milling/grinding, precautions should be taken to ensure

	that the heating of the sample is kept to a minimum since heating can have a negative impact on the quality of the extracted DNA. • Milling/grinding techniques with a high risk of cross-contamination (such as the combined use of liquid nitrogen and mortar) shall be avoided as far as possible. As a rule of good practice, any dust-producing methodological step should be contained from all other analytical steps. If salts, spices, powdered sugars and/or other substances that could potentially interfere with the extraction or analytical method are present, appropriate purification steps should be considered according to the selected method. For example, in samples from composite matrices, the target matrix (e.g., the breading layer of meat patties) can be isolated for DNA extraction.
Method of analysis	**DNA extraction/purification** **General** The following considerations apply for the design of extraction methods. The quality and yield of nucleic acid extracted using a given method on a given matrix should be both repeatable and reproducible in terms of analysis, provided sufficient nucleic acid is present in the matrix from which it has been extracted. In order to obtain a good quality DNA, it is advisable, where relevant, to remove the following: ▪ Polysaccharides (pectin, cellulose, hemi-cellulose, starch, thickeners, etc.) using appropriate enzyme treatments (e.g., pectinase, cellulase, hemi- cellulase, α-amylase) or organic extraction (e.g. CTAB/chloroform); ▪ RNA and/or proteins using an appropriate treatment, such as enzymatic treatment by RNase and proteinase, respectively; ▪ The lipid fractions, using enzyme treatments, or solvents (e.g., n-hexane); ▪ Salts (e.g., from the extraction/lysis buffer, from the precipitation step) able to interfere with the subsequent analysis. In particular for solid or dried samples, the volume of lysis/extraction buffer shouldbe adapted to guarantee the DNA is dissolved. **NOTE 1**: DNA purification can be performed by different means such as fractionated precipitation, using solvents like phenol, chloroform, ethanol, isopropanol, and/or by adsorption on solid matrices (anion exchange resin, silica orglass gel, diatomaceous earth, membranes, etc.). Several DNA purification principlesmay also be combined. If appropriate, extraction and purification can be performed within the same step. Should a DNA co-precipitant such as glycogen, PEG or t-RNA be used to improve theDNA recovery during the precipitation steps, it should neither contain any detectable level of nuclease activity or PCR inhibitors/competitors, nor bear any sequence similarity with the potential PCR target under study. For genetically modified plants, a carrier DNA may be used (e.g., salmon or herring sperm DNA). When using vacuum freeze dryers to dry the DNA pellets obtained after a

	precipitation step, the risk of cross contamination should be taken into account. Re-suspend the DNA in water or in a buffer solution that prevents DNA from degradation. When setting up a new type of DNA extraction, or when applying one of the methods described in FSSAI 05.022:2024 to a new matrix, the potential quality and integrity of the extracted DNA using the chosen protocol should be estimated by the following approach. A known quantity of a tracer DNA is added to the lysis buffer plus sample used for DNA extraction. When the chosen tracer is a predetermined amount of DNA or represents a predetermined number of copies of a particular DNA-sequence mixed to a matrix at start of DNA extraction, attention shall be paid to ascertain the lack of DNA sequence similarity between the tracer DNA and the target DNA sequence under study. The use of a tracer DNA is a good approximation to a real situation where DNA of a given matrix, complexed to other components (e.g., proteins) is expected. Such a method may also be used to estimate the presence of soluble and trans-acting PCR inhibitors in the extracted DNA (ISO 21571). However, tracer DNA may give a misleading impression of recovery, since tracer DNA may be much easier to separate from matrix than the target DNA. **Controls** The controls to be included are described in Table 1 of ISO 20813. Negative DNA target control should be prepared from DNA extracted from non-target species prevalent in the sample (e.g. for a horse assay in cattle meat, the non-target species is cattle). As a rule of practice the following controls may be incorporated; a. The use of *environment controls* helps the laboratory to identify sources of contamination at an early stage and can even be used to identify in which work area the contamination is present. This can be demonstrated in various ways, e.g. if negative samples included in the series of homogenized samples showed negative results, starting at the first step of the process (e.g. grinding step if relevant). b. At least one *extraction blank control* shall be included each time DNA is extracted from one or more samples. The tube shall always be the last in each series. It may be appropriate to put one extraction blank on, for example, a rack of eight tubes or a microplate of 96 wells for automated extraction. c. A *positive extraction control* shall be included regularly. This control reveals if something is wrong with the reagents or the performance of the extraction protocol. d. The *positive DNA target control* demonstrates the ability of the nucleic acid amplification procedure to detect the target DNA sequence at a low copy number in order to confirm the LOD. e. The *negative DNA target control* demonstrates the ability of the nucleic acid amplification procedure to avoid false positive amplification in the

	absence of the target DNA sequence. f. The *PCR reagent control* demonstrates the absence of contaminating nucleic acid in the PCR reagent batches used. The PCR reagent control can be omitted when the extraction blank control is used. g. The *PCR inhibition control* can be used to demonstrate the absence of soluble inhibitors. This can also be demonstrated by serial dilutions of the template nucleic acid. However, some type of assessment of the effect of soluble inhibitors on the results of the analysis of the sample shall be made. h. A *PCR inhibition control* is mandatory, if all PCR test on the sample give negative results. These should as a minimum include an extraction blank control and a positiveextraction control, but may also include an environment control. **Control of DNA purity (Internal PCR control)** When setting up a new type of extraction, the presence of PCR inhibitors in the extracted DNA may be estimated using DNA spikes. The amount of added DNA shall not exceed the maximum level supported by PCR and shall contain a definite number of target sequence copies. This number should be determined individually for each target sequence and indicated as a multiple of the existing lower limit of detection. Ideally, the target concentration of the positive control PCR should correspond to the sensitivity needed in the analysis. Care shall be taken when using highly concentrated cloned target DNA. As far as possible, the positive controls shall conform to the conditions of the test material with regard to the nucleic acids they contain.
Calculation with units of expression	Calculation with units of expression is carried out according to purpose and choice of your interest using the methods for the quantitation of the extracted DNA (Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements).
Inference **(Qualitative Analysis)**	Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for theidentification and the Detection Of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction 3. ISO 21568: 2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Sampling
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Method No.	**Chapter 1.2**	**Revision No. & Date**	**0**
Scope	The scope of this document pertains to providing the general requirements to the methods for the quantitation of the extracted DNA from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.		
Caution	Only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder-free gloves. The use of aerosol-protected pipette tips (protection against cross-contamination) is recommended.		
Principle	Quantitation of extracted DNA could be useful for subsequent PCR analysis. — It may be performed by either physical (e.g. measure of absorbance at a specific wavelength), chemical-physical (e.g. use of intercalating or binding agents able to emit fluorescence), enzymatic (e.g. bioluminescence detection) methods, capillary electrophoresis or by quantitative PCR. The latter method is especially suitable for composite matrices or for samples with a low DNA content or whose DNA is degraded. — There are several methods available to quantify the DNA present in a solution, as described in FSSAI 05.024:2024. It is for the user to choose the most appropriate one to be applied, depending on the amount and quality of DNA to be quantified and, consequently, on the matrix from which the DNA has been extracted. — Alternative protocols are suitable, provided that the method has been validated on the respective matrix under investigation.		
Apparatus/Instruments	Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.		
Materials and Reagents	Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.		
Preparation of Reagents	Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.		
Sample Preparation	Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.		
Method of analysis	**General** The quality, integrity and amount of the nucleic acid template influences the performance of the analytical method, and hence the analytical results obtained. The limit of detection of a specific method may therefore depend on the amount of nucleic acids used. Quantitation of DNA is helpful — to compare the efficiency of different DNA extraction protocols for a given matrix (repeatability), and — to measure the concentration of nucleic acids prior to analysis.		

	Range of application Each method of quantitation shall be applied within its dynamic range, also considering its level of precision. **Quantity standards** 1. The accuracy of the quantitation methods depends on the nucleic acid standards used to calibrate the method. 2. If using a method that is sensitive to the size and/or quality of the nucleic acid fragments, then the nucleic acid standards that match the size and/or quality of the expected nucleic acid as extracted from the sample shall be used. 3. The reference material used should ensure traceability to stated references, usually national or international Standards, through an unbroken chain of comparison [see ISO 17034]. 4. When a method using intercalating agents is employed, high molecular mass DNA standard should be used when high molecular mass DNA is to be quantified. Low molecular mass DNA should be used when low molecular mass DNA is to be quantified. 5. High molecular mass nucleic acid usually also contains a certain amount of lower molecular mass fragments. This means that many methods for DNA quantitation suffer from a certain degree of inaccuracy, which should be taken into account. 6. At least three points (preferably replicated) are required for the construction of a good calibration curve. The amount of standard DNA used for each calibration point depends on the sensitivity of the method and on the dynamic range under consideration.
Calculation with units of expression	Calculation with units of expression is carried out according to purpose and choice of your interest using the methods for the quantitation of the extracted DNA (Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements).
Inference (Qualitative Analysis)	Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.
Reference	ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Nucleotide sequencing-based methods: General requirements and sample preparation**		
Method No.	**Chapter 1.3**	**Revision No. & Date**	**0**
Scope	The scope of this document is to provide the minimum requirements of performance characteristics for the detection and identification of animal species in foods and feed products by nucleotide sequencing methods (DNA sequencing). The DNA sequencing methods described in this document are Sanger and Next Generation Sequencing (NGS), including second and third-generation sequencing, for analysis of single-species food products and multispecies products. Single species products that are made from one piece (e.g., fish fillet, beef tenderloin) are appropriate to be analyzed by Sanger sequencing whereas NGS is the appropriate method for simultaneous multispecies identification. For the identification of the species' DNA, sequences are compared with specific reference databases. Results can be obtained at different taxonomic levels (e.g., order, species, genus, family, etc.) depending on the type of database and DNA data analysis performed. This document applies to DNA sequences for mammals, birds, fish, mollusk's, crustaceans, amphibians, reptiles and insects and the validation of the applicable methods.		
Caution	During the analysis, unless otherwise stated, use only reagents of recognized molecular biology grade and distilled or demineralized water or water of equivalent purity, according to EN ISO 24276. Regarding laboratory organization, see EN ISO 24276.		
Principle	Method of identifying organisms based on short, standardized DNA fragments containing both conserved and variable sequences from a specific region or regions of the genome. The principle of DNA barcoding is that by comparison with a reference database, the sequence from this DNA can be used to uniquely identify an organism or link it to a specific taxon.		
Apparatus/Instruments	Refer method FSSAI 05.029:2024 for specific requirements.		
Materials and Reagents	Refer method FSSAI 05.029:2024 for specific requirements.		
Preparation of Reagents	Refer method FSSAI 05.029:2024 for specific requirements.		
Sample Preparation	The requirements needed for sample preparation depend on the type of sample to be analyzed. Samples can be divided into three different categories as shown below; **Category A**: Single species sample composed of a single piece (ex. 1 sample = 1 fish fillet or 1 beef steak), there is no need for grinding or homogenization. The sample is composed of a single piece and a part of that piece can be taken and analyzed. This category can be analyzed by Sanger sequencing or NGS. **Category B**: Single species product composed of several pieces or units of the same type of tissue (ex: 1 sample = package with 10 fish fillets or 20 meat pieces). Depending on the sample preparation either NGS or Sanger sequencing can be used as described below; Preparation of a representative test sample for NGS analysis only. Take at least one portion of each fish fillet/meat piece (same type of tissue and portion size). All portions collected are mixed to produce a composite sample of the food product received. Therefore, the composite sample should be correctly homogenized to guarantee the		

	representativeness of each portion collected. Homogenize the totality of the laboratory sample. This type of test sample can't be analyzed by Sanger. **Category C**: Multiple species product that can be composed by several species (both animals and/or plants) or different types of tissues as ingredients (ex: lasagne, pizza, seafood cocktail, minced meat) or as unwanted contamination (food containing trace species amounts and/or cross-contamination). Samples are totally grinded and homogenized. Category C products must be analyzed by NGS. This type of test sample can't be analyzed by Sanger.
Method of Analysis	**DNA extraction** (Refer methods FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements) **DNA sequencing workflow** **1. General** According to the method used (Sanger or NGS) and the different types of instruments, the instructions provided by the instrument manufacturer should be followed. Sequences are exploitable if they are complying with the quality control parameters of the instrument used and with the quality parameters established for each sequence obtained. **2. Sanger method** This method produces a single electropherogram. It can only be used when a sample contains a single species as its content (e.g. one fish fillet, one steak, one shrimp, etc). Therefore, Sanger sequencing is appropriate for foods included in category A and B, as the case may be. After DNA extraction, Sanger workflow includes: ▪ PCR ▪ Purification of PCR product ▪ Asymmetric amplification with dideoxy termination (PCR) ▪ Purification of sequencing fragments ▪ DNA sequencing **2.1. PCR** Genes that are known to be useful for species identification, namely mitochondrial genes (e.g., COI, cytB,12S rRNA) or nuclear genes (e.g., 18S rDNA). The use of universal primers will enable to amplify many different species. These primers usually anneal in highly conserved regions in the DNA that are identical or highly similar in a large group of taxa (species, genus, family, order, domain). This strategy is commonly named as DNA barcoding. The degree of universality is established according to the DNA regions (genes) where the primers anneal and is assessed in a first step by bioinformatic tools. It is very common that primers contain degenerated positions to increase their universality. There are many universal primers already published for many taxa. However additional primers can be designed using primer design bioinformatic tools. Nevertheless, a in silico universality analysis of the primers used should be done to compile the list of taxa where the primers can anneal. After PCR amplification, the amplicon is visualized by agarose gel electrophoresis (optionally). Other visualization systems can be used. This step enables to confirm the

	expected size of the amplicon and theabsence of additional unwanted non-specific amplicons that can be produced during the PCR reaction. **2.2. PCR purification** In this step the purpose is to clean up the amplicon from the excess of unused PCR reagents. This is normally done using commercially available kits. However, a classical ethanol-based precipitation and purification procedure can also be used. After PCR amplification, the amplicon is visualized by agarose gel electrophoresis (optionally). **2.3 Asymmetric amplification (PCR)** This step is done using a kit from the DNA sequencer manufacturer. Depending on the Sanger sequencing instrument, each brand has its own consumables and instructions. Therefore, this is done following the manufacturer's instructions. Basically, in this step, a single primer is used for the amplification of a single DNA strand, only the forward or the reverse primer is used for the amplification. Additionally, the nucleotides used are a mixture of common dNTPs with fluorescence-labelled ddNTPs, and when these are incorporated, they stop the DNA copy at random positions creating a mixture of amplified fragments from the same template but with different lengths.
	2.4 DNA sequencing The mixture of fragment lengths produced during the asymmetric amplification are separated using a high-resolution (1 nucleotide) electrophoresis system. Usually, this is done by capillary electrophoresis producing an electropherogram that incorporates the DNA sequence obtained. There are different DNA sequencer providers, and the instruments need to be used following the manufacturer's instructions. **2.5 Assessing raw sequence quality** This is usually done by visual examination of the electropherogram obtained. Each corresponding sequenced nucleotide should present a unique curve. Background noise should be clearly distinguishable from the real curves obtained for each nucleotide. In the case of background interference, the sequence should be discarded. **3.Next Generation Sequencing method** This method originates a file containing multiple DNA sequences (usually thousands/millions of sequences). Because by NGS each DNA molecule originates a unique DNA sequence, this method is appropriate for samples that may contain multiple species providing a species identification result for the species present in the mixture. Therefore, NGS can be used for any kind of samples regardless of whether or not they contain single or multiple species in their composition. As shown in Annex A NGS is appropriate for foods included in all categories, as described above. After DNA extraction, NGS workflow includes: • DNA library preparation • NGS sequencing • Assessing raw read data quality

3.1. DNA library preparation

DNA libraries are prepared from the DNA extracted. This process may include a first step of PCR targeting specific gene(s) regions or can be performed without using PCR. Therefore, the two main criteria previously established by the user, include:

- The inclusion of a first step of PCR targeting specific regions in the DNA
- The type of NGS technology used. This can be a short-read technology or a long- read technology depending on the NGS instrument available.

Since there are many different brands of sequencing machines it is important to match each defined quality requirements from the manufacturer (DNA library concentration, average amplicon size, range of amplicon size, etc.).

3.1.1. DNA library preparation without PCR

The DNA library construction (with no PCR) may include the following steps:

- DNA fragmentation
- Ligation of indexes/adapters/barcodes
- Quantitation, normalization, and quality control of the resulting library
- Pooling of libraries for multiplexed sequencing runs

➢ All these steps are performed with the reagents available for each NGS instrument and following manufacturer instructions. Appropriate controls and adjustments to these steps should be made as recommended by each instrument brand and according to the technology used (e.g., long read technology optionally requires DNA fragmentation and ligation of indexes)

➢ Because the NGS sequencing method enables simultaneous and independent sequencing of millions of DNA molecules, the pooling of libraries can be used to maximize the output obtained by NGS. Pooling of libraries is a strategy to run more than one DNA library coming from different samples in parallel. This is done by using an index/adaptor/barcode that adds a unique identifier for each DNA coming from different libraries and samples. Therefore, multiplexing samples requires the assignment of a unique identifier to identify individual samples and is typically documented to allow the association of sequence data obtained for each identifier with the correct metadata.

3.1.2. DNA library preparation with PCR

The DNA library construction including PCR includes the following steps:

- Target-specific PCR (single or multiplex)
- Addition of indices/adapters/barcodes
- Quantitation and purification
- Pooling of libraries for multiplexed sequencing runs

When a PCR-based approach is used, universal primers are used as described for Sanger sequencing. Multiplexing strategy can be incorporated during the DNA library preparation and can be done at

the following levels:

- Sample multiplexing (pooling more than one sample in the same sequencing run)
- Genes/amplicons multiplexing (e.g., pooling more than one gene-specific amplicon in the same sequencing run)
- Sample multiplexing and genes/amplicons multiplexing (pooling more than one sample and more than one specific gene/amplicon in the same sequencing run)

➢ When using a gene-specific PCR-based approach there are two ways of incorporating unique identifiers to the amplicons for sequencing. This can be done as a specific step after performing PCR. In this case the amplicons can be mixed with specific reagents that add a unique identifier to each amplicon. On the other hand, this can also be done by a second PCR reaction containing the identifiers. This unique identifier is commonly called a barcode and all this process is made with specific kits that are specific for each type of NGS instrument used. Therefore, this is a post-PCR process to be performed according to the manufacturer instructions, including all the quality controls mentioned on those instructions.

➢ Alternatively, the identifiers can be added during the PCR reaction by using fusion primers for amplification. Fusion primers are long primers that have an additional sequence on the 5' end of the primer sequence to be used. Usually, the length of this additional sequence depends on the type of NGS instrument used but is typically 30-50 nucleotides long. Therefore, the fusion primers need to be designed to fit with the NGS instrument brand to be used.

3.2. NGS sequencing

Regardless of the type of pre-treatment of DNA with/without PCR or fragmentation, each specific piece of equipment to be used has its own specific manufacturer instructions both for 2nd or 3rd generation sequencing.

3.3. Raw read processing and sequence quality assessment

Generation of sequence read files should use instrument-specific software and/or instrument specific pipelines. Several physical measures such as signal-to- noise ratio shall be considered and their measurements should be monitored during the sequencing experiment. Sequence read files should be configured in the appropriate file format, which contains the compilation of individual sequence reads, each with its own identifier, and an associated base quality score for each nucleotide. FASTQ format is one of the most common file formats obtained at the end of a NGS run and contains the reads and all quality metrics. The quality should be evaluated and using appropriate software (e.g., FASTQC tool is commonly used to evaluate the quality of sequencing results for FASTQ (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw data pre- processing may include removal/trimming of low-quality

	sequences/bases, demultiplexing when mixtures of samples are used in the same run, removal of adapters/primer, and trimming of reads to a fixed length (when a PCR-based approach is used). Additional computational steps can be added according to the equipment/technology used. Quality assessment includes but is not limited to Quality scores (both nucleotide and full sequence Q scores), length distribution, and GC content (not needed for a PCR-based NGS). A defined Q-score threshold should be established (e.g., Q-score of 20). Based on the processing of raw data and the quality criteria defined, sequences not complying should be discarded. A fasta file is commonly originated containing all the good-quality sequences. The number of reads of the file used for database comparison should be recorded. When using a multiplex approach based on a barcoding PCR-based NGS by mixing different samples and/or different DNA regions, the number of reads for each combination sample/gene should be recorded. It is preferable to establish a minimum number of reads for each combination (e.g., 1000 reads/sample/region). However, this threshold should be established according to the criteria defined by each laboratory. The use of a non-PCR NGS approach will not enable obtaining this type of information at this stage. However, after a comparison of the reads obtained with the database, these parameters can be calculated.
Calculation with units of expression	Species identification results obtained by DNA sequencing are only qualitative. The name of the species/taxonomic level is the result with no additional unit ofmeasurement.
Inference (Qualitative Analysis)	Refer method FSSAI 05.029:2024 for specific requirements
Reference	1.ISO 22949-1:2020, Molecular biomarker analysis — Methods of analysis for thedetection and identification of animal species in foods and food products (nucleotide sequencing-based methods) — General requirements and definitions. 2. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for theidentification and the Detection Of animal species from foods and food products —General requirements and definitions 3.ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**Chapter 1.4**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide the minimum requirements of performance characteristics for the detection of nucleic acid sequences (DNA) by molecular methods, such as polymerase chain reaction (PCR), including different post-PCR detection methods, real-time PCR, single and/or multiple probe-based detection techniques as well as the combination of such methods. The document is applicable to the detection, identification and quantification of DNA from animal species of higher and lowers taxonomic groups in foodstuffs, and the validation of applicable methods. It is applicable to mammals, birds, reptiles, amphibians, fishes, mollusk's, crustaceans and insects.		
Caution	See at Material and Reagents and Preparation of reagents.		
Principle	Principle for the detection of animal derived DNA by real-time PCR method is provided under the respective methods. Refer methods FSSAI 05.030:2024 to FSSAI 05.039:2024 for specific requirements.		
Laboratory set-up	The work area in the laboratory should be designed to prevent accidental DNA contamination originating from, for example, dust, human material and spreading aerosols, including consideration of: • Systematic containment of the methodological steps involved in the production of the results • A forward flow principle for sample handling. For DNA-based methods, separation (temporal and/or physical) of work is required to prevent contamination. Designated contained/dedicated work areas with their own apparatus are recommended, as follows: • A work area for grinding and homogenization; • A work area for extraction of the nucleic acid from the test material; • A work area dedicated to the setup of PCR/amplification reactions; • A work area dedicated to subsequent processing, including analysis andcharacterization of the amplified DNA sequences, if applicable. If human DNA is detected by the method, appropriate contamination prevention measures (e.g., use of masks, gloves and disposable coats) should be taken in order to prevent false-positive results by contamination with the operator or other human DNA during analysis. Physical separation through the use of different rooms is the most effective and preferable way of ensuring separate work areas, but other methods can be used as protection against contamination, provided their effectiveness is comparable. The airflow system should be set up and directed in a way that prevents intrusion of dust/amplicons from work areas with higher contamination risk to work areas with lower contamination risk.		
Materials and Reagents	In order to avoid contamination, a sterile technique should be adopted in the PCR set-up area, e.g., powder-free gloves, sterilized plastic ware, autoclaved reagents; disposable plasticware and aerosol-protected, DNA/RNA-free and DNase/RNase- free filtered pipette tips should be		

	used. Materials and all containers and disposables containing reagents shall be preserved from any contaminating agent (e.g., dust). Manufacturers' recommendations for the use of reagents should be followed. Appropriate controls can be used to assess the integrity of reagents and the absence of DNase. No unintended enzyme activities (e.g., exonuclease) that might interfere with PCR shall be present in the preparation. The reaction buffer shall be suitable for the polymerase used.
Preparation of Reagents	For the analysis, unless otherwise stated, only analytical grade reagents suitable for molecular biology, free from DNA and DNases, should be used. Reagents and solutions should be stored at room temperature unless otherwise specified. PCR reagents should be stored in small aliquots to minimize the risk of contamination. The water used shall be double-distilled, deionized or of comparable quality. Solutions should be prepared by dissolving the appropriate reagents in water and autoclaved, unless specified differently. Sterile filtration devices (possibly 0.22 µm pore size) may be used when autoclaving is not possible.
Sample Preparation	A representative sample should be tested. It shall be ensured that the test samples used for DNA extraction are representatives of the laboratory sample, such as by homogenizing the sample or appropriate portions thereof. At least two aliquots should be taken from the homogenized laboratory sample as test portions for DNA extraction and subsequent analysis. If possible, the sample material should not be taken from the surface of the laboratory sample in order to minimize the risk of the amplification of adhering contaminants. If the analytical method to be used for the sample detects human DNA, special contamination prevention measures should be taken. Concerning the preparation of DNA from the test portion, the general instructions and measures described in ISO 21571 should be followed. One of the DNA extraction methods described in ISO 21571:2005, Annex A, should be considered. Alternatively, commercial kits can be used for the extraction and purification of DNA.
Method of analysis for Performance Characteristics	**1. Applicability** When assessing if a method is fit for purpose, the following aspects regarding thenature of the target should be considered: • The location of the target (nuclear or mitochondrial) • The copy number per cell • The length of the target sequence For quantitative species-specific methods, a nuclear gene, excluding mitochondrial DNA, shall be targeted. The target sequence shall be present as a single copy per haploid genome, or the copy number shall be determined/known. The following aspects regarding the matrix should be considered: • The nature of the potential sample matrices • The degree of processing of the sample constituents • The different species and animal tissue types involved • The preparation of the sample matrix **NOTE** Mitochondrial PCR targets cannot be used for reliable quantification of haploid genome copy number ratios of different species, because the amount of mitochondrial targets differs with

	tissue type. **2. Specificity** The specificity should be assessed in a two-step procedure: theoretical and experimental evaluation of the inclusivity and exclusivity. In silico testing of the specificity of primers and probes with available bioinformatics tools shall be performed. If sequence data are used for verification of animal speciation results, they should be based on appropriate databases with due consideration of the timing of submission of individual entries and any subsequent changes in the taxonomic classification or naming. **2.1. Requirements for inclusivity testing** • Experimental results from testing the method with the target animal species should be provided. This testing should include relevant breeds of the animal species according to the scope of the method. • Material for experimental inclusivity testing should contain approximately 100 target DNA copies. Each sample material shall be at a minimum tested in duplicate. Sequence variants of the target animal species should be detected with comparable amplification efficiency if they occur. • The target animal species for inclusivity testing are normally more than five breeds **2.2. Requirements for exclusivity testing** • Experimental results from testing the method with non-target animal species shall be provided. This testing should include both taxonomically close and not closely related animal species. Animal species or taxonomic groups relevant with regard to the scope of the method shall be tested, e.g. species commonly used in food in general and particularly in matrices considered in the scope of the method. The method should clearly distinguish between target and non- target animal species. • Sufficient DNA should be used for experimental exclusivity testing. A number of 2500 target copies ensure that cross-reactivity can be identified. Select a minimum of 10 species that could cause interference with the target animal species present in the food test material. • Other species should be included if relevant, e.g., if there are sequence homologies of oligonucleotides to nucleic acid sequences. • The cross-reactivity of the matrix should be characterized. • The suitability of the DNA used for amplification should be confirmed by an amplification control, e.g. by a single copy (chromosomal) DNA consensus PCR system (e.g. myostatin or actin). **3. Sensitivity** • Experimental results from testing the method at different concentrations in order to test the range of use of the method shall be available. They shall be described in the validation report. • If applicable, detailed information about how a cut-off value can be established and used in the laboratory should be provided. • Animal species that require qualitative testing should be detected at levels relevant to the interested party, e.g., the

consumer.

3.1. Limit of detection (LOD)

3.1.1. Absolute LOD

- The absolute LOD (LOD_{abs}) shall be indicated in copy numbers of the target sequence per reaction with the confidence level (typically 95 %) specified.
- Twenty copies or less can be applied for single-copy genes, and an appropriate number of haploid genome equivalents for high copy number genes.
- If for the LOD determination, a DNA with a known copy number of the target sequence is not available, plasmid DNA can be used.
- The LOD_{abs} of the method is determined experimentally by preparing a dilution series of the target material with dilutions in the range of the expected/targeted limit of detection.

3.1.2. Relative LOD

- The relative LOD (LOD_{rel}) shall be determined in relevant non-target animal species DNA as background. Depending on test requirements, the LOD_{rel} is adjusted to this value. The LOD_{rel} expresses the relative c/c % of the target animal species DNA in other animal species DNA which is detected with 95 % confidence.
- The LOD_{rel} should be determined experimentally by preparing one or more defined reference samples with defined percentage content of the target DNA in the range of the limit of detection. Each reference sample is analyzed in at least 10 replicates. The percentage of the reference sample where at least 95 % of the replicates give positive results is considered the LOD_{rel}

3.1.3. Asymmetric LOD (for multiplex methods only)

- In the case of multiplex methods where the detection of different targets is restricted by competitive effects, as in the case of multiplex real-time PCR methods, the LOD for the single targets in an asymmetric target situation expressed as target ratio needs to be validated. Different contents of the specific animal target sequence are mixed to obtain defined copy ratios (i.e., ratios of 1:1 000 and 1 000:1; 1:100 and 100:1). The ratio where each target animal is detected with 95 % confidence is determined experimentally with an appropriate number of replicates for the defined reference sample.

4. Specific requirements for quantitative methods

- The upper and lower limit of the linear range of the method shall be determined. The assessment of these limits and the linear range shall be carried out on samples containing animal non-target DNA relevant to the food item.

4.1. Limit of quantification (LOQ)

- The absolute LOQ (LOQ_{abs}) shall be indicated as copy numbers of the target sequence. It shall be equal to the smallest amount included in the dynamic range.
- The relative LOQ (LOQ_{rel}) shall be determined in DNA of other relevant animal species. Depending on the test requirements,

	the LOQrel should be adjusted to this value. The LOQ rel expresses the ratio of the target animal species DNA copy number to other animal species DNA copies or to the DNA copies of a reference gene representative for the whole taxonomic rank. The LOQrel should be equal to the smallest concentration included in the dynamic range. • If, for the LOQ determination, a DNA with known copy number of the target sequence is not available, plasmid DNA should be used. This plasmid can also serve as a calibrator. • A minimum of 15 replicates with a target concentration of the expected LOQ shall be tested. The criteria for precision and trueness shall be fulfilled for the results. The LOQ values reported from collaborative study data generally refer to the lowest level of the analyte that was observed to have a relative standard deviation of reproducibility of 25 % or less. **4.2. Dynamic range** • The dynamic range should cover the percentage values as well as the copynumbers according to the expected use and scope of the method. • In order to define the relevant minimum copy number, the desired dynamic range in terms of target copy percentages shall be determined. It should be considered that the genome size of the species in the expected sample material restricts the maximum copy number that can be used for the analysis (e.g., 100 ng to 200 ng, depending on the method). **Note:** For example, for cattle, a genome size of 4 pg. can be assumed, which resultsin a maximum copy number of 25000 in 100 ng of sample DNA material. The copy numbers of the dynamic range for both, the target and referencesequence, shall be then determined as follows: For the reference sequence, the maximum number of copies can be calculated considering genome sizes and the amount of sample DNA used for analysis as described above; • For the target, the lowest copy number should be the absolute LOQ; as a prerequisite, the lowest possible value considering the ratio compared to the maximum number of copies of total/reference DNA should be taken into consideration. • The minimum copy number of the reference sequence and the maximum copy number for the target sequence should be given by the ratio of the minimum and maximum, respectively, percentage values. **Note:** The dynamic range is established on the basis of a standard curve with a minimum of four concentration levels evenly distributed at least in duplicate. **Note:** For a desired upper limit of the percentage dynamic range of 100 %, the minimum copy number of the reference can be equal to the lower limit of the copy number range of the target sequence, and for a desired LOQ of 0.1 % at an absolute LOQ of 30 copies, the upper limit of the reference target is 30,000 copies. **4.3. Determination of precision and trueness for quantitative methods** The precision should be determined with the relative

repeatability standarddeviation (RSDr).

A sufficient number of replicates (at least 15) for at least three DNA materials with different target percentages covering the whole dynamic range should be analyzed. Note: Mitochondrial DNA is not adequate for the targets of quantitative methods.

- The RSDr for all replicates shall be ≤ 25 % over the whole dynamic range of the method.
- The trueness shall be within 25 % of the accepted reference value for all replicates over the whole dynamic range of the method.

4.4. Robustness

Results from the empirical testing of the method against small but deliberate variations in method parameters (e.g. variation in concentration of kit components,variation in apparatus) should be provided, if available.

4.4.1. Robustness determination by inter-laboratory study

Robustness can be determined by performing an interlaboratory study. The robust method shall be selected by considering that the results from different laboratoriesdo not vary significantly.

4.4.2. Robustness determination by a multifactorial orthogonal test design

- The test should be carried out in a multifactorial approach where several alterations, including, but not limited to, master mix concentration, reaction volume, primer and probe concentration, annealing temperature and thermocycler platform, are assessed.
- For qualitative methods, at least three replicates should be tested. The target animal species copy number used in the test should be in a concentration threefold of the LODabs (95 % confidence) of the method.
- For quantitative methods, three defined target concentrations over the whole dynamic range of the method should be tested in three replicates each.

Note: The method is considered to be robust if all reactions give the expected results.

5. Data analysis

5.1. Control

Each control shall have a valid value, and, if the observed result for any control is different from the valid value, the analysis shall be repeated. Environmental controls with a positive result shall always initiate measures to remove and prevent contamination of the laboratory environment. If a non-valid result for any of the other controls is obtained repeatedly, measures shall be taken to locate and remove/replace the source(s) responsible for the error, and the analysis is then repeated. Analytical results shall only be reported when all controls yield valid values, and the valid values for the controls are as follows:

- Extraction blank control shall always be negative
- Positive extraction control shall always be positive
- Negative results shall always be negative (negative sample results are valid, even if the negative DNA target control is not valid if all other controls are valid)
- Positive DNA target control shall always be positive;

- Negative DNA target control should be negative;
- PCR reagent control shall always be negative;
- PCR-inhibition control shall not show significant inhibitory effects in the case of samples with negative qualitative results and for samples with quantitative results.

The above controls shall be used for interpreting/reporting the test sample result.

5.2. Conventional PCR

The amplicons generated by conventional PCR shall have the expected length (e.g., gel visualization). To avoid false-positive results, verification of the obtained amplicon can be performed by hybridization, sequencing, restriction enzyme analysis or another suitable sequence-specific method of verification in addition to the length confirmation.

Note: Melting curve analysis is sometimes used for amplicon verification but is not sequence specific.

5.3. Real-time PCR amplification curves

Real-time PCR amplification curves should be visually checked for a sigmoid shape in order to exclude artefacts/false-positive results.

Note: Melting curve analysis is sometimes used for amplicon verification but is not sequence specific.

6. Expression of results

- Results shall be described to show the detection of target-derived DNA.
- EXAMPLE For target analyte X, the presence of DNA derived from the state- specific target sequence and animal species or taxonomic group was detected.

Note: The scope of this analysis is only to show the presence/absence of DNA of thenamed animal species, not to show the presence/absence of tissue of the animal species (e.g. DNA derived from egg, gelatine).

- Results for all test portions of one sample in one analysis shall be consistent. When at least one test portion gives a positive result and at least one gives a negative result, the PCR analysis shall be repeated.
- If the PCR results of the second analysis are not identical for all test portions, DNA extraction and PCR analysis shall be repeated.
- If at least two repetitions of the procedure (beginning with the DNA extraction) give ambiguous results, such as a positive and a negative result, the report shall state that the sample is negative at the limit of detection.
- The result shall provide the specificity (species or taxonomic group or groups) and target (nuclear, mitochondrial or other) of the method in order to allow an unambiguous interpretation and comparability of the results.

6.1. Expression of negative results

- Negative results shall be described to show no detection of target-derived DNA.
- EXAMPLE For target analyte X, animal species-derived DNA was not detected.

6.2. Expression of quantitative results

The results of quantitative methods shall state the unit of

	measurement. • The result shall provide the measurement uncertainty and also the calibrators and the calculation method used, where applicable. • The applicability of the measured result with regard to the mass/mass percentage of the target species in the sample shall be explained **7. Single-laboratory validation** An analysis method should have been sufficiently tested within a laboratory to disclose the required specification prior to the interlaboratory study, see ISO 13495. Reference materials or certified reference materials (CRMs) should be considered to be used for the validation of detection and quantification methods of nucleic acids. **7.1. Interlaboratory study (collaborative study)** • Information about the collaborative study (organizer, protocol, number of participating laboratories, etc.) and the performance data obtained by the study shall be reported with appropriate references to the relevant documents. Collaborative studies for the validation of PCR methods for detection, identification and quantification of specific DNA sequences can be performed according to other relevant documents (e.g., Codex Alimentarius CAC/GL 74- 2010). **Note:** Small-scale collaborative study (a pre-validation study involving, for example, two to four laboratories) can be performed to test the general transferability of the method before the expense of organizing a large-scale study is incurred. • For precise validation, data are collected from multiple laboratories having facilities and proficiency in molecular biology testing. In ISO 13495:2013, the required number of laboratories is eight and four for the international and national levels of validation, respectively. According to AOAC International (2002), the required number is eight laboratories. Statistical analysis is calculated based on ISO 5725-1:1994, 6.3. **7.1.1. Qualitative methods** A collaborative validation study of a qualitative PCR method shall be designed by considering the probability of detection (POD) (see ISO/TS 16393) within the range of the method. **Note:** Traditional nonparametric 5 % false positive and 5 % false negative rates reflect PODs of 5 % and 95 %. **7.1.2. Quantitative methods** The relative reproducibility standard deviation (RSDR) should be ≤ 25 % over the whole dynamic range of the method. Note: At levels of 0,1 % (copy/copy), an RSDR of 50 % can be acceptable
Calculation with units ofexpression	Results shall be described to show detection of target derived DNA. Negative results shall be described to show no detection of target derived DNA. Quantitative results shall be expressed in the ratio of target DNA copy numbers ortarget species mass fractions.
Inference	Qualitative analyses indicate the presence or absence (lack of detection) of a certaintarget.

	In quantitative analyses, the measured value is calculated as the ratio of DNA copy numbers. The use of this ratio should examine possible influences, including the number of DNA copies with regard to the target in the genome. Other units (e.g., the ratio of masses) can be employed. The principles of calculation of the ratio shall be reported. If a quantitative method is intended to judge the mass/mass ratio of different animal species ingredients in a sample, it should be indicated that the values measured for the DNA copy number ratio cannot reflect in all cases the mass/mass ratio of animal constituents in the sample.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for the Preparation of PCR-quality DNA using phenol/chloroform-based DNA extraction methods**		
Method No.	**FSSAI 05.022:2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document pertains to providing the phenol/chloroform-based method for DNA (PCR-quality) extraction from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.		
Caution	A fume hood is necessary for handling organic chemicals. Use gloves and make it a point to discard used gloves and wear a new pair frequently during experiments. Use of ungloved hands could easily result in nuclease contamination.		
Principle	The method consists of a lysis step (thermal lysis in presence of sodium dodecyl sulfate and a high EDTA content) followed by the removal of contaminants (such as lipophilic molecules, polysaccharides and proteins) and nucleases from the DNA-containing aqueous phase using phenol and chloroform. A final DNA precipitation with ethanol concentrates the DNA and eliminates salts and residual chloroform. The critical step of the method is the lysis step.		
Apparatus/Instruments	1. **Centrifuge**, capable of achieving a minimum acceleration of 10 000 *g*. 2. **Water bath or incubator**, working in a temperature range from 60 °C to 70 °C. 3. **Vacuum dryer** (optional) 4. **Freeze dryer** (optional) 5. **Mixer**, e.g., Vortex 6. **Reaction vessels**, resistant to freezing in liquid nitrogen		
Materials and Reagents	1. **Ethanol**, volume fraction (C2H5OH) = 96 % 2. **Glacial acetic acid** (CH3COOH) 3. **Potassium acetate** (C2H3O2K) 4. **Hydrochloric acid**, (HCl) = 37 % 5. **Isoamyl alcohol** [(CH3)2CHCH2CH2OH] 6. **Phenol** (C6H5OH) 7. **Chloroform** (CHCl3) 8. **Tris(hydroxymethyl)-aminomethane** (Tris) (C4H11NO3) 9. **Ethylenediaminetetraacetic acid dipotassium salt** (K2EDTA) 10. **Potassium hydroxide** (KOH) 11. **Potassium chloride** (KCl) 12. **Sodium dodecyl sulfate** (SDS) (C12H25O4SNa) 13. **Proteinase K**, approximately 20 Units/mg lyophilizate 14. **RNase-A**, DNase-free, from bovine pancreas, approximately 50 Kunitz Units/mg of lyophilizate 15. **Equilibrated phenol** 16. **Chloroform-isoamyl alcohol** 17. **Phenol-chloroform-isoamyl alcohol** 18. **Extraction/lysis buffer** 19. **TE buffer** 20. **Proteinase-K solution**		

	21. **RNase-A solution** 22. **Ethanol solution**, (C_2H_5OH) = 70 %. Store and use at −20 °C. 23. **Potassium acetate solution**
Preparation of Reagents	1. **Chloroform-isoamyl alcohol** Mix 24 volume parts of chloroform with 1 volume part of isoamyl alcohol. 2. **Equilibrated phenol**, pH > 7.8. Use phenol equilibrated against extraction buffer without SDS, or prepared according to standard protocol, or according to the manufacturer's recommendations. 3. **Phenol-chloroform-isoamyl alcohol** Mix 1 volume part of equilibrated phenol with 1 volume part of thechloroform-isoamyl alcohol solution. 4. **TE buffer**, *c*(Tris) = 0.010 mol/l, *c*(K2EDTA) = 0.001 mol/l. Adjust the pH to 8.0 with HCl or KOH. 5. **Proteinase-K solution**, ρ = 20 mg/ml, dissolved in sterile water. Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing. 6. **Potassium acetate solution**, c($C_2H_3O_2K$) = 3 mol/l. Adjust the pH to 5.2 with glacial acetic acid. Do not autoclave. If necessary,filter through a 0.22 µm filter. 7. **RNase-A solution**, ρ = 10 mg/ml lyophylizate. Store at −20 °C, but avoid repeated freezing and thawing **Extraction/lysis buffer**, substance concentration c(Tris) = 0.050 mol/l,c(K2EDTA) = 0.050 mol/l, mass concentration ρ(SDS) = 30 g/l.
Sample Preparation	Refer chapter 1.1 of this manual.
Method of analysis	**Step 1:** Weigh 0.25 g of the test sample into a microtube and add 1.6 ml of extraction buffer and, when necessary (e.g., in protein-rich matrices), 50 µL ofproteinase K solution **Step 2:** Incubate at 60 °C to 70 °C, usually for between 30 min to 2 h (overnightincubation is also possible) **Step 3:** Add RNase A up to a final concentration of 0.1 µg/ml and centrifuge at 5000 *g* for 30 min and recover the supernatant in a fresh tube and recover the supernatant in a fresh tube **Step 4:** Add 1 volume of equilibrated phenol to the supernatant, then mix gently and thoroughly and centrifuge at 5000 *g* for 15 min and recover the upper aqueousphase in a fresh tube. **Step 5:** Add 1 volume of phenol-chloroform isoamyl alcohol to the supernatant, andthen mix gently and thoroughly. **Step 6:** Centrifuge at 5000 *g* for 15 min and recover the aqueous phase in a freshtube. Repeat this step once or more times (depending on the matrix) until the interface between the phases are clean. **Step 7:** Add 1 volume of chloroform/isoamyl alcohol to the supernatant, then mixgently and thoroughly. **Step 8:** Centrifuge at 5000 *g* for 10 min and recover the upper aqueous phase in afresh tube. Repeat, if necessary, until the interface between the phases is clear. **Step 9:** Mix the supernatant with 0.1 volume of potassium acetate solution and 2.5 volumes of 96 % ethanol, then mix

	thoroughly by inversion. **Step 10:** Incubate for at least 5 min in liquid nitrogen, or 1hr at −80 °C, or overnightat −20 °C. **Step 11:** Centrifuge at 10000 *g* (or up to 13000 *g*) at 4 °C for at least 15 min, then carefully discard the supernatant. **Step 12:** Carefully wash the DNA pellet with 2 volumes of 70 % ethanol solution Centrifuge at 10000 *g* to 13000 *g* at 4 °C for 15 min, then discard carefully the supernatant. This step is essential for the removal of the precipitating salts that could interfere with the subsequent analysis (e.g., PCR). **Step 13:** Dry the pellet and re-dissolve it in 100 µL of water or appropriate buffer, e.g., TE buffer. This is the DNA master stock. Add RNase-A up to a final concentration of 0.1 µg/ml. **Note**: For some matrices, it is helpful to perform different enzymatic steps. Alpha- amylase is added to the lysis buffer to digest the starches in case of amylaceous matrices. Treatment of samples with proteinase-K is necessary in a variety of matrices to eliminate proteins. Also, treatment with RNase is usually recommended for those matrices where RNA co-precipitation may disturb the subsequent analytical test.
Calculation with units ofexpression	Calculation with units of expression is carried out according to purpose and choice of your interest using the methods for the quantitation of the extracted DNA- Refer methods FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.
Inference (Qualitative Analysis)	Refer method FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction 3. ISO 21568: 2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Sampling
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for the Preparation of PCR-quality DNA using the CTAB-based DNA extraction methods**		
Method No.	**FSSAI 05.023:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document pertains to providing the CTAB-based method for DNA extraction/purification from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.		
Caution	A fume hood is necessary for handling organic chemicals. Room temperature should not drop below 16°C (Maintained 16-25°C). Use gloves and make it a point to discard used gloves and wear a new pair frequently during experiments. Use of ungloved hands could easily result in nuclease contamination.		
Principle	The method consists of a lysis step (thermal lysis in the presence of CTAB), followed by several extraction steps in order to remove contaminants, such as polysaccharides and proteins. The salt concentration during the extraction steps is very important for the removal of the contaminants, since a CTAB-nucleic acid precipitate will occur if the salt concentration drops below approximately 0.5 mol/l at room temperature and/or if the temperature drops below 16 °C. By increasing the salt concentration (e.g., addition of sodium chloride), the removal of denaturated proteins and polysaccharides complexed to CTAB is achieved, while the nucleic acids are solubilized. Chloroform is used to further separate the nucleic acids from CTAB and polysaccharide/protein complexes.		
Apparatus/Instruments	1. **Oven or incubator,** preferably with a shaker. 2. **Centrifuge,** e.g., microcentrifuge, capable of achieving an acceleration of up to 12000 g. In some steps a refrigerated centrifuge is required 3. **Vacuum dryer** (optional). 4. **Mixer**, e.g., Vortex 5. **Reaction vessels**, resistant to freezing in liquid nitrogen		
Materials and Reagents	1. **α-Amylase** (optional), type IIa from *Bacillus* species, 1500 to 3000 units/mg of protein. 2. **Chloroform** ($CHCl_3$). 3. **Ethanol**, (C_2H_5OH) = 96 %. 4. **Ethylenediaminetetraacetic acid disodium salt** (Na2-EDTA) 5. **Hexadecyl-trimethyl-ammonium-bromide** (CTAB) ($C_{19}H_{42}BrN$). 6. **Hydrochloric acid**, (HCl) = 37 %. 7. **Isopropanol** [$CH_3CH(OH)CH_3$]. 8. **Proteinase-K** (optional), approximately 20 Units/mg of lyophilizate 9. **RNase A, DNase-free**, (optional) from bovine pancreas, approximately 50 Units/mg of lyophilizate. 10. **Sodium chloride** (NaCl). 11. **Sodium hydroxide** (NaOH). 12. **Tris(hydroxymethyl)-aminomethane** (Tris) ($C_4H_{11}NO_3$). 13. **α-Amylase solution** (optional) 14. **CTAB extraction buffer** 15. **CTAB-precipitation buffer** 16. **Sodium chloride solution** 17. **Ethanol solution**, $\varphi(C_2H_5OH)$ = 70 %		

	18. **Proteinase-K solution** (optional) 19. **RNase-A solution** (optional) 20. **TE buffer**
Preparation of Reagents	1. **CTAB extraction buffer**, ρ(CTAB) = 20 g/l, c(NaCl) = 1.4 mol/l, c(Tris) = 0.1mol/l, c(Na2EDTA) = 0.02 mol/l. Adjust the pH to 8.0 with HCl or NaOH 2. **CTAB-precipitation buffer**, ρ(CTAB) = 5g/l, *c*(NaCl) = 0.04 mol/l 3. **Sodium chloride solution**, *c*(NaCl) = 1.2 mol/l. 4. **Proteinase-K solution**, ρ= 20 mg/ml, dissolved in sterile water. Do notautoclave. Store at −20 °C, but avoid repeated freezing and thawing. 5. **RNase-A solution**, ρ (RNase A) = 10 mg/ml. Store in aliquots at −20 °C. 6. **TE buffer**, c(Tris) = 0.01 mol/l, c(Na2-EDTA) = 0.001 mol/l. Adjust the pH to 8.0with HCl or NaOH. 7. **α-Amylase solution** (optional), c(α-amylase) = 10 mg/ml. Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.
Sample Preparation	Refer chapter 1.1 of this manual.
Method of analysis	**Step 1:** Weigh 200 mg to 300 mg of the test sample into a tube and add 1.5 ml of pre-warmed (65 °C) CTAB extraction buffer and mix. (In some cases, a higher amount of buffer may be required to suspend the matrix.) **Step 2:** Add 10 µL of proteinase-K solution, smoothly mix the tubes and incubate for 30 min at 65 °C, under agitation (optional). **Step 3:** Centrifuge for 10 min at approximately 12 000 *g*. Transfer the supernatant to a new tube, add 0,7 to 1 volume of chloroform and mix thoroughly. Centrifuge for 15 min at approximately 12 000 *g*. Transfer the upper phase (aqueous) to a new tube. **CTAB-precipitation** **Step 4:** Add 2 volumes of the CTAB precipitation buffer. Incubate for 60 min atroom temperature without agitation. **Step 5:** Centrifuge for 15 min at 12000 *g*. Discard the supernatant. Dissolve the precipitated DNA by adding 350 µL of NaCl solution. **Step 6:** Add 350 µL of chloroform and mix thoroughly. Centrifuge for 10 min at 12000 *g*. Transfer the aqueous phase into a new tube. **NOTE**: CTAB-precipitation is not necessary for all matrices, only for protein- and polysaccharide-rich matrices. Alternatively, a solid-phase purification of the DNA (e.g., by the use of spin columns) is possible assuming the results are equivalent. **DNA precipitation** **Step 7:** Add 0.6 volume of isopropanol, mix smoothly by inverting the tube and keep the tube at room temperature for 20 min. Centrifuge for 15 min at 12000 *g*. **Step 8:** Discard the supernatant. Add 500 µL of ethanol solution to the tube and invert several times. This is the critical step ensuring complete removal of CTAB. **Step 9:** Centrifuge for 10 min at 12000 *g*. Discard the supernatant. Dry the DNApellet and redissolve it into 100 µL of water or an appropriate buffer, e.g., TE buffer. This is the DNA master stock. **Note**: For some matrices, it is helpful to perform different enzymatic steps. Alpha- amylase is added to the lysis buffer to digest the starches in case of amylaceous matrices. Treatment of samples with proteinase-K is necessary in a variety of matrices to eliminate

	proteins. Also, treatment with RNase is usually recommended for those matrices where RNA co-precipitation may disturb the subsequent analytical test.
Calculation with units ofexpression	Calculation with units of expression is carried out according to purpose and choice of your interest using the methods for the quantitation of the extracted DNA (Refer method FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements).
Inference (Qualitative Analysis)	Refer method FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction 3. ISO 21568: 2005, Foodstuffs — Methods of analysis for the detection of genetically 4. modified organisms and derived products — Sampling
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Method for the Preparation of PCR-quality DNA using VLB digestion and silica-based DNA extraction methods</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.024:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">The scope of this document pertains to providing the VLB digestion and silica-basedmethod for DNA (PCR-quality) extraction from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.</td></tr>
<tr><td>Caution</td><td colspan="3">A fume hood is necessary for handling organic chemicals. Room temperature should not drop below 16°C (Maintained 16-25°C). Use gloves and make it a point todiscard used gloves and wear a new pair frequently during experiments. Use of ungloved hands could easily result in nuclease contamination.</td></tr>
<tr><td>Principle</td><td colspan="3">The method consists of a lysis step using Vertebrate lysis (thermal lysis in the presence of SDS, EDTA and Proteinase K in a buffered solution) followed by a purification step by means of silica membrane, in the presence of high concentrations of chaotropic salt Guanidine thiocyanate (GuSCN). The principle of this method is the binding of high affinity of negatively charged nucleic acids to positively charged silica particles through hydrogen bonding. All the contaminants are removed by the subsequent washing with buffers. A final elution step with water permits DNA recovery.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">1. Refrigerated centrifuge capable of achieving a minimum 14000 rpm
2. Water bath or incubator, working in a temperature range from 60 °C to 70 °C.
3. Mixer, e.g., Vortex
4. Micro centrifuge tubes (2 ml & 1.5ml)
5. Spin columns
6. Homogenizer BEAD BUG-6 (Bead bug, Benchmark scientific, USA)</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">1. Zirconium beads
2. Proteinase K
3. Sodium chloride (NaCl)
4. Tris HCl
5. Ethylenediaminetetraacetic acid (EDTA)
6. SDS
7. Glycerol
8. Guanidine thiocyanate (GuSCN)
9. 4% Triton X-100
10. Milli Q water
11. Lysis buffer
12. Vertebrate Lysis Buffer (VLB)
13. Binding membrane buffer
14. Protein wash buffer
15. Wash buffer</td></tr>
<tr><td>Preparation of Reagents</td><td colspan="3">Vertebrate Lysis Buffer (VLB) (200 ml)<table><tr><td>100 mM NaCl</td><td>:</td><td>20 ml (1 M NaCl)</td></tr><tr><td>50 mM Tris HCl pH 8</td><td>:</td><td>10 ml (1 M Tris HCl pH 8)</td></tr><tr><td>10 mM EDTA pH 8</td><td>:</td><td>4 ml (0.5 M EDTA pH 8)</td></tr><tr><td>05% SDS</td><td>:</td><td>1 g</td></tr><tr><td></td><td></td><td>Make the volume to 200 ml with dd water</td></tr></table></td></tr>
</table>

Proteinase K

(20 mg/ml) in 10 mM Tris HCl, pH 7.4 and 50% glycerol v/w

Proteinase K	:	100 mg
Mili Q water	:	2 ml
1 M Tris HCl pH 7.4	:	0.05 ml (50 µl)
Milli Q water	:	0.5 ml
Glycerol	:	2.5 ml
		Mix well and store

Lysis mix

Vertebrate Lysis Buffer	:	5 ml
Proteinase K	:	0.5 ml

Binding buffer (300 ml)

6 M Guanidine thiocyanate (GuSCN)	:	21.276 g
20 mM EDTA pH 8	:	1.2 ml (0.5 M EDTA)
10 mM Tris HCl pH 6.4	:	3 ml (0.1 M Tris pH 6.4)
4% Triton X-100	:	1.2 ml
		Make up the volume to 300 mlHeat to dissolve at 56°C

Wash buffer

Ethanol (96%)	:	6 ml
50 mM NaCl	:	0.2375 ml (1 M NaCl 0.475)
10 mM Tris HCl pH 7.4	:	0.095 ml 1 M Tris HCl pH 7.4
0.5 mM EDTA pH 8	:	0.0095 ml (0.5 M EDTA pH 8)
		Make up to 9.5 ml with water

BM buffer (Binding Membrane buffer)

Binding buffer	:	15 ml
Ethanol	:	15ml
		Store in dark at room temperature (1 month)

PWB buffer (Protein wash buffer)

Binding buffer	:	13 ml
Ethanol	:	35 ml
Water	:	2 ml
		Store in dark for up to onemonth

EDTA (0.5M)

EDTA	:	18.614g
	:	Add of in 50ml Milli Q water
	:	Then dissolve it by adding 10M NaOH
	:	Adjust the pH to 8.0 by adding conc. HCl
		Make up the volume to 100ml and filter through filter paper

Sample Preparation	Refer chapter 1.1 of this manual
Method of analysis	**Step-1:** weigh 50 mg of meat sample in to Zirconium bead micro tubes and add 60 µL of lysis mix (Vertebrate lysis buffer (VLB) +

	Proteinase k) **Step-2:** Homogenize in Bead Bug homogenizer for 3 min and place the tubes inwater bath at 56°C for overnight **Step-3:** Centrifuge lysed sample at 5000 rpm for 3 min and take clear solution in tofresh tubes **Step-4:** Add 100 µL of Binding Membrane buffer to the supernatant, then mix gently by pipetting and the vortex mix **Step-5:** Transfer lysate to spin columns (about 150 µL) and Centrifuge at 7800 rpm for 5 min at 25°C (DNA binds to the membrane) **Step-6:** Place the spin columns in new 2 ml tube, add 180 µL of Protein Wash Buffer and centrifuge at 7800 rpm for 2 min at 25°C **Step-7:** Again, place spin columns in new 2 ml tubes, add 375 µL of Wash Buffer and centrifuge at 14000 rpm for 5 min at 25°C **Step-8:** Repeat the above step for one more time **Step-9:** Place the columns in heat block/water bath at 56°C for 30 min for evaporation of residual ethanol **Step-10:** Place the spin columns in to fresh 1.5 ml tubes and dispense the 50 µL of double distilled water (pre-warmed at 56°C) into the column membrane and incubate for 5 min. **Step-11:** Centrifuge at 7800 rpm for 5 minutes at 4°C **Step-12:** Place the eluted samples in heat block for 10 min at 60°C
Calculation with units ofexpression	Calculation with units of expression is carried out according to purpose and choice of your interest using the methods for the quantitation of the extracted DNA (Refer method FSSAI 05.026:2024., FSSAI 05.027:2024., FSSAI 05.028:2024., for specific requirements).
Inference (Qualitative Analysis)	Refer method FSSAI 05.026:2024., FSSAI 05.027:2024., FSSAI 05.028:2024., for specific requirements.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction 3. ISO 21568: 2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Sampling
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Method for the Preparation of PCR-quality DNA using Hexane-based DNA extraction method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.025:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">The scope of this document pertains to providing the Hexane based method for DNA (PCR-quality) extraction from fat rich products especially Ghee (clarified butter fat)/tallow (rendered ruminant body fat). It has been established for Ghee/tallow but could also be applicable to other fat rich products.</td></tr>
<tr><td>Caution</td><td colspan="3">A fume hood is necessary for handling organic chemicals. Room temperature should not drop below 16°C (Maintained 16-25°C). Use gloves and make it a point to discard used gloves and wear a new pair frequently during experiments. Use of ungloved hands could easily result in nuclease contamination.</td></tr>
<tr><td>Principle</td><td colspan="3">The method consists of a lysis step (thermal lysis in presence of sodium dodecyl sulfate and a high EDTA content) followed by removal of fats and oil using organic solvent like Hexane and DNA is precipitated using isopropanol.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">1. Centrifuge, capable of achieving a minimum acceleration of 14000 rpm.
2. Water bath or incubator, working in a temperature range from 60 °C to 70 °C.
3. 50 ml centrifuge tubes
4. 2 ml micro centrifuge tubes
5. Mixer, e.g., Vortex</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">1. Proteinase K
2. DNA extraction buffer
3. Hexane
4. Chloroform: Isoamyl alcohol (24:1)
5. Ice-cold Isopropanol
6. Ethanol (70%)
7. Nuclease free water</td></tr>
<tr><td>Preparation of Reagents</td><td colspan="3">DNA extraction buffer
<table><tr><td>Buffer volume</td><td>:</td><td>100ml</td></tr><tr><td>Tris 10mM pH 8.0</td><td>:</td><td>121mg</td></tr><tr><td>EDTA 0.1M</td><td>:</td><td>3.722g</td></tr><tr><td>SDS (0.5%)</td><td>:</td><td>0.5g</td></tr><tr><td></td><td></td><td>Make up the volume to 100ml and filterthrough filter paper</td></tr></table>
Proteinase K (20 mg/ ml) in 10 mM Tris HCl, pH 7.4 and 50% glycerol v/w
<table><tr><td>Proteinase K</td><td>:</td><td>100 mg</td></tr><tr><td>Mili Q water</td><td>:</td><td>2 ml</td></tr><tr><td>1 M Tris HCl pH 7.4</td><td>:</td><td>0.05 ml (50 µL)</td></tr><tr><td>Milli Q water</td><td>:</td><td>0.5 ml</td></tr><tr><td>Glycerol</td><td>:</td><td>2.5 ml</td></tr><tr><td></td><td></td><td>Mix well and store</td></tr></table>
Chloroform: Isoamyl alcohol (24:1)
1. Mix 24 volume parts of chloroform with 1 volume part of isoamyl alcohol.</td></tr>
</table>

Sample Preparation	Refer chapter 1.1 of this manual
Method of analysis	**Step 1:** Take 15 ml of sample (ghee/tallow) in a 50 ml centrifuge tube (Melt the solid samples) **Step 2:** Add 7.5 ml of DNA extraction buffer, 7.5 ml of hexane & 30 µL of proteinase K (20 mg/ml). Vortex and incubate at 65 °C for 20 min. **Step 3:** Centrifuge at 13000 rpm for 15 min. Discard the supernatant. **Step 4:** Collect the aqueous layer along with precipitate (approximately 6 – 7 ml) into a fresh centrifuge tube & add 10 ml of ice-cold chloroform: isoamyl alcohol (24: 1). Vortex 15s and centrifuge at 13000 rpm for 15 min. **Step 5:** Transfer aqueous phase quantitatively and add 0.8 volume of ice-cold isopropanol. Mix gently by inverting the tubes and keep at – 20 °C overnight. **Step 6:** Centrifuge at 13000 rpm for 30 min. **Step 7:** Collect the precipitate & wash the pellet with 70% ethanol. Centrifuge at 13000 rpm for 10 min. **Step 8:** Collect the pellet and dry at 37 °C. Dissolve in 100 µL of nuclease-free water and keep it in heating block for 10 min at 60 °C.
Calculation with units of expression	Calculation with units of expression is carried out according to purpose and choice of your interest using the methods for the quantitation of the extracted DNA(Refer method FSSAI 05.026:2024., FSSAI 05.027:2024., FSSAI 05.028:2024., for specific requirements).
Inference (Qualitative Analysis)	Refer method FSSAI 05.026:2024, FSSAI 05.027:2024, FSSAI 05.028:2024, for specific requirements.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for theidentification and the Detection of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction 3. ISO 21568: 2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Sampling
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for the quantitation of extracted DNA: Basic ultraviolet spectrometric method**		
Method No.	**FSSAI 05.026:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document pertains to providing the basic ultraviolet spectrometric method for the quantitation of the DNA extracted from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.		
Caution	Refer chapter 1.2		
Principle	Nucleic acids in solution absorb ultraviolet (UV) light in the range from 210 nm to 300 nm with an absorption maximum at 260 nm. Since DNA, RNA and nucleotides have their absorption maximum at 260 nm, RNA and nucleotide contamination of DNA solutions cannot be determined by UV spectrometry. For this reason, RNA must be removed enzymatically during DNA extraction before DNA determination. Also, oligonucleotides and nucleotides derived from RNA hydrolysis should be eliminated (e.g., by silica treatment, as outlined in FSSAI 05.024:2024). Oligonucleotides and nucleotides generated by RNase treatment, if not removed (e.g., by silica treatment) can lead to an overestimation of the DNA content of the sample. Moreover, double-stranded DNA absorbs less UV light compared to single- stranded DNA. Since the proportion of single-stranded DNA in the solution is unknown, to avoid overestimation of the DNA content, all the DNA in the test sample is converted to its single-stranded form by using the denaturing agent sodium hydroxide. Since nucleic acids do not absorb at 320 nm, reading at 320 nm is informative for the determination of background absorption due to light scattering and UV-active compounds. **Note 1**: The production of a calibration curve is not necessary, provided that an appropriate molar extinction coefficient is chosen as a function of the type of nucleic acid under study and/or its integrity. However, the calibration of the spectrometer should be verified periodically by measuring the concentration of reference DNA solutions. **Note 2**: The method is applicable to DNA concentrations in the range from 2 µg/ml to 50 µg/ml. Before quantitation, suitable dilutions of the extracted DNA to be quantified should be made, in order to be in the linear range of the spectrometric measurement (optical density between 0.05 to 1). Occasionally, residual compounds (e.g. CTAB from the DNA extraction procedure) may interfere with the UV spectrometric detection at 260 nm, because they absorb at this wavelength.		
Apparatus/Instruments	1. **UV-spectrometer**, single-beam, double-beam or photodiode array instrumentsare suitable. 2. **Mixer/shaker**, e.g., Vortex 3. **Measurement vessels**, for example quartz cells/cuvettes or plastic cells/cuvettes suitable for UV detection at a wavelength of 260 nm. The size of the measurement vessels used determines the volume for measurement: half-micro cells (1000 µL), micro cells (400 µL), ultra-micro cells (100 µL) and quartz capillaries (3 µL to 5 µL). The optical path of standard cell is usually 1 cm.		

Materials and Reagents	1. **Tris(hydroxymethyl)-aminomethane** (Tris) (C4H11NO3). 2. **Sodium hydroxide** (NaOH). 3. **Hydrochloric acid**, φ(HCl) = 37 %. 4. **Carrier DNA**, e.g., Herring Sperm DNA11), or Calf Thymus DNA11). 5. **DNA reference solution** 6. **Sodium hydroxide solution**, *c*(NaOH) = 2 mol/l. 7. **Dilution buffer**, *c*(Tris) = 0,01 mol/l.
Preparation of Reagents	1. **DNA reference solution** Prepare a DNA stock solution with 10 mg/ml by dissolving 100 mg carrier DNA in 10 ml of dilution buffer. DNA dissolves at these concentrations only slowly and the resulting solution is very viscous. Afterwards dilute this prepared stock reference DNA-solution further with dilution buffer up to the desired working concentration (e.g., 25 µg/ml). 2. **Dilution buffer**, *c*(Tris) = 0.01 mol/l. Adjust the pH to 9.0 with HCl.
Sample Preparation	Mix the DNA reference solution with the dilution buffer. Use Nuclease-free water or 1X TE buffer as a solvent to suspend the nucleic acids, and place each sample in a quartz cuvette. Zero the spectrophotometer with a sample of solvent (Blank).
Method of analysis	1. The correct calibration of the spectrometer can be verified by the use of areference DNA solution, as follows: • for blank measurement only dilution buffer is used to fill the measurementvessel; • the measurement vessel is filled with the reference DNA solution. 2. Absorption is measured for both the blank and reference DNA solutions at wavelengths of 260 nm and 320 nm. 3. For the blank solution, mix dilution buffer plus sodium hydroxide solution, so that a final NaOH substance concentration of 0.2 mol/l is reached. This mixture is used to fill the measurement vessel. 4. Mix the test DNA solution with sodium hydroxide solution and, if needed, with dilution buffer, to obtain a final NaOH substance concentration of 0.2 mol/l. This mix is used to fill the measurement vessel. 5. Measure the absorption after 1 min incubation time for both the blank and reference DNA solutions at wavelengths of 260 nm and 320 nm. The reading is stable for at least 1 h. **EXAMPLE 1** For the blank solution, mix 90 µL of dilution buffer and 10 µL of sodium hydroxide solution and transfer to a 100 µL measurement vessel. **EXAMPLE 2** For the test DNA solution, mix 80 µL of dilution buffer or water, 10 µL of sodium hydroxide solution and 10 µL of a DNA solution of unknown concentration and transfer to a 100 µL measurement vessel.
Calculation with units of expression	➢ The absorption (OD) at 320 nm (background) is subtracted from the absorption at 260 nm, resulting in the corrected absorption at 260 nm. ➢ If the corrected OD at 260 nm equals 1, then the estimated DNA concentration is 50 µg/ml for double stranded DNA, or 37 µg/ml for single-stranded DNA (i.e., denatured with sodium hydroxide),

	respectively. ➢ Reliable measurements require OD values at a wavelength of 260 nm to begreater than 0.05. ➢ Finally, calculate the mass concentration, ρ, of the double-stranded test DNA solution, taking into consideration the denaturation and the dilution factor applied according to Equation (1): ρ DNA = F × (OD260 −OD320) × 37(1) Where, F is the dilution factor; OD260 is the absorbance at 260 nm;OD320 is the absorbance at 320 nm; 37 is the conversion factor, in micrograms per millilitre. **EXAMPLE** For a calculation with a dilution factor of 10 and an OD260 of 0.658 andan OD320 of 0.040: ρDNA = 10 × (0.658 – 0.040) × 37 µg/ml = 229 µg/ml.
Inference (Qualitative Analysis)	• Pure DNA has an A260/A280 ratio of approximately 1.8. If the ratio is less than the mentioned value, it is due to the contamination of proteins/phenol. • Strong absorbance at A280 resulting in a low A260/A280 ratio indicatesthe presence of contaminants, such as proteins. • Strong absorbance at 270 nm and 275 nm may indicate the presence of contaminating phenol.
Reference	ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
Approved by	Scientific Panel on Methods of Sampling and Analysis

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for the quantitation of extracted DNA: Agarose gel electrophoresis and ethidium bromide staining method**		
Method No.	**FSSAI 05.027:2024**	**Revision No. & Date**	0.0
Scope	The scope of this document pertains to providing the Agarose gel electrophoresis and Ethidium Bromide staining method for the quantitation of the DNA extracted from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.		
Caution	Refer chapter 1.2		
Principle	DNA separates electrophoretically, on the basis of its charge and molecular mass, when loaded onto a molecular sieve (agarose gel) and subjected to an electric field in the presence of a buffer solution. Ethidium bromide (EtBr) intercalates into the DNA and, when excited by ultraviolet light, emits orange fluorescence. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescence produced by the unknown sample with that of a series of quantity standards. The molecular mass of such standards must be similar to that of the DNA under quantitation, because EtBr intercalation, and thus fluorescence emission, also depends on the length of the DNA fragments. EtBr also stains single-stranded DNA and RNA. For a more precise estimation of the DNA content, RNA must be removed enzymatically.		
Apparatus/Instruments	**1. Microwave oven or boiling water bath** **2. Equipment for agarose gel electrophoresis**, with accessories and power supply. **3. Ultraviolet (UV) trans-illuminator or lamp**, preferably with wavelength of 312 nm. Alternatively, equipment for column chromatography of nucleic acids and the according detection system or other similar suitable systems may be used. **4. Recording instrument**, for example a photo documentation system with 3000 ASA films and UV filter adequate for EtBr-emitted fluorescence. As an alternative, a video-documentation system with a CCD camera, adequate UV filter and (optional) quantitative analysis software may be used.		
Materials and Reagents	**1.Ethidium bromide** (EtBr) ($C_{21}H_{20}N_3Br$). **2.Glycerol** ($C_3H_8O_3$). **3.Sodium acetate** ($C_2H_3O_2Na$), for the TAE buffer system only. **4.Hydrochloric acid**, φ(HCl) = 37 %. **5.Sodium hydroxide** (NaOH). **6.Tris(hydroxymethyl)-aminomethane** (Tris) ($C_4H_{11}NO_3$). **7.TAE buffer solution (1x)** **8.Tris/borate (TBE) buffer solution (0.5x)** **9.Sample loading buffer solution (5x)** **10. Ethidium bromide solution**		
Preparation of Reagents	**1.TAE buffer solution (1x)** *c*(Tris) = 0.050 mol/l, *c*($C_2H_3O_2Na$) = 20 mmol/l, *c*(Na2-EDTA) = 0.001 mol/l. Adjust the pH to 8.0 with glacial acetic acid or NaOH. It is advisable to prepare the TAE buffer solution as a concentrated stock solution (maximum 50-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono) - distilled or deionizedwater.		

	2. **Tris/borate (TBE) buffer solution (0.5x)**, *c*(Tris) = 0.055 mol/l, *c*(boric acid) = 0.055 mol/l, *c*(Na2EDTA) = 0.001 mol/l. Adjust the pH to 8.0 with HCl or NaOH.It is advisable to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if precipitation is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water. 3. **Sample loading buffer solution (5x)**, φ(glycerol) = 50 %, ρ (bromophenol blue) = 2.5 g/l and/or ρ (xylene cyanol) = 2.5 g/l, dissolved in electrophoresis buffer solution (**TAE buffer solution (1x)** or **Tris/borate (TBE) buffer solution (0.5x)**). 4. **Ethidium bromide solution, c(EtBr) = 0.5 mg/l.** It is advisable to store the ethidium bromide solution as a concentrate (e.g., 10mg/ml) at 5° C in the dark (EtBr is light-sensitive). It is also advisable to avoid weighing EtBr. The stock solution should be prepared by dissolving an appropriate amount of water in the vessel already containing the EtBr powder, or alternatively, by employing pre- weighed EtBr tablets. Solubilization of EtBr should be carried out protected from light, under agitation at room temperature. This usually takes approximately 1 h.
Sample Preparation	Use Nuclease-free water or 1X TE buffer as a solvent to suspend the nucleic acids. Mix the sample DNA solutions with the loading buffer, and mix and apply the mixture to the sample slots (wells) with a micropipette.
Method of analysis	**Agarose gel preparation** ▪ Gels should not be thicker than 1 cm. ▪ The agarose concentration and quality determine the resolution capacity of the gel. For high molecular mass DNA quantitation, agarose concentrations between 8 g/l and 10 g/l are used. For low molecular mass DNA (e.g., degraded or restricted) higher agarose concentrations are used (up to 40 g/l). ▪ Weigh an appropriate amount of agarose and add it to the electrophoresis buffer solution. Allow the solution to boil in a microwave oven or in a water bath, until the agarose is completely dissolved. ▪ Replace the volume lost by evaporation with an equivalent amount of water, mix by swirling (avoid air bubbles trapping), cool down the solution to about 60° C and keep it at this temperature until usage. ▪ Prepare a gel support (gel tray) with a suitable sample comb placed in right position. Pour the agarose solution onto the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended). **DNA sample preparation** ▪ Mix the sample DNA solutions (e.g., 5 µL to 10 µL) with approximately 20 % (with respect to the final sample volume) of loading buffer (e.g., add 2.5 µL of loading buffer to 10 µL of DNA sample), mix and apply the mixture to the sample slots (wells) with a micropipette. ▪ If the unknown samples are suspected to be too concentrated, also provide some dilutions of them to be loaded onto the gel. To determine the size of the extracted DNA fragments, add the sample loading buffer in the proportion of 20 % with respect to the sample volume) to a suitable amount of the DNA molecular mass standard

	and carry out electrophoresis in parallel. ▪ To estimate the concentration of the unknown sample, run standard DNA quantity samples in parallel. Such samples contain known amounts (within the dynamic range of the method, i.e., 5 ng to 500 ng) of the DNA quantity standard diluted in water or in electrophoresis buffer. It is recommended to use quantitation standards containing at least 5 calibration points (i.e., different amounts of DNA). **Submarine electrophoresis** ▪ Carefully remove the samples comb from the gel. Transfer the gel (with its gel tray) to the electrophoresis cell, so that the wells reside closer to the cathode (negative electrode). ▪ Fill the cell with the electrophoresis buffer. Overlay the gel with approximately 2 mm of the same buffer and load the samples using a micropipette. ▪ Carry out the electrophoresis at room temperature at the appropriate voltage and power intensity (generally a maximum constant voltage of 5 V/cm, with respect to the distance between the electrodes, is recommended). ▪ Under the described conditions, DNA is negatively charged, so it migrates from the cathode to the anode. The electrophoresis time depends on the migration distance required, on the current generated by the power supply, the buffer used, the electro-endosmosis and the concentration of the agarose in the gel. **Staining** ▪ After completing the electrophoresis, incubate the gel for 15 min to 50 min in the ethidium bromide solution at room temperature, possibly in the dark (and/or in a stainless-steel tank with a cover) with gentle shaking. ▪ If necessary, reduce the background staining by de-staining the gel in water for 10 min to 30 min. As an alternative to post-electrophoresis staining, EtBr can be added to the gel before pouring it. ▪ In this case, EtBr is added to the gel to a final concentration of 0.01 mg per milliliter of gel when the gel has been cooled to a temperature of 60 °C. If the gel is cast with ethidium bromide, load the unknown sample and the DNA quantity standard into separate slots produced with the same comb on the same gel. ▪ Otherwise, the quantity of ethidium bromide will be different for the two, so yielding erroneous quantitation results. To minimize the problems of EtBr movement in the gel, some EtBr can also be added to the electrophoresis (tank) buffer. After the gel electrophoresis, no de-staining step is usually required. **Gel recording** Transfer the gel to the trans-illuminator surface, switch on the UV light and record the DNA fluorescence by photography or video documentation.
Calculation with units of expression	The DNA content of the sample is estimated by comparing the unknown samples with the DNA quantity standard samples that underwent electrophoresis in parallel. This evaluation can be carried out visually or, better, with the aid of quantitation software able to calculate an adequate calibration curve.

Inference (Qualitative Analysis)	Compare the obtained band intensity of the fragment to the DNA ladder with known concentrations, which is closest in size to your piece of DNA.
Reference	ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
Approved by	Scientific Panel on Methods of Sampling and Analysis

<table>
<tr><td>एफएसएसएआई
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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Method for the quantitation of extracted DNA: Real-time PCR method for the quantitation of the extracted DNA</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.028:2024</td><td>Revision No. & Date</td><td>0</td></tr>
<tr><td>Scope</td><td colspan="3">The scope of this document pertains to providing the Real-Time PCR method for the quantitation of the DNA extracted from meat & meat products. It has been established for meat & meat products but could also be applicable to other food matrices and feed.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.2</td></tr>
<tr><td>Principle</td><td colspan="3">- Quantitation of the extracted DNA using a Real-time PCR assay specific for mammals and poultry (e.g., myostatin gene);
- Amplification of the animal-specific DNA sequence of the myostatin gene (MSTN) in a real-time PCR., which is present as a single copy diploid nuclear gene and the length of the target sequence is 87 bp.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer chapter 1.4 of this manual), the following equipment is required;
Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides
<table>
<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Myostatin gene as the target sequence</td></tr>
<tr><td>Myostatin gene - F</td><td>5’-GTGCAAATCCTGAGACTCAT -3’</td><td>600 nmol/l</td></tr>
<tr><td>Myostatin gene - R</td><td>5’-ATACCAGTGCCTGGGTTCAT -3’</td><td>600 nmol/l</td></tr>
<tr><td>Myostatin gene - P</td><td>5’-[FAM]-CCATGAAAGACGGTACAAGGTATACTG-BHQ1 -3'</td><td>250 nmol/l</td></tr>
</table>
B. PCR master mix
In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td colspan="3">For DNA quantitation using a real-time method only chemicals/consumable of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder-free gloves. The use of aerosol-protected pipette tips (protection against cross-contamination) is recommended.</td></tr>
<tr><td>Sample Preparation</td><td colspan="3">For preparation of the test portion/sample, follow general requirements and specific methods described in chapter 1.1 of this manual.
Use Nuclease-free water or 1X TE buffer as a solvent to suspend the nucleic acids.</td></tr>
<tr><td>Method of analysis</td><td colspan="3">Reaction mixes
The method is for a total volume of 10 µL per PCR and the reaction setup is given in Table 2.</td></tr>
</table>

Table 2 — Reaction setup for the amplification

Component	Volume (µL)
2 X Probe PCR Master Mix	5
Forward Primer	0.3
Reverse Primer	0.3
Probe	0.2
Sample DNA or controls	1 µL
Water	to 10 µL

The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow ISO 20813:2019.
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 9 µl into each reaction vial;
- Add 1 µl of each sample DNA or positive DNA target control or blank/other controls; mix and centrifuge briefly.

Temperature-time programme

The temperature-time programme as outlined in Table 3 was used in the validation study. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.

Table 3 — Temperature-time programme

Protocol	**Conditions**	**Fluorescence measurement**
Initial denaturation	95 °C 3 min	No
Denaturation	95 °C 15 S	No
Annealing/extension	60 °C 60 Sec	Yes
GOTO 35X cycles		

Calculation with units of expression

General

Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (*C*t) or cycle quantification (*C*q). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as "undetermined", "no amplification" or the maximum number of reaction cycles performed.

Inference (Qualitative Analysis)	- The quantification of DNA by qPCR relies on the detection of amplified product ("amplicon") at each cycle of the PCR. - PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification; - compare the Cq value of your sample against the standard curve (a set of samples with a known DNA quantity) - Amplification at lower Cq values determines the high concentration of the DNA, where higher Cq values determines the lower concentration of the DNA.
Reference	1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions 2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction 3. ISO 20224 (Series):2020 Molecular biomarker analysis — Detection of animal- derived materials in foodstuffs and feedstuffs by real-time PCR
Approved by	Scientific Panel on Methods of Sampling and Analysis

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Method No.	**FSSAI 05.029.2024**	**Revision No. & Date**	**0.0**
Scope	The scope of this document is to provide a procedure for the identification of single mammalian and poultry meat species to the level of genus or species using DNA barcoding. The identification of meat species is carried out by PCR amplification of either a segment of the mitochondrial cytochrome b gene (cytb, Syn MCB) or the cytochrome c oxidase I gene (cox1, syn COI) or both, followed by sequencing of the PCR products and subsequent sequence comparison with entries in databases. The methodology allows the identification of a large number of commercially important mammalian and poultry meat species. This method has been successfully validated on game meat species as well; however, laboratory experience is available that it can also be applied tocommercial meat species as well as processed samples (e.g., comminuted, emulsified, cooked, fried, deep-fried, cold-smoked, hot-smoked, salted and frozen).		
Caution	This document is usually unsuitable for the analysis of highly processed foods, e.g., canned luncheon meat, with highly degraded DNA where the fragment lengths are not sufficient for the amplification of the targets. Furthermore, it is not applicable for complex meat products containing mixtures of two or more mammalian and poultry meat species. During the analysis, unless otherwise stated, use only reagents of recognized molecular biology grade and distilled or demineralized water or water of equivalentpurity, according to ISO 20813. Regarding laboratory organization, see ISO 20813.		
Principle	DNA is extracted from meat and meat products by applying a suitable method. Segments of approximately 472 base pairs of the MCB gene and/or approximately 658 base pairs of the COI gene are amplified by PCR. In the further course, the nucleotide sequence of the PCR product is determined by a suitable DNAsequencing method (e.g., Sanger sequencing). The sequence is evaluated by comparison to sequence entries in databases, thus allowing the assignment to a meat species or genus according to the degree of identity with stored sequences.		
Apparatus/Instruments	Apart from the usual laboratory equipment, the following equipment is required: 1. **UV-spectrophotometer or fluorometer, to determine the concentration ofDNA** 2. **Thermocycler** 3. **Gel electrophoresis device** 4. **Gel documentation system** 5. **DNA sequencer**		
Materials and Reagents	**PCR reagents** 1. Thermostable DNA polymerase (for hot start PCR) 2. PCR reaction buffer (including MgCl2 or with separate MgCl2 solution) 3. Deoxy nucleoside triphosphate mix (dATP, dCTP, dGTP and dTTP)		

4. Oligonucleotides (see Tables 1 and 2)
5. Agarose
6. Suitable DNA length standard for assessing the amplification product length
7. Sequencing primers (see Table 3)
8. 70% ethanol
9. Sodium acetate (NaOAc) (pH 4.6)
10. Formamide
11. EDTA - 125mM

Oligonucleotides

Table 1 — Oligonucleotides for amplification of the MCB gene region

Name	DNA Sequence of oligonucleotide
mcb 398	5'-TACCATGAGGACAAATATCATTCTG-3'
mcb 869	5'- CCTCCTAGTTTGTTAGGGATTGATCG -3'

Table 2 — Oligonucleotides for amplification of the COI gene region

Name	DNA Sequence of oligonucleotide
LepF1_t1	5'TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG-3'
VF1_t1	5'-TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG-3'
VF1d_t1	5'-TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG-3'
VF1i_t1	5'-TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG-3'
LepR1_t1	5'-CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA-3'
VR1d_t1	5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA-3'
VR1_t1	5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA-3'
VR1i_t1	5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA-3'
Note: Forward and Reverse primer ratio in the cocktail is 1:1, except VF1i_t1 & VR1i_t1 ratio is 3:3 (F denotes the forward and R denotes the reverse primer).	

Sequencing primers

For PCR products from MCB gene amplification, use the forward and reverseprimers individually for bidirectional Sanger sequencing. For PCR products from COI gene amplification; use the primers mentioned in below

Table 3 — Sequencing primers for COI PCR products

Name	DNA Sequence of oligonucleotide
M13F (−21)	5'-TGT AAA ACG ACG GCC AGT-3'
M13R (−27)	5'-CAG GAA ACA GCT ATG AC-3'

<table>
<tr><td>Preparation of Reagents</td><td>During the analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving and distilled or demineralized water or water of equivalent purity, according to ISO 20813. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) is recommended. Regarding laboratory organization, see ISO 20813.</td></tr>
<tr><td>Sample Preparation</td><td>It should be ensured that the test portion used for DNA extraction is representative for the laboratory sample. In composed samples (e.g., ready-to-use meals), single pure meat pieces have to be separated and analyzed. With the analysis of samples composed of several pieces (e.g., bags with different pieces), test portions for every putative meat species are taken and analyzed separately. To minimize the risk of amplifying adhering contaminants, test sample material shall not be taken from the surface of the laboratory sample. For further information regarding sample preparation, see chapter 1.1</td></tr>
<tr><td>Method of analysis</td><td>
1. PCR Setup
The method was validated for a total volume of 25 µL (for both cox1 & cytb) per PCR. The reagents given in Table 4 and Table 5 should be used for the cytb and cox1PCR, respectively.
Reagents are completely thawed at room temperature and should be centrifuged briefly before usage. A PCR reagent mixture is prepared containing all PCR components in the given concentrations except for the DNA extract. The amount of PCR mixture depends on the total volume per PCR and the total number of the reactions including a sufficient pipetting reserve.
Positive PCR results are expected when using a DNA concentration of approximately 1 ng/µL reaction solution. If it is necessary to improve the PCR result, the inserted DNA quantity may be increased (e.g., to increase the yield of PCR product) or decreased (e.g., to avoid PCR inhibition).

Table 4 — Components for the MCB PCR

<table>
<tr><td>Reagent</td><td>Final concentration in the reaction solution</td></tr>
<tr><td>PCR buffer</td><td>10x - 2.5 µL</td></tr>
<tr><td>MgCl2[a]</td><td>25 mM – 1.25 µL</td></tr>
<tr><td>dNTP mix [a]</td><td>10 mM – 0.5 µL</td></tr>
<tr><td>mcb 398</td><td>10pm/µL - 1 µL</td></tr>
<tr><td>mcb 869</td><td>10pm/µL - 1 µL</td></tr>
<tr><td>Platinum Taq DNA Polymerase</td><td>0.75 Units – 0.25 µL (3units/ µL)</td></tr>
<tr><td>Water</td><td>Add to obtain the final volume</td></tr>
<tr><td>Sample DNA</td><td>About 5 µL</td></tr>
<tr><td colspan="2">[a] Use reagent only if not already included in the PCR buffer</td></tr>
</table>
</td></tr>
</table>

Table 5 — Components for the CO1 PCR

Reagent (stock solution)	Final concentration in the reaction solutionHSP buffer 10x – 2.5 µL
MgCl2	50mM – 1.25 µL
dNTP mix	10mM – 0.5
Cocktail forward Primer	0.25 µL
Cocktail reverse Primer	0.25 µL
Hot-start DNA Polymerase	0.6 units - 0.12 µL (5 Units/ µL)
Water	Add to obtain final volume
Sample DNA	5 µL

Positive PCR results are expected when using a DNA concentration of approximately 1 ng/µL reaction solution. If it is necessary to improve the PCR result, the inserted DNA quantity may be increased (e.g., to increase the yield of PCR product) or decreased (e.g., to avoid PCR inhibition).

1.1. Temperature-time program

The temperature-time programs as outlined in Table 6, have been successfully used. The use of different reagent conditions and PCR cyclers can require specific optimization. The time for initial denaturation depends on the hot-start polymerase used.

Table 6 — Temperature-time program for the MCB or COI PCR

After the PCR is finished, store samples in the refrigerator until further analysis.

Step	Parameter	Temperature	Time	Cycles
1	Initial denaturation/ activation of the hot-start polymerase	95 °C	5 min	1
2	Denaturation	94 °C	30s or 45s	45
3	Annealing	55.4 °C	30s or 45s	
4	Elongation	72 °C	1 min	
5	Final elongation	72 °C	10 min	1
6	Holding step	4 °C	∞	1

1.2. PCR controls

In addition to the reaction setups for the samples to be analyzed, an amplification reagent control and an extraction blank control (see EN ISO 24276) have to be included.

A positive DNA target control (see EN ISO 24276) can be helpful to demonstrate the ability of the PCR to amplify the target sequence. As positive control material, genomic DNA extracted from a known meat species or an available plasmid containing the target sequence can be used. If a sample shows no amplification in both targets, it may be helpful to exclude an inhibition of the PCR by performing an inhibition control

reaction (see EN ISO 24276). This can be done either by dilution of sample DNA or by using an internal inhibition control assay.

Regarding the PCR controls, also see ISO 20813

2. Evaluation

2.1. Evaluation of PCR products

The PCR product can be assessed, and quality and quantity can be estimated, e.g., byagarose gel electrophoresis.

A volume of 4 µl to 10 µl of each PCR product is separated in, for example, an agarose gel of suitable concentration [e.g., 1 % to 2 % (w/v)] and evaluated with a gel documentation system. In one lane, an appropriate DNA size standard is included for comparison. For the cytb PCR, a product of approximately 460 bp PCR and for the coxI PCR, a product of approximately 650 bp should be clearly visible after gel electrophoresis.

No amplicons should be visible for the amplification reagent control and the extraction blank control. For the positive DNA target control, PCR products of the expected size should be visible.

2.2. Evaluation of the PCR results

The cytb PCR and/or the cox1 PCR can show a positive or a negative result for the amplification of the target sequence(s).

Depending on the outcome of the PCR, the next step is to consider the following:

- If the sample is positive for one or both targets (cytb and/or cox1), sequencing of one or both PCR products should be performed as the next step.
- If the sample is negative for both targets, it is necessary to carry out an inhibition control (see EN ISO 24276). If no inhibition is exhibited, it may be possible that:
- Both PCR systems do not match sufficiently to the target sequence of the DNA extracted from the meat species under analysis. In this case, species identification of the sample is not possible with this method and analyses with further universal primer pairs (i.e., 16S rRNA primers) may follow the tests; or
- The DNA extracted was degraded or not of sufficient quantity for PCR.

2.3 Sequencing of PCR products

Sequencing of PCR products is carried out according to the method available for thetesting laboratory.

2.1.1. Primary Purification:

PCR products obtained from a sample show a single band in the gel, the (remaining) PCR reaction mixture can directly be purified using a suitable commercial kit and then go for the cyclic sequencing.

2.1.2. Cyclic Sequencing:

Components for the cyclic sequencing of the PCR are provided in the Table 7.

Table 7 — Components for the cyclic sequencing PCR

Reagent	volume
Master mix	0.5 µL
Sequencing buffer	1.75 µL
Forward Primer	1 µL

Reverse Primer	1 µL
DNA template	1 µL
Sterile Nuclease free water	Add to obtain the final volume of 10 µL
Note: For sequencing of the MCB PCR products, the primers used for the generation of the amplicons serve as sequencing primers. Concerning the COI PCR products, the sequencing primers bind only to the M13 tail and therefore differ from those used in the PCR amplification (Table 5).	

The details of the PCR conditions for the cyclic sequencing are provided in Table 8.

Table 8 — Temperature-time program for the Cyclic sequencing PCRconditions

Stage	Step	Temperature	Time	Cycles
1.	Initial denaturation	96 °C	1 min	1
2.	Denaturation	96 °C	0.10 min	25
3.	Annealing	Based on primer	0.10 min	
4.	Extension	60 °C	0.10 min	
5.	Final Extension	60 °C	0.40 min	1
6.	Hold	4 °C	∞	1

Then go for the secondary purification of the obtained product

2.1.3.Secondary Purification:

1. Transfer the reaction product into a 1.5ml tube
2. Make a master mix I of 10 µL Milli-Q water and 2 µL of 125Mm EDTA per reaction
3. Make master mix II of 2 µL of 3M NaOAc (pH 4.6) and 50 µL of ethanol per reaction.
4. To 1.5ml centrifuge tube, add 12 µL of master mix I and then add 10 µL of PCR product
5. Add 52 µL of master Mix II to the same tube and incubate for 15 min at room temperature (25 °C)
6. Spin it at 12000x g for 20 min and discard the supernatant
7. Add 250 µL of 70% ethanol and spin at 12000x g for 10 min at room temperature
8. Discard the supernatant and kept it for air dry, then add 12-15 µL of formamide. Mix it, then spin down and transfer to wells of the PCR plate and cover with septa.
9. Denature at 95 °C for 4 min and snap chill at -20°C. Then proceed to capillary electrophoresis

   The DNA fragments from the sequencing reaction are subsequently separated by means of a DNA sequencer, e.g., using capillary electrophoresis. Fluorescence signals are recorded and analyzed with the device software.

2.4. Evaluation of sequence data

- The sequence trace data (or chromatogram) shall be checked visually to ensure the sequence reaction has worked sufficiently, and base calling is correct. Based on experience, the length of the determined sequence should be in general, approximately 80 % of the expected read length.
- In case of misassigned nucleotides to chromatogram peaks, sequences have to be edited using appropriate software and evaluating the fluorescent peak data.
- A sequence analysis should be preferably performed of both DNA strands. These complementary/overlapping sequences should be combined into a consensus sequence. This serves as an important way of checking the accuracy of the sequence and can help remove any ambiguous bases.
- The sequences of the primers are excluded from the determined sequences before the comparison to database sequences.

2.5. Comparison of the sequence with public databases

2.5.1. General

- Cytb and/or cox1 DNA sequences are evaluated with regard to the taxon by comparison to sequence entries in the nucleotide collection (nr/nt) by BLAST. For cox1 DNA sequences, the cox1 sequence database of the Barcode of Life (BOLD) project is used in parallel.
- Prior to queries in public databases, it is important to gather information about the taxon under investigation (e.g., from NCBI taxonomy browser and/or BOLD Taxonomy section)
  - Additional species in the same genus
  - Presence of declared and related species in GenBank and BOLD
  - Amount of cytb and/or cox1 sequences of respective species in GenBank/BOLD
- It is recommended to use the FASTA format when pasting sequences into the query boxes of BLAST and BOLD so that the query results are displayed together with the names of the sequences.

2.5.2. Sequence comparison of MCB and/or COI DNA sequences with GenBank

- The edited cytb and/or cox1 DNA sequences are subjected to a comparison with sequences from the nucleotide collection (nr/nt) of GenBank by BLAST, optimized for highly similar sequences (Megablast) in order to identify what species, the sequences originate from.
- The obtained matches are displayed as a list and are sorted by default by maximum score. Before assigning a species, re-sort the hits by maximum identity to check for inconsistencies. The hits are additionally presented as alignments with the query sequence at the end of the search result.
- In cases with more than 100 hits with ≥ 98 % identity, it is required to increase the number of maximal target sequences (under algorithm parameters) to identify all relevant species.
- The query result should be saved as pdf-file (or similar) to document the output of the database at the time of

	comparison and should include the following: — Species of sequence entries with identities ≥ 98 % (including gaps); — Degree of identity, in percentage; — Degree of query coverage, in percentage — Search database used and the date of search. In case of non-compliance of the species of top BLAST hits with the declared species, the BLAST search is repeated with restriction to database entries belonging to the declared species. This can be done by entering the species name under "Organism" in the BLAST form. The result is recorded, like above. **2.5.3. Sequence comparison of MCB and/or COI DNA sequences with BOLD** • In order to assign the species with COI sequences, the COI sequence database of the Barcode of Life (BOLD) project can be used. • The web page "Identification Request/Animal identification" acts as a portal allowing the edited COI query sequence to be compared with the BOLD reference database. • Various search options are possible that relate to different collections of reference data, but the default settings, selecting only records at the species level, provide an excellent initial step for identifying the species. The search result provides the following information: ▪ At the top of the query result sheet, a species is assigned, based on the identity values from the 99 top hits. ▪ The per cent similarity for 99 top matching records in the database against the query sequence obtained from the sample is displayed in a graph, full records for these corresponding top 99 matches can be shown. ▪ BOLD can also produce a simple neighbour-joining tree to display the results of the homology search graphically. The query sequence is highlighted in the tree diagram. In cases where BOLD returns more than one species or none at all, and a species match could not be made, an additional search is possible, selecting a different set of reference data e.g. the "Public Record Barcode Database" (this restricts the search to sequences that have been published) or "All Records on BOLD" (this is thebroadest database). In cases where the BOLD database is unable to identify the query sequence, other publicly available reference databases could be searched, e.g., GenBank The query result should be saved as a pdf file (or similar) to document the output of the database at the time of comparison and has to be recorded as follows: ▪ Species, to which BOLD has matched the sequence ▪ Species name and similarity of hits with ≥ 98 % ▪ Query sequence clusters with sequences from a single species in the tree: Yes or No? Search database used and the date of search
Calculation with units ofexpression	Species identification results obtained by DNA sequencing are only qualitative. The name of the species/taxonomic level is the result

	with no additional unit ofmeasurement.
Inference (Qualitative Analysis)	For species identification, two specimens with a sequence similarity of 97% or above are considered to be a single species. A high Identity value generally falls in the range of 98-100% sequence similarity. The identity reports on the percentage of base pairs that are the same between the sequence of your specimen and that of the reference specimen. If 99 out of 100 base pairs match, then you have a 99% identity value in your results. If there is a large number of reference sequences that fall into the 98-100% range in your results. If more than one reference species has 98-100% sequence similarity with your specimen, you would identify your specimen conclusively at the genus level, but not at a species level.
Reference	1. ISO 22949-1:2020, Molecular biomarker analysis — Methods of analysis for the detection and identification of animal species in foods and food products (nucleotide sequencing-based methods) — General requirements and definitions 2. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions 3. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction ISO 24276, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions
Approved by	Scientific Panel on Methods of Sampling and Analysis

<table>
<tr><td>एफएसएसएआई
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भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR— BovineDNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.030:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Bovine (Bos taurus and Bos indicus) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of bovine materials derived from Bos taurus and Bos indicus. The assay also detected species bison (Bison bison) and yak (Bos mutus).

The target sequence is a partial fragment of the bovine nuclear beta actin gene (ACTB) (e.g., GenBank accession number: NC_037352.1), which is present as a single copy per haploid genome and the length of the target sequence is 62 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitablemethod Refer method Refer method FSSAI 05.022:2024, FSSAI 05.023:2024, FSSAI 05.024:2024, FSSAI 05.025:2024 for specific requirements.
The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a PCR assay specific for mammals (e.g., myostatin gene);
- Detection of the bovine species-specific DNA sequence of the beta actin gene (ACTB) (e.g., GenBank accession number: NC_037352.1) in a real-timePCR.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer Chapter 1.4 of this manual), the following equipment is required;
Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides

Table 1 — Oligonucleotides
<table>
<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Bovine beta actin gene as the target sequence (GenBank accession number: NC_037352.1)</td></tr>
<tr><td>Bovine – 62bp - F</td><td>5’-GGCCTCGGAGTGTGTATTCAG-3’</td><td>400 nmol/l</td></tr>
<tr><td>Bovine – 62bp - R</td><td>5’-GCCCCAGAATGAGGTTCACTT-3’</td><td>400 nmol/l</td></tr>
<tr><td>Bovine – 62bp - P</td><td>5’-[FAM]-AGGTGCACAGTACGTTC-[NFQ-MGB]-3'</td><td>200 nmol/l</td></tr>
</table>
B.PCR master mix</td></tr>
</table>

<table>
<tr><td></td><td>- In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) is recommended.</td></tr>
<tr><td>Sample Preparation</td><td>Preparation of the test portion/sample
The test sample used for DNA extraction shall be representative of the laboratory sample and homogeneous, e.g., by grinding or homogenizing the laboratory sample to a fine mixture. For preparation of test portion/sample, follow general requirements and specific methods described in chapter 1.4 of this manual
Preparation of DNA extracts
The extraction/purification and quantification of DNA from the test portion shall follow the general requirements and methods provided in chapter 1.1.</td></tr>
<tr><td>Method of analysis</td><td>Reaction mixes
The method is for a total volume of 25 µL per PCR and reaction setup is given in Table 2.

Table 2 — Reaction setup for the amplification
<table><tr><td>Component</td><td>Volume (µL)</td></tr><tr><td>2 X Probe PCR Master Mix</td><td>12.5</td></tr><tr><td>Forward Primer</td><td>1.0</td></tr><tr><td>Reverse Primer</td><td>1.0</td></tr><tr><td>Probe</td><td>0.5</td></tr><tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or controls</td><td>5</td></tr><tr><td>Water</td><td>to 25 µL</td></tr></table>
The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagentmixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into eachreaction vial;
- Add 5 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA targetcontrol or blank/other controls; mix and centrifuge briefly.
Temperature-time programme
The temperature-time programme as outlined in Table 3 was used in the validationstudy. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial</td></tr>
</table>

<table>
<tr><td></td><td>denaturation depends on the master mix used.

Table 3 — Temperature-time programme
<table>
<tr><th>Protocol</th><th>Conditions</th><th>Fluorescence measurement</th></tr>
<tr><td>Initial denaturation</td><td>95 °C 10 min</td><td>No</td></tr>
<tr><td>Denaturation</td><td>95 °C 15 S</td><td>No</td></tr>
<tr><td>Annealing/extension</td><td>60 °C 60 Sec</td><td>Yes</td></tr>
<tr><td></td><td>GOTO 45X cycles</td><td></td></tr>
</table></td></tr>
<tr><td>Calculation with units of expression</td><td>General
Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Bovine-specific primers Bovine-62bp-F and Bovine-62bp-R and the probe Bovine-62bp-P produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
3. ISO 20224-1:2020 Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR — BuffaloDNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.031:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Buffalo (Bubalus bubalis) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of buffalo materials derived from Bubalus bubalis.
The target sequence is a partial fragment of the buffalo melanocortin-1- receptor gene MC1R (e.g., GenBank accession number: MN687828), which is present as a single copy diploid nuclear gene and the length of the target sequence is 87 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method (Refer method FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a real-time PCR assay specific for mammals (e.g., myostatin gene);
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<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer chapter 1.4 of this manual), the following equipment is required;

Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides
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<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Myostatin gene as the target sequence</td></tr>
<tr><td>Myostatin gene - F</td><td>5’-GTGCAAATCCTGAGACTCAT -3’</td><td>600 nmol/l</td></tr>
<tr><td>Myostatin gene - R</td><td>5’-ATACCAGTGCCTGGGTTCAT -3’</td><td>600 nmol/l</td></tr>
<tr><td>Myostatin gene - P</td><td>5’-[FAM]-CCATGAAAGACGGTACAAGGTATACTG-BHQ1 -3'</td><td>250 nmol/l</td></tr>
</table></td></tr>
</table>

<table>
<tr><td></td><td>B. PCR master mix
- In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) is recommended.</td></tr>
<tr><td>Sample Preparation</td><td>Preparation of the test portion/sample
The test sample used for DNA extraction shall be representative of the laboratory sample and homogeneous, e.g., by grinding or homogenizing the laboratory sample to a fine mixture. For preparation of test portion/sample, follow general requirements and specific methods described in chapter 1.4 of this manual.

Preparation of DNA extracts
The extraction/purification and quantification of DNA from the test portion shall follow the general requirements and methods provided in chapter 1.1.</td></tr>
<tr><td>Method of analysis</td><td>Reaction mixes
The method is for a total volume of 10 µL per PCR and reaction setup is given in Table 2.

Table 2 — Reaction setup for the amplification
<table>
<tr><td>Component</td><td>Volume (µL)</td></tr>
<tr><td>2 X Probe PCR Master Mix</td><td>5</td></tr>
<tr><td>Forward Primer</td><td>0.3</td></tr>
<tr><td>Reverse Primer</td><td>0.3</td></tr>
<tr><td>Probe</td><td>0.2</td></tr>
<tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or controls</td><td>1 µL</td></tr>
<tr><td>Water</td><td>to 10 µL</td></tr>
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The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 9 µl into each reaction vial;
- Add 1 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA target control or blank/other controls; mix and centrifuge briefly.</td></tr>
</table>

<table>
<tr><td></td><td>Temperature-time programme

The temperature-time programme as outlined in Table 3 was used in the validation study. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.

Table 3 — Temperature-time programme
<table>
<tr><th>Protocol</th><th>Conditions</th><th>Fluorescence measurement</th></tr>
<tr><td>Initial denaturation</td><td>95 °C 10 min</td><td>No</td></tr>
<tr><td>Denaturation</td><td>95 °C 10 S</td><td>No</td></tr>
<tr><td>Annealing/extension</td><td>60 °C 30 Sec</td><td>Yes</td></tr>
<tr><td></td><td>GOTO</td><td></td></tr>
<tr><td>Melt Curve</td><td colspan="2">65.0 to 95 °C. increase 0.5 °C for 0.05 +Plate read</td></tr>
</table></td></tr>
<tr><td>Calculation with units of expression</td><td>General
Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- buffalo-specific primers Buffalo-87bp-F and Buffalo-87bp-R and the probe Buffalo-87bp-P, produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
3. ISO 20224 (SERIES) Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

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भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR — Chicken DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.032:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of chicken (Gallus gallus) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of chicken materials derived from Gallus gallus domesticus and Gallus gallus.
The target sequence is a partial fragment of Gallus gallus transforming growth factor beta 3, intron 4 (TGF-β3) gene (e.g., GenBank accession number AY685072.1), which is present as a single copy per haploid genome and the length of the target sequence is 77 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer to chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method(Refer method FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a PCR assay specific for poultry (e.g., myostatin gene);
- Detection of the chicken species-specific DNA sequence of the transforminggrowth factor beta 3, intron 4 gene (TGF-β3) (e.g., GenBank accession number AY685072.1) in a real-time PCR.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory (Refer chapter 1.4 of this manual), the following equipment is required;

Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides

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<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Chicken TGF- β3 gene as the target sequence (GenBank accession number: AY685072.1)</td></tr>
<tr><td>Chicken – 77bp - F</td><td>5’-CAGCTGGCCTGCCGGC-3’</td><td>400 nmol/l</td></tr>
<tr><td>Chicken – 77bp - R</td><td>5’-GCCCAGTGGAATGTGGTATTCA-3’</td><td>400 nmol/l</td></tr>
<tr><td>Chicken – 77bp - P</td><td>5’- [FAM] - TGCCACTCCTCTGCACCCAGTGC- [TAMRA] - 3'</td><td>200 nmol/l</td></tr>
</table>

B. PCR master mix</td></tr>
</table>

<table>
<tr><td></td><td>- In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) isrecommended.</td></tr>
<tr><td>Sample Preparation</td><td>Preparation of the test portion/sample
The test sample used for DNA extraction shall be representative of the laboratory sample and homogeneous, e.g., by grinding or homogenizing the laboratory sample to a fine mixture. For preparation of test portion/sample, follow general requirements and specific methods described in chapter 1.4 of this manual.
Preparation of DNA extracts
The extraction/purification and quantification of DNA from the test portion shall follow the general requirements and methods provided in chapter 1.1.</td></tr>
<tr><td>Method of analysis</td><td>Reaction mixes
The method is for a total volume of 25 µL per PCR, and reaction setup is given in Table 2.

Table 2 — Reaction setup for the amplification
<table>
<tr><td>Component</td><td>Volume (µL)</td></tr>
<tr><td>2 X Probe PCR Master Mix</td><td>12.5</td></tr>
<tr><td>Forward Primer</td><td>1.0</td></tr>
<tr><td>Reverse Primer</td><td>1.0</td></tr>
<tr><td>Probe</td><td>0.5</td></tr>
<tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or contro</td><td>5</td></tr>
<tr><td>Water</td><td>to 25 µL</td></tr>
</table>
The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into eachreaction vial;
- Add 5 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA target control or blank/other controls; mix and centrifuge briefly.
Temperature-time programme
The temperature-time programme as outlined in Table 3 was used in the validationstudy. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.</td></tr>
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<table>
<tr><td></td><td>

Table 3 — Temperature-time programme

<table>
<tr><th>Protocol</th><th>Conditions</th><th>Fluorescence measurement</th></tr>
<tr><td>Initial denaturation</td><td>95 °C 10 min</td><td>No</td></tr>
<tr><td>Denaturation</td><td>95 °C 15 S</td><td>No</td></tr>
<tr><td>Annealing/extension</td><td>60 °C 60 Sec</td><td>Yes</td></tr>
<tr><td colspan="3">GOTO 45X cycles</td></tr>
</table>

</td></tr>
<tr><td>Calculation with units of expression</td><td>General
Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Chicken-specific primers Chicken-77bp-F and Chicken-77bp-R and the probe Chicken-77bp-P produce a sigmoid-shaped amplification curve and aCt value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
3. ISO 20224-4:2020 Molecular biomarker analysis — Detection of animal-derivedmaterials in foodstuffs and feedstuffs by real-time PCR</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
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Food Safety and Standards Authority of India
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Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR — Porcine DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.033:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Porcine (Sus scrofa) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of porcine materials derived from Sus scrofa domesticus and Sus scrofa.
The target sequence is a partial fragment of the porcine beta actin gene (ACTB) (e.g., GenBank accession number: DQ452569.1), which is present as a single copy per haploid genome and the length of the target sequence is 97 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method, (Refer method FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a PCR assay specific for mammals (e.g., myostatin gene);
- Detection of the Porcine species-specific DNA sequence of the beta actin gene (ACTB) (e.g., GenBank accession number: DQ452569.1) in a real-time PCR.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer FSSAI 05.025:2024), the following equipment is required;

Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides
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<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Porcine beta actin gene (ACTB) as the target sequence (GenBank accession number: DQ452569.1)</td></tr>
<tr><td>Porcine – 97bp - F</td><td>5’-CGTAGGTGCACAGTAGGTCTGAC-3’</td><td>400 nmol/l</td></tr>
<tr><td>Porcine – 97bp - R</td><td>5’-GGCCAGACTGGGGACATG-3’</td><td>400 nmol/l</td></tr>
<tr><td>Porcine – 97bp - P</td><td>5’-[FAM]-CCAGGTCGGGGAGTC-[NFQ-MGB]-3'</td><td>200 nmol/l</td></tr>
</table></td></tr>
</table>

<table>
<tr><td></td><td>B.PCR master mix
In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognizedanalytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) isrecommended.</td></tr>
<tr><td>Sample Preparation</td><td>Preparation of the test portion/sample
The test sample used for DNA extraction shall be representative of the laboratory sample and homogeneous, e.g., by grinding or homogenizing the laboratory sample to a fine mixture. For preparation of test portion/sample, follow general requirements and specific methods described in chapter 1.4 of this manual.
Preparation of DNA extracts
The extraction/purification and quantification of DNA from the test portion shall follow the general requirements and methods provided in chapter 1.1.</td></tr>
<tr><td>Method of analysis</td><td>Reaction mixes
The method is for a total volume of 25 µL per PCR and reaction setup is given in Table 2.

Table 2 — Reaction setup for the amplification
<table><tr><td>Component</td><td>Volume (µL)</td></tr><tr><td>2 X Probe PCR Master Mix</td><td>12.5</td></tr><tr><td>Forward Primer</td><td>1.0</td></tr><tr><td>Reverse Primer</td><td>1.0</td></tr><tr><td>Probe</td><td>0.5</td></tr><tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or controls</td><td>5 µL</td></tr><tr><td>Water</td><td>to 25 µL</td></tr></table>
The following points to be considered;

All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagentmixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.

Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into eachreaction vial;
- Add 5 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA targetcontrol or blank/other controls; mix and centrifuge briefly.

Temperature-time programme
The temperature-time programme as outlined in Table 3 was used in the validation study. The use of different reaction conditions and</td></tr>
</table>

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<tr><td></td><td>real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.

Table 3 — Temperature-time programme
<table>
<tr><th>Protocol</th><th>Conditions</th><th>Fluorescence measurement</th></tr>
<tr><td>Initial denaturation</td><td>95 °C 10 min</td><td>No</td></tr>
<tr><td>Denaturation</td><td>95 °C 15 S</td><td>No</td></tr>
<tr><td>Annealing/extension</td><td>60 °C 60 Sec</td><td>Yes</td></tr>
<tr><td colspan="3">GOTO 45X cycles</td></tr>
</table></td></tr>
<tr><td>Calculation with units of expression</td><td>General
Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Porcine-specific primers Porcine-97bp-F and Porcine-97bp-R and the probe Porcine-97bp-P, produce a sigmoid-shaped amplification curve and aCt value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
3. ISO 20224-3:2020 Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR</td></tr>
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स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR — Ovine DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.034:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Ovine (Ovis aries) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of ovine materials derived from Ovis aries.

The target sequence is a partial fragment of the ovine nuclear prolactin receptor gene (PRLR) (e.g., GenBank accession number: AF041979.1), which is present as a single copy haploid genome and the length of the target sequence is 88 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method(Refer chapter 1.1 of this manual).
The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a PCR assay specific for mammals (e.g., myostatin gene);
- Detection of the ovine species-specific DNA sequence of the nuclear prolactin receptor gene (PRLR) (e.g., GenBank accession number:AF041979.1) in a real-time PCR.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer chapter 1.4 of this manual), the following equipment is required;
Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides
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<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Ovine PRLR gene as the target sequence (GenBank accession number: AF041979.1)</td></tr>
<tr><td>Ovine – 88bp - F</td><td>5’-CCAACATGCCTTTAAACCCTCAA-3’</td><td>400 nmol/l</td></tr>
<tr><td>Ovine– 88bp - R</td><td>5’-GGAACTGTAGCCTTCTGACTCG-3’</td><td>400 nmol/l</td></tr>
<tr><td>Ovine – 88bp - P</td><td>5’- [FAM]-TGCCTTTCCTTCCCCGCCAGTCTC-[TAMRA] - 3'</td><td>200 nmol/l</td></tr>
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B. PCR master mix
- In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration,</td></tr>
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<tr><td>Probe</td><td>0.5</td></tr>
<tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or controls</td><td>5</td></tr>
<tr><td>Water</td><td>to 25 µL</td></tr>
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The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial;
- Add 5 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA target control or blank/other controls; mix and centrifuge briefly.

Temperature-time programme
The temperature-time programme as outlined in Table 3 was used in the validation study. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.</td></tr>
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<tr><td>Calculation with units of expression</td><td>General
Suitable instrument-specific data analysis software shall be used for resultinterpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- ovine-specific primers Ovine-88bp-F and Ovine-88bp-R and the probe Ovine-88bp-P, produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 16577, Molecular biomarker analysis — Terms and definitions
2. ISO 20813, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
3. ISO 21571, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
4. ISO 20224-2:2020, Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR
5. ISO 24276, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — General requirements and definitions</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
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Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR —Goat DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.035:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Goat (Capra hircus) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrixand can be applied to the detection of Goat materials derived from Capra hircus.

The target sequence is a partial fragment of goat chromosome 9 DNA sequence (e.g., GenBank accession number: NC_030816.1), which is present as a single copy haploid genome and the length of the target sequence is 87 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method (Refer method FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a PCR assay specific for mammals (e.g., myostatin gene);
- Detection of the goat species-specific DNA of goat chromosome 9 DNAsequence (GenBank accession number NC_030816.1) in a real-time PCR</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer chapter 1.4 of this manual), the following equipment is required;

Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides
<table>
<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Specific DNA sequence in goat chromosome 9 DNA as the target sequence (GenBank accession number: NC_030816.1)</td></tr>
<tr><td>Goat – 87bp - F</td><td>5’-GGAAGGAAAGAGAATGGGGATATGG-3’</td><td>400 nmol/l</td></tr>
<tr><td>Goat – 87bp - R</td><td>5’-TCTCCACACACAGCCAAAACC-3’</td><td>400 nmol/l</td></tr>
<tr><td>Goat – 87bp - P</td><td>5’[FAM]-ATCCATCTCTCCCTCCACTCCCTGCCTAA-[TAMRA]-3'</td><td>200 nmol/l</td></tr>
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<tr><td></td><td>B. PCR master mix
-In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP), MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) is recommended.</td></tr>
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Table 2 — Reaction setup for the amplification
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<tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or controls</td><td>5 µL</td></tr>
<tr><td>Water</td><td>to 25 µL</td></tr>
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The following points to be considered;

All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial;
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The temperature-time programme as outlined in Table 3 was used in the validation study. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.

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<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Goat-specific primers Goat-87bp-F and Goat-87bp-R and the probe Goat- 87bp-P, produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
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<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
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3. ISO 20224-5:2020 Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR</td></tr>
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Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR — Horse DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.036.2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Horse ((Equus caballus) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of Horse materials derived from (Equus caballus).
The target sequence is an Equus caballus isolate (e.g., GenBank accession number:
NC_009171.3), which is present as a single copy diploid nuclear gene and the length of the target sequence is 87 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer FSSAI 05.022:2024 and FSSAI 05.025:2024.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method (Refer method FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using a real-time PCR assay specific for mammals (e.g., myostatin gene);
- Detection of the horse species-specific DNA sequence of the Equus caballus (e.g., GenBank accession number: NC_009171.3) in a real-time PCR.</td></tr>
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Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
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<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration inPCR</th></tr>
<tr><td colspan="3">Specific DNA sequence in Equus (GenBank accession number: NC_009171.3)</td></tr>
<tr><td>Horse – 125bp - F</td><td>5’-ACTCATCAAACGCCGCTCTC-3’</td><td>400 nmol/l</td></tr>
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<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) isrecommended.</td></tr>
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The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagentmixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual
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<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- horse-specific primers Horse-125bp-F and Horse-125bp-R and the probe Horse-125bp-P, produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
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3. ISO 20224-6:2020 Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR</td></tr>
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Ministry of Health and Family Welfare</td><td colspan="3">Molecular Biomarker Analysis - Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR —Turkey DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.037:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Turkey (Meleagris gallopavo) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of turkey materials derived from Meleagris gallopavo.
The target sequence is a partial fragment of the Meleagris gallopavo chromosome Z DNA sequence (e.g., GenBank accession number: NC_015041.2), which is present as a single copy per haploid genome and the length of the target sequence is 118 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable (Refer method FSSAI 05.022:2024., FSSAI 05.023:2024., FSSAI 05.024:2024., FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:
- Verification of the quality and amplifiability of the extracted DNA using aPCR assay specific for poultry (e.g., myostatin gene);
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The following points to be considered;

All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.

Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial;
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<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Turkey-specific primers Turkey-118bp-F and Turkey-118bp-R and the probe Turkey-118bp-P, produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
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3. ISO 20224-8:2020 Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR</td></tr>
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<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of Donkey (Equus asinus) specific DNA derived from food and feed. It requires the extraction of an adequate quantity of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of donkey materials derived from Equus asinus.

The target sequence is a partial fragment of the Equus asinus isolate (e.g., GenBank accession number NW_014638576.1), which is present as a single copy diploid nuclear gene and the length of the target sequence is 95 bp.</td></tr>
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Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into eachreaction vial;
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<tr><td>Calculation with units ofexpression</td><td>General
Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Donkey-specific primers Donkey-95bp-F and Donkey-95bp-R and the probe Donkey-95bp-P, produce a sigmoid-shaped amplification curve and aCt value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
2. ISO 21571:2005, Foodstuffs — Methods of analysis for the detection of genetically modified organisms and Derived products — Nucleic acid extraction
3. ISO 20224-7:2020 Molecular biomarker analysis — Detection of animal-derivedmaterials in foodstuffs and feedstuffs by real-time PCR</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
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<tr><td>एफएसएसएआई
fssai
भारतीय खाद्य सुरक्षा और मानक प्राधिकरण
Food Safety and Standards Authority of India
स्वास्थ्य और परिवार कल्याण मंत्रालय
Ministry of Health and Family Welfare</td><td colspan="3">Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR— Goose DNA detection method</td></tr>
<tr><td>Method No.</td><td>FSSAI 05.039:2024</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This document specifies a real-time PCR method for the qualitative detection of goose (Anser cygnoides domesticus and Anser anser domesticus) specific DNA derived from food and feed. It requires the extraction of an adequate quantity ofPCR amplifiable DNA from the relevant matrix and can be applied to the detection of turkey materials derived from Anser cygnoides domesticus and Anser anser domesticus.
The target sequence is a partial fragment of the goose unplaced genomic scaffold (e.g., GenBank accession number: NW_013185870.1), which is present as a single copy per haploid genome and the length of the target sequence is 121 bp.</td></tr>
<tr><td>Caution</td><td colspan="3">Refer chapter 1.1 and chapter 1.4 of this manual.</td></tr>
<tr><td>Principle</td><td colspan="3">DNA extraction from the test portion to be performed by applying a suitable method (Refer method FSSAI 05.022:2024, FSSAI 05.023:2024, FSSAI 05.024:2024, FSSAI 05.025:2024 for specific requirements).

The DNA analysis consists of two parts:

- Verification of the quality and amplifiability of the extracted DNA using PCRassay specific for poultry (e.g., myostatin gene);
- Detection of the goose species-specific DNA sequence of the unplaced genomic scaffold (e.g. GenBank accession number NW_013185870.1).</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">In addition to the usual molecular laboratory equipment (Refer chapter 1.4 of this manual), the following equipment is required;

Real-time thermocycler instrument: A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">A. Oligonucleotides
Table 1 — Oligonucleotides
<table>
<tr><th>Name</th><th>DNA sequence of the oligonucleotide</th><th>Final concentration in PCR</th></tr>
<tr><td colspan="3">Specific sequence in Anser cygnoides domesticus unplaced genomic scaffold as the target sequence (GenBank accession number: NW_013185870.1)</td></tr>
<tr><td>Goose–121bp - F</td><td>5′-ACGAGGATAGGTTGTGACAGC-3′</td><td>400 nmol/l</td></tr>
<tr><td>Goose–121bp - R</td><td>5′-GAATCTCTGTGTCGTCTTCTCTATATG-3′</td><td>400 nmol/l</td></tr>
<tr><td>Goose–121bp - P</td><td>5′- [FAM]-ACTCTGTTCAGCCTTGCGAAGACCTTATGC-[TAMRA] - 3′</td><td>200 nmol/l</td></tr>
</table>
B. PCR master mix
- In general, Real-time PCR master mix contains thermostable DNA polymerase, the four dNTPS (dATP, dGTP, dTTP, dCTP),</td></tr>
</table>

<table>
<tr><td></td><td>MgCl2, KCl, and buffer as a dilutable concentration, which is ready to use.</td></tr>
<tr><td>Preparation of Reagents</td><td>For molecular biomarker analysis, only chemicals/consumables of recognized analytical grade, appropriate for molecular biology, shall be used. All prepared solutions should be sterilized by autoclaving. Use only powder free gloves. The use of aerosol protected pipette tips (protection against cross-contamination) is recommended.</td></tr>
<tr><td>Sample Preparation</td><td>Preparation of the test portion/sample
The test sample used for DNA extraction shall be representative of the laboratory sample and homogeneous, e.g., by grinding or homogenizing the laboratory sample to a fine mixture. For preparation of test portion/sample, follow general requirements and specific methods described in chapter 1.4 of this manual.
Preparation of DNA extracts
The extraction/purification and quantification of DNA from the test portion shall follow the general requirements and methods provided in provided in chapter 1.1.</td></tr>
<tr><td>Method of analysis</td><td>Reaction mixes
The method is for a total volume of 25 µL per PCR and reaction setup is given in Table 2.

Table 2 — Reaction setup for the amplification
<table>
<tr><td>Component</td><td>Volume (µL)</td></tr>
<tr><td>2 X Probe PCR Master Mix</td><td>12.5</td></tr>
<tr><td>Forward Primer</td><td>1.0</td></tr>
<tr><td>Reverse Primer</td><td>1.0</td></tr>
<tr><td>Probe</td><td>0.5</td></tr>
<tr><td>Sample DNA (20 ng/µl to 200 ng/µl) or controls</td><td>5 µL</td></tr>
<tr><td>Water</td><td>to 25 µL</td></tr>
</table>
The following points to be considered;
All reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagentmixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the numberof reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow chapter 1.4 of this manual.
Set up the PCR as follows:
- Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into eachreaction vial;
- Add 5 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA target control or blank/other controls; mix and centrifuge briefly.
-
Temperature-time programme
The temperature-time programme as outlined in Table 3 was used in the validationstudy. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.</td></tr>
</table>

<table>
<tr><td></td><td>
Table 3 — Temperature-time programme
<table>
<tr><th>Protocol</th><th>Conditions</th><th>Fluorescence measurement</th></tr>
<tr><td>Initial denaturation</td><td>95 °C 10 min</td><td>No</td></tr>
<tr><td>Denaturation</td><td>95 °C 15 S</td><td>No</td></tr>
<tr><td>Annealing/extension</td><td>60 °C 60 Sec</td><td>Yes</td></tr>
<tr><td></td><td>GOTO 45X cycles</td><td></td></tr>
</table>
</td></tr>
<tr><td>Calculation with units ofexpression</td><td>General
Suitable instrument-specific data analysis software shall be used for result interpretation. If amplification of the DNA target sequence in a sample occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated and reported as cycle threshold (Ct) or cycle quantification (Cq). In the absence of detectable PCR products (e.g., negative controls), the result shall be expressed as “undetermined”, “no amplification” or the maximum number of reaction cycles performed.</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td>Identification
The target sequence is considered as detected if:
- Goose-specific primers Goose-121bp-F and Goose-121bp-R and the probe Goose-121bp-P produce a sigmoid-shaped amplification curve and a Ct value or Cq value can be calculated;
- PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;
- The amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and Ct values or Cq values.</td></tr>
<tr><td>Reference</td><td>1. ISO 20813: 2019, Molecular biomarker analysis — Methods of analysis for the identification and the Detection Of animal species from foods and food products — General requirements and definitions
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</table>

Note 1: The brand / model of equipments/ accessories/ column / Chemicals & reagents given in the methods are for reference purpose only. The end user may use equivalent specifications of equipments/ accessories/ column / Chemicals & reagents.

FSSAI does not endorse / promote any particular brand/ model of equipments/ accessories/ column / Chemicals & reagents.

Note 2: Alternate Rapid kits/equipments may be used to get quick results for screening and surveillance purposes, provided the kit/equipment is approved by FSSAI. Details of the rapid food testing kit/equipment approved by FSSAI are available at https://www.fssai.gov.in/cms/raft.php**.**

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