TERMS OF REFERENCE FOR R&D PROJECT

Food and Agriculture Department

Biotechnology for Food and Agriculture Sectional Committee, FAD 23

1 Title of the Project

Validation of method of test for detection of Buffalo DNA in foodstuff and feedstuffs by real-time PCR.

2 Background

2.1 Animal-derived foods hold substantial global economic significance, particularly in India where livestock farming thrives. Notably, India boasts the world's largest buffalo population at 109.9 million (DAHD 2022), driving significant economic input. Water buffalo meat, a premier agricultural export, constitutes 19.05% of the nation's meat production. Indigenous buffalo contribute 35% and non-descript buffaloes 14% to milk production, growing annually at 6.5% (DAHD 2022). Exploiting high demand and processing conditions, opportunistic fraudsters adulterate with inferior substitutes, such as mixing buffalo ghee in cow ghee and buffalo meat in mutton/chevon, yielding subtle economic gains. These covert manipulations pose a risk of economically motivated adulterations, warranting vigilance in supply chain vulnerability assessment.

2.2 Remarkably, after the horse meat scandal of Europe, scientists at the global level and regulators were keen on developing and adopting molecular assays, especially DNA techniques, to tackle such unobtrusive incidents. Further, ISO TC/34 SC/16 (An ISO sub-Committee on Horizontal methods for biomarker analysis) has come up with an ISO/TS 20224-Series of ISO standards, which is based on real-time PCR, for the detection of many of the economically important meat species. However, this series does not provide a method for the detection of buffalo DNA. Considering the economic potential of buffalo-derived foods in India, ICAR-NMRI has developed a real-time PCR-based Buffalo DNA detection assay in parallel with the style of ISO 20224-series. The method is validated by single laboratory validation process and submitted to BIS (FAD 23) for developing as a national standard. Multi Laboratory Validation of this assay, through a collaborative ring trail, may elevate this method for formulating Indian Standard which may be proposed to be taken up by the concerned ISO Sub-Committee.

3 Objective

To validate the given method of test for buffalo DNA detection using real-time PCR for various performance criteria, i.e., robustness, reproducibility, sensitivity and specificity as detailed under method validation protocol.

4 Scope

4.1 Validation of the method of test for detection of Buffalo DNA using real-time PCR, as given under Research Methodology covering 'false-positive and false-negative rate' and 'limit of detection of qualitative assays' through a properly designed and well-participated collaborative ring trial model, by at least 12 number of participating laboratories proficient in molecular biology testing as per the criteria and method mentioned in these ToR.

4.2 The validation of the test method shall include various performance criteria, i.e., robustness, reproducibility, sensitivity and specificity as detailed under method validation protocol.

5 Research Methodology

5.1 The proposer/project leader should have the experience and competence in the field of animal DNA Detection, evident from peer reviewed publications.

5.2 Laboratories engaged in the project shall have experience and infrastructure in molecular biomarker analysis, (Real-time PCR, Digital PCR, Sequencing etc.) and method validation as per relevant ISO Standards (IS 20813 and ISO 20224 series of standards) and requirements of ISO/IEC 17025: 2017.

5.3 The validation protocol of this Buffalo DNA detection method (qualitative) should be planned following a comprehensive approach consisting of two main phases: single laboratory validation (Phase 1) and collaborative trial validation (Phase 2) (as per ISO 20813:2019).

5.4 Phase 1: Single Laboratory Validation

This Buffalo DNA detection method has already been sufficiently validated (single lab validation) for basic PCR parameters and other performance characteristics as per the requirement of ISO 20813:2019. The parameters for validation include, but are not limited to, inclusive and exclusive specificity, assay robustness, limit of detection of the assay (both absolute and relative) etc. In-house quality control materials (QCM) developed as per ISO Guide 80 have been utilized for this single laboratory validation. The optimized PCR conditions for buffalo DNA detection are provided in Annexure 1. The proposer shall undertake single laboratory validation of this test method in his/her laboratory including the analysis of robustness as given below:

5.4.1 Robustness

The robustness shall be primarily tested by changing reaction conditions for the following factors: 1) real-time PCR instruments; 2) reaction volume; 3) annealing temperature and 4) primer or probe concentration. For each factor, the PCRs will be analyzed in triplicate, each with 20 copies of the target sequence and with 100 copies of the non-target sequence as negative controls.

5.5 Phase 2: Collaborative Ring Trail

5.5.1 Number of Participating Laboratories (Min.): Twelve labs which are proficient in molecular biology testing (*see* **5.1** and **5.2**).

5.5.2 Protocol to be Followed

5.5.2.1 This collaborative validation study for 'qualitative buffalo DNA detection using real-time PCR' shall be designed using DNA solutions and by considering the probability of detection (POD) within the range of the method. Traditional nonparametric 5 % false positive and 5 % false negative rates reflect PODs of 5 % and 95 %.

a) Robustness

Robustness shall also be tested for collaborative ring trial as given under **5.4.1** by using different types of real-time PCR equipment.

b) Measurement of Reproducibility

Evaluation of False-Positive & False-Negative Rates

i) A minimum of 6 DNA samples of buffalo and 6 DNA samples of beef, which are labelled with randomized coding numbers, shall be issued to each participant laboratory (buffalo and beef genomic DNA will be extracted from meat), and the traceability of the meat has to be established through bi-directional Sanger sequencing in-house.
ii) Each DNA shall be issued in 6 replicates.
iii) Buffalo DNA (positive target) solution with 10 copies/µl and beef DNA (non-target) solution with 20 copies/µl shall be used for this study (copy numbers has to be measured using digital droplet PCR and serial dilution to be done using 0.2X TE buffer containing 20 ng/ µL of sonicated salmon-sperm DNA).
iv) The PCR master mix and the oligonucleotides (primers and probes) shall be issued to the collaborative centers to conduct the PCR experiments (Annexure 1). Each DNA sample shall be tested by the participants in a single PCR test with 1-5 µl of the respective DNA solution (as per the final reaction volume opted), using the procedure and the conditions given in Annexure 1. The results shall be used to calculate false-positive & false-negative rates and results shall be expressed as percentage (%). The performance criteria set shall be that neither the false negative rate nor the false positive rate should exceed 5 %.

c) Measurement of Sensitivity

Absolute Limit of Detection (LOD 95%)

i) Construction of plasmid vector: The buffalo target DNA sequence shall be synthesized and cloned into the plasmid vector (pUC57-Amp) and shall be used as the standard to evaluate the LoD.
ii) The plasmid solution (with 1000 copies/µL) containing yet another untargeted background DNA (with 20 ng/ µL of sonicated salmon-sperm DNA) shall be issued to each participant laboratory (copy numbers has to be measured using digital PCR and serial dilution has to be done using 0.2X TE buffer contains background DNA).
iii) Participants shall be instructed to dilute the plasmid target in the range of 0.02 copies/µL to 4 copies/µL using 0.2X TE buffer containing 20 ng/µL of sonicated salmon-sperm DNA.

This dilution shall help to ensure the total plasmid target copy/reaction of 20, 10, 5, 2, 1, 0.5 and 0.1.

iv) The laboratories shall be performing the buffalo DNA detection PCR using each dilution series and using a minimum 6 replicates.

v) The lowest plasmid copy number per reaction at which all the replicates from all the participant laboratory generates a positive signal shall be fixed as LoD.

vi) Probability of detection (POD) is the probability that PCR amplification shall take place at a given number of copies of the target sequences. First, the results submitted by the laboratories shall be checked for deviations and outliers. Then, the qualified qualitative data generated across all laboratories and dilution levels shall be used to determine the POD=0.95 of the detection method. Mean amplification probability, slope parameter 'b' and standard deviation shall be calculated using the data generated across all laboratories using a POD curve.

6 Expected Deliverables

Detailed project report of the work done, in hard copy and digital formats, as per the scope specified under **4**, with the following as appendices:

a) A validated protocol for buffalo DNA detection using real-time PCR for the given method of test;

b) Report of analysis of all the experiments conducted by the participant laboratories along with the data obtained and statistical analysis of the data collected during the collaborative ring trial.

7 Timeline and Method of Progress Review

7.1 Timeline for the project is 6 months from the date of award of the project.

7.2 Stages for Progress Review

Stage	Timeline
Stage I An interim report comprising of in-house verification of basic parameters by the collaborating laboratories using the SOPs provided.	End of second month
Stage II A report on the progress of collaborative ring trail experiments for method validation.	Fourth month
Stage III Draft report submission – Sectional Committee will evaluate the draft report and provide feedback/recommend changes, if required.	End of fifth month

At the end of 6th month, project allottee to submit final project report incorporating recommendations/feedback of the Committee, if any.

Note: The timelines given above are indicative and calculation of time will start from the date of award of sanction letter for the project to the Project leader.

8 Support BIS will Provide

8.1 Access to Indian and International Standards.

8.2 Letters from BIS to concerned stakeholders, wherever required for support in research project.

9 Nodal Officer

Smt. Varsha Gupta, Sc. D/Joint Director, FAD, BIS, may be contacted at fad23@bis.gov.in for any queries on the research project.

Annexure-1

Real Time PCR, Conditions and Protocol to be Followed by the Participating Laboratories Under R&D Project on 'Validation of Test Method - Detection of Animal-Derived Materials in Foodstuffs and Feedstuffs by Real-Time PCR —Buffalo DNA Detection Method'

Table 1 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in PCR
Buffalo MC1R gene as the target sequence (GenBank accession number: MN687828)		
Buffalo – 87bp - F	5' - CTGGTAGTGGCTGCCAACG	300 nmol/l
Buffalo – 87bp - R	5 '- CAGCAGGTCAGACACAGCC	300 nmol/l
Buffalo – 87bp - P	5'- [FAM] – CCAAGAACCGCAACCTGCACTCCCCC – [BHQ]-3'	200 nmol/l

Table 2 — Reaction setup for the amplification

Component	Volume (µL)
2 X Probe PCR Master Mix	12.5
Forward Primer	0.75
Reverse Primer	0.75
Probe	0.5
DNA Solution	5 µL
Water	to 25 µL

Table 3 — Temperature-time programme

Protocol	Conditions	Fluorescence measurement
Initial denaturation	95 °C 10 min	No
Denaturation	95 °C 10 S	No
Annealing/extension	60 °C 30 Sec	Yes
GOTO 45X cycles		
Melt Curve	65.0 to 95 °C. increase 0.5 °C for 0.05 +Plate read	