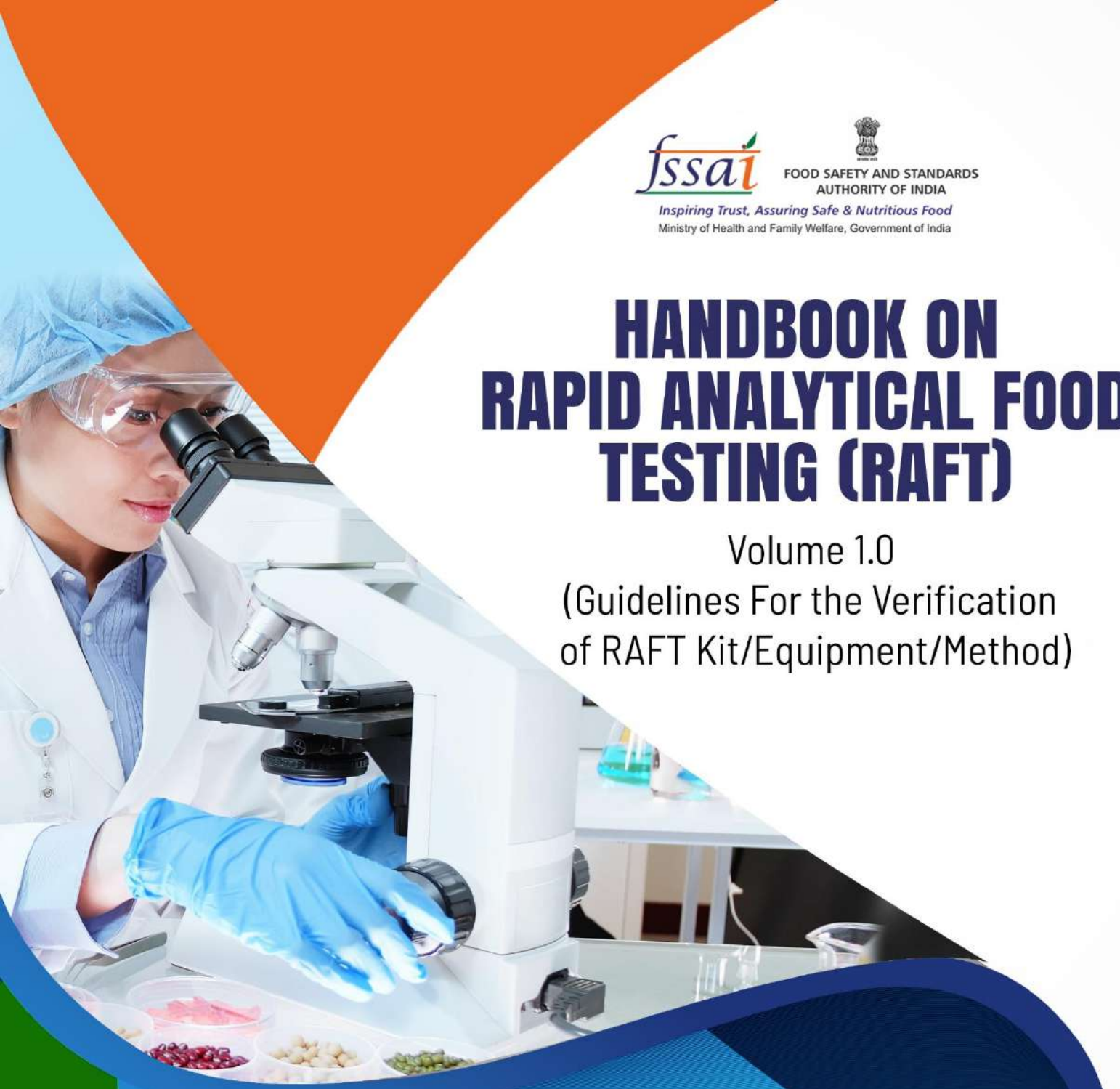
FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA

Inspiring Trust, Assuring Safe & Nutritious Food

Ministry of Health and Family Welfare, Government of India

HANDBOOK ON RAPID ANALYTICAL FOOD TESTING (RAFT)

Volume 1.0
(Guidelines For the Verification of RAFT Kit/Equipment/Method)



हिन्दी का मान : राष्ट्र का सम्मान

सत्यमेव जयते

Rajesh Bhushan, IAS
Chairman

भारतीय खाद्य सरंक्षा एवं
मानक प्राधिकरण
स्वास्थ्य एवं परिवार कल्याण मंत्रालय
भारत सरकार
एफडीए भवन, कोटला रोड नई दिल्ली-110002
FOOD SAFETY AND
STANDARDS AUTHORITY OF INDIA
Ministry of Health & Family Welfare
Government of India
FDA Bhavan,Kotla Road, New Delhi-110002
Website:- fssai.gov.in
Helpline No:- 1800112100

FOREWORD

Food Safety and Standards Authority of India (FSSAI) has introduced a policy to validate & approve developed kits/devices under the FSS (Laboratory and sample analysis) Regulation, 2011. FSSAI is facilitating rapid food testing methods to reduce the screening time of food products at the field level and accelerate surveillance activities. The goal of these rapid testing devices and equipment is to provide results in 'real time'. Rapid Analytical Food Testing kits are advanced, easy to use, portable hand-held devices and they don't require any sophisticated equipment or conventional reagents to conduct the tests. They facilitate our collective resolve to achieve the goal of creating a safe food testing ecosystem in the country. The tests can be performed anywhere by anyone without any specific/ advanced training requirements. These rapid kits/equipment/methods are expected to become an integral part of quality assurance/quality control programs in the food industry as well as for use in regulatory and surveillance purposes in future.

I am pleased to launch this Handbook on "Rapid Analytical Food Testing Kits (RAFT)" Volume 1.0 (Guidelines for the Verification of RAFT Kit/Equipment Method). This handbook would provide guidance to desirous manufacturers/method developers, Food Laboratories, Research organizations/institutions, Start-ups, etc. for obtaining approval from FSSAI for such Rapid Analytical Food Testing Kits.

I am hopeful that this Handbook will enhance the understanding of technology and encourage Research Organizations/Institutes/Start-ups to develop indigenous Rapid Food Testing Kits/Equipment/Method.

17th September, 2022

(Rajesh Bhushan)

PREFACE

Food safety is a public health priority as serious outbreaks of foodborne diseases have been documented globally in the past decade. As India increases its demand for food ingredients and raw materials, the need to safeguard public health throughout the entire value chain must be met. The current focus of Food Safety and Standards Authority of India (FSSAI) is to cover all scopes of the global food supply chain from farm to fork. FSSAI is working to address the challenges of an ever-increasing Indian population and ever-increasing food needs.

Food testing is paramount in light of the adverse effects of poor safety and quality on human health. The evaluation and use of traditional and sophisticated techniques for detecting food contaminants is a major challenge. Such techniques are relatively expensive, time-consuming, and labor-intensive. Moreover, the availability of such high-level analyses is limited in developing countries, such as India.

Rapid kits for food testing are the need of the hour to ensure "*faster, better, cheaper*" real-time testing of food products throughout the supply chain. FSSAI has made a rapid progress towards the use of state-of-the-art food testing technologies since 2019. FSSAI has framed a policy for approval of Rapid Analytical Food Testing (RAFT) Kits/Equipment/Methods for the purpose of facilitating on-the-spot field-testing by the Food Safety Officers (FSOs) or for their use in the Mobile Food Testing Labs. The RAFT Kits/Equipment/Methods are carefully reviewed and considered by the RAFT Committee, Scientific Panel on Methods of Sampling & Analysis and finally approved by the Competent Authority.

This Handbook on "Rapid Analytical Food Testing Kits (RAFT)" has been developed for the ease of understanding the procedure for approval of rapid kits, equipment, and methods. The criteria for validating and verifying these kits and equipment are under development by subject matter experts. These validations and checks are very important to recommend an appropriate quick method in India to safeguard the interests of food business operators, consumers and regulators.

Shri Arun Singhal
Chief Executive Officer
Food Safety and Standards Authority of India
FDA Bhawan, Kotla Road, New Delhi

September, 2022

ACKNOWLEDGEMENT

Food Safety and Standards (Laboratory and Sampling Analysis) First Amendment Regulations, 2020 stipulates a system for approving Rapid Analytical Food Testing Kits/Equipment/Methods meant for screening and surveillance purposes. FSSAI constantly supports and encourages all such startups/research institutions that are making efforts to create rapid food testing kits.

The creation of this guideline document incorporating validation/verification requirements for these "Rapid Analytical Food Testing Kits (RAFT)" would further simplify the food testing ecosystem and promote ease of doing business.

I extend my heartfelt gratitude to all members of the Committee on Rapid Analytical Food Testing Kits/Equipment/Methods Committee who have played a pivotal role in coming out with the "HANDBOOK ON RAPID ANALYTICAL FOOD TESTING (RAFT) Volume 1.0," especially Dr. Lalitha Ramakrishna Gowda whose able guidance, knowledge and insight contributed to the successful creation of this Handbook.

I owe my deepest appreciation to the Chairperson, FSSAI and CEO, FSSAI for their cooperation, support and constant encouragement without which the work would not have been complete.

September 2022

Dr. Harinder Singh Oberoi
Advisor (QA),
Food Safety and Standards Authority of India,
FDA Bhawan, Kotla Road,
New Delhi – 110002

LIST OF CONTRIBUTORS

NAME	ORGANIZATION
Dr. Lalitha Ramakrishna Gowda	CSIR-CFTRI, Mysuru (Chairperson)
Dr. Ajit Dua	Punjab Biotechnology Incubator, Department of Science and Technology and Environment, Govt. of Punjab), Mohali
Dr. Anoop A. Krishnan	Export Inspection Agency, Kochi
Dr. Deepak Sharma	CSIR – Institute of Microbial Technology, Chandigarh
Dr. Kaushik Banerjee	National Referral Laboratory, National Research Centre for Grapes, Pune
Dr. Kiran N Bhilegaonkar	ICAR-Indian Veterinary Research Institute Regional Station, Pune
Dr. SK Panda	ICAR - Central Institute of Fisheries Technology, Kochi
Dr. Bhaskar Datta	Indian Institute of Technology, Gandhinagar
Dr. Harinder Singh Oberoi	Food Safety and Standards Authority of India
Mr. Rakesh Kr Yadav	Food Safety and Standards Authority of India
Dr. Dinesh Kumar	Food Safety and Standards Authority of India
Ms. Kanika Aggarwal	Food Safety and Standards Authority of India
Mr. Pravesh Shanker Chaubey	Food Safety and Standards Authority of India
Ms. Sunaina Verma	Food Safety and Standards Authority of India

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CHAPTER 1: Introduction

Access to quality and safe food has been man's main endeavour from the earliest days of human existence. A safe food is one that is free of microbiological, chemical or physical hazards and does not cause illness or injury when consumed as intended. A sea of change in consumer awareness about food safety and its relation to health has occurred. Concerns about food safety and awareness have emanated from, changes in life style and food habits, food handling practices, availability of exotic products and processes and the globalization of trade in food. The development and application of analytical methods and techniques in food analysis has grown in parallel to the consumer's concern.

Modern food-safety management standards operate on the precepts of quality assurance and safety throughout the food chain: from farm to fork. The goal of food analysis has traditionally been, and still is, to ensure quality and safety of food. There exist several well-established analytical techniques such as spectrophotometry, high performance liquid chromatography (HPLC), gas chromatography (GC), liquid and gas chromatography couple to mass spectrometry (LC-MS/MS and GC-MS/MS), atomic absorption spectrometry, quantitative real time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA). Nevertheless, these techniques are unsuitable for direct application in the premises of food businesses as they are relatively expensive, time-consuming and labour intensive, impeding their use as point-of-need tests or rapid tests. In addition, accessibility to such high-end analytical techniques is limited.

The term "rapid testing" is used to indicate a series of analytical methods that generally share common features, such as being easy to operate, quick, and inexpensive, and above all enable on-site application. The "point-of-need-/point-of-care-test" highlights the key feature of rapid tests, i.e., the ability to provide a response to an analytical demand exactly where the demand is posed e.g., non -laboratory settings such as milk collection point/raw material testing in manufacturing unit/food being sold at shops. The point-of-need/rapid testing approach bases on simplifying and shortening the analytical process by cutting most of the steps required by traditional laboratory-based analysis yet providing the required degree of sensitivity and reliability of the result. To this end, there is a high demand for cost-effective and robust analytical devices/kits for food safety monitoring in remote settings in order to create effective prevention and control strategies.

Several emerging technologies for rapid testing offer numerous advantages such as being rapid, affordable, sensitive, specific, user-friendly, robust, equipment free and economically profitable. Rapid methods are usually categorized according to the technique on which they are based. A rapid method can be an assay based upon colorimetric, fluorescent, immunochemical or electrochemical reactions that gives instant or real time results, but on the other hand it can also be a simple modification of a procedure that significantly reduces the

time of analysis. Colorimetric detection represents the most common detection method mainly due to its simplicity. Immunology- based methodologies are based on the specific binding of antigens with antibodies and imply the choice of an appropriate antibody as the determining factor. Other rapid methods are based on 1) modifications and automation (mechanical devices to automate tedious steps) of conventional methods, 2) use of biosensors, 3) nucleic acid-based assays, and 4) miniaturisation.

Manufacturers have developed and put on the market a gamut of commercial kits/devices that meet the constraints of conventional/classic reference methods that are described in regulations. A large number of commercial rapid analytical kits are available for food analysis. Lateral flow test strips (LFS) have been developed for the identification of food contaminations such as foodborne pathogens, chemicals and food adulterations. Innovative culture media that reduce the time to detect pathogens, enumerate quality indicators or identify bacteria are based on chromogenic substrates, that enable a very simple reading of bacterial growth by colour change and are considered as a rapid method. Similarly, commercially available ready-to-use agar plates not only save time but provide consistent results in hygiene and pathogen analysis. In techniques that involve the use of several reagents the most time intensive step is the pipetting of the reagents. Assembled rapid kits are available wherein all the reagents are in the form of tablets and require only the addition of the sample extract.

The most commonly used rapid test methods are for microbiological analysis followed by mycotoxins and allergens. Rapid microbiological tests provide a valuable resource for validating and verifying the effectiveness of sanitation practices to minimize the risk of cross-contact contamination during food manufacture. Similarly, rapid test kits for mycotoxins provide the food business operator the means to ensure that vendors supply materials that comply with specification.

Globally there are a large number of commercial manufacturers of kits. Given the potential application of the commercial rapid test kits as screening tools, it is important that the performance of these rapid methods should, be validated against established reference methods to assess their reliability before adapting a new alternative or rapid method for regulatory use. Validation of the rapid test method/kit consists in demonstrating the capability to accomplish detection and quantification at the same level of accuracy and sensitivity as the reference method and evaluated by collaborative studies.

In the past years an array of new or improved rapid kits/ equipment/methods for the detection of foodborne pathogens, toxins and other contaminants etc. have inundated the market. Having recognised the potential to revolutionize the food testing landscape by increasing the outreach of testing at the ground-level and reducing time taken to test food items the Food Safety and Standards Authority of India (FSSAI) has introduced a policy for approval of rapid food testing kits/equipment/methods benefitting the food industry and surveillance activities in the country. FSSAI, as a regulatory agency tasked with ensuring the safety of the nation's food supply has ensured that such laboratory methods needed to support regulatory compliance, and

enforcement actions meet the highest analytical performance standards appropriate for their intended purposes. FSSAI has published the policy as a regulation in the Gazette, namely "Food Safety and Standards (Laboratory and Sample Analysis) First Amendment Regulations, 2020". The rapid food testing kits/equipment approved by FSSAI are to be used only for screening and surveillance purposes only

SCOPE: The scope of this handbook is to outline the policies and procedures laid down by FSSAI essential for the approval of new or improved rapid kits/ equipment/methods. The handbook details the application format and the review process including the criteria by which kits/methods etc., shall be evaluated. The validation/ verification criteria for these kits/equipment for chemical, microbiological and nucleic acid/protein- based assay are also described.

1.1. Rapid Analytical Food Testing (RAFT) Scheme:

The RAFT scheme is included in Food Safety and Standards (Laboratory and sample Analysis) First Amendment Regulations, 2020 which includes

"*2.4 Approval of Rapid Analytical Food testing Kit, Equipment or Method-*

2.4.1 Food Authority may approve Rapid Analytical Food Testing Kit, Equipment or Methods for the purpose of testing of Food.

2.4.2 *The procedure for approval of Rapid Analytical Food Testing Kit, Equipment or Methods shall be as per the guidelines that may be framed by the Food Authority from time to time."*

FSSAI has constituted a committee for scrutinization of applications received by FSSAI under this scheme. The recommendations of RAFT committee are ratified by the Scientific Panel on Methods of Sampling and Analysis (SPSMA) and approved by the Competent Authority before adoption/implementation. Thereafter, the status of all the rapid kit/equipment is placed before the Food Authority for ratification (https://fssai.gov.in/cms/raft.php)

The rapid food testing kits/equipment approved by FSSAI are to be used for screening and surveillance purposes only. For enforcement purpose, the method as described in the FSSAI Manuals of Methods of Analysis of Foods and/ or the method as described in tables of Appendix B of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 are to be used.

Application processing fee

Application processing fee of Rs. 2,000 + GST @18% (not included in the fees prescribed against each category) per application/product in favour of Sr. Accounts Officer, FSSAI payable at New Delhi by Demand Draft or through online mode.

(i) The manufacturer/method developer shall pay a fee of Rs. 25,000 + GST @18% for the issuance of the Conformance Certificate (CC)

(ii) Subsequent renewal, if recommended by RAFT Committee & SPMSA and approved by the competent authority, will attract a renewal fee of Rs. 10,000 + GST @18%.

Bank account details of FSSAI are mentioned below:

Name: Senior Accounts Officer, FSSAI, New Delhi
Bank: Bank of Baroda, Nirman Bhawan
Account No: 26030100008653
IFSC Code: BARB0(Zero)NIRDEL
The GST No. of FSSAI is 07AAAGF0023K1ZV (0 is Zero).

CHAPTER 2: GUIDELINES FOR APPROVAL OF RAPID ANALYTICAL FOOD TESTING KIT/EQUIPMENT OR METHOD

1. Purpose & Scope -

To lay down a general procedure for approving test methods, kit(s) or portable (hand-held) equipment designed to perform rapid analytical food testing vis-a-vis corresponding official FSSAI/ Standard method.

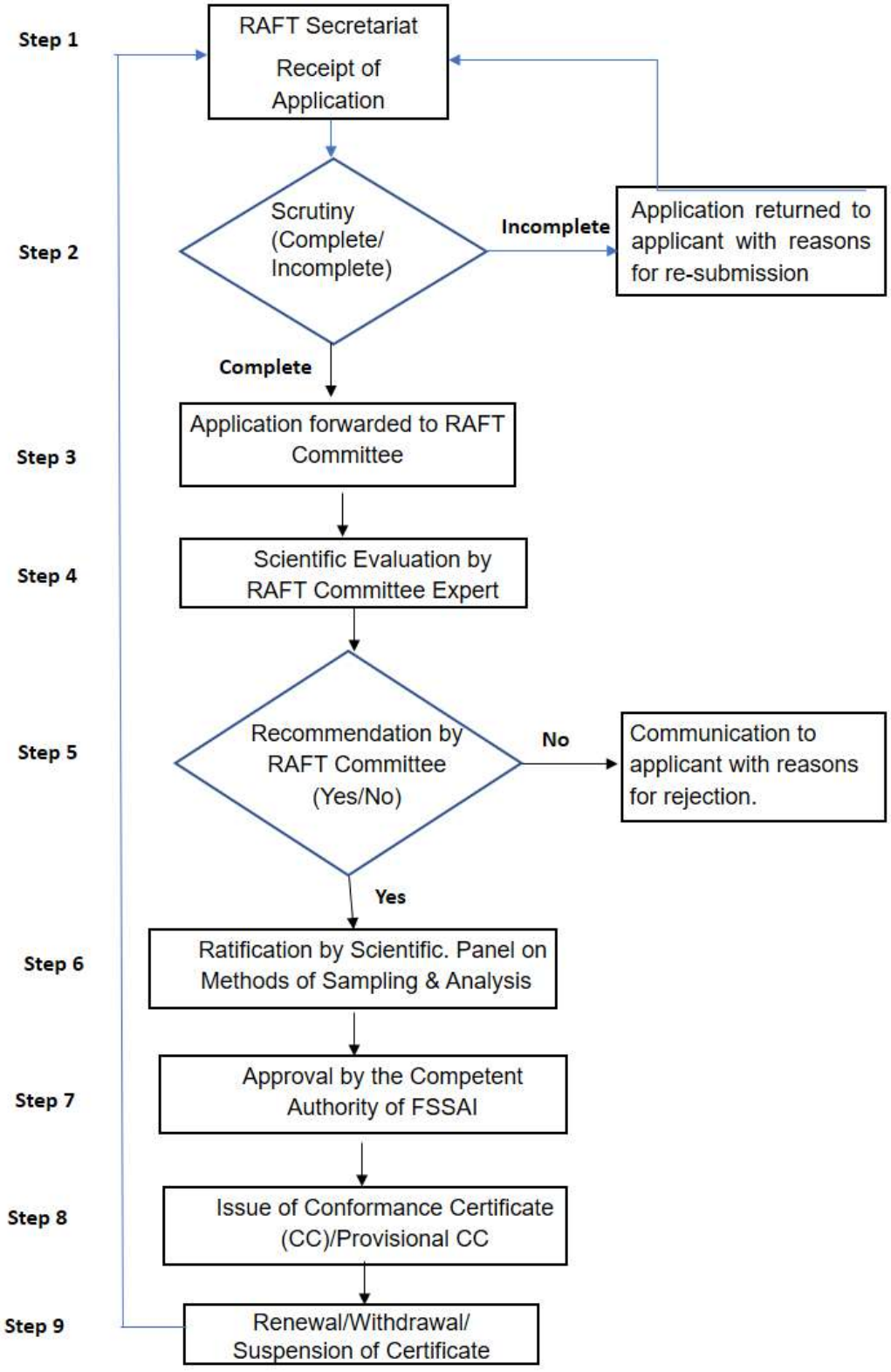
2. Definitions -

Applicant	The manufacturer(s) of test kit(s)/ hand held equipment or individual/ group that have developed the rapid method/ protocol.
Hand held equipment	Any portable equipment that requires no or minimum electrical connections, easy to use and operate at field level. Preferable such equipment should have reference to conventional methods or parameters that they can analyse.
Official Methods	Methods approved by FSSAI.
Qualitative method	A method that identifies analyte(s) based on chemical, biological, or physical properties; method of analysis whose response is either the presence or absence of the analyte detected either directly or indirectly in a certain amount of sample. Most qualitative methods are or can be made at least "semi-quantitative" to provide rough estimates of the amount of analyte present.
Quantitative method	A method that measures analyte(s) based on chemical, biological, biophysical or physical properties in terms of a numerical value.
Rapid method(s) / Protocol(s)	Any method of analysis that reduces the time period of testing and gives instantaneous results which may be useful for preliminary screening and surveillance purposes at the field level.
Rapid test kits	Any kits intended to be applicable at the field level with a precision closer to that of conventional methods by reducing the time period of testing.

Standard method	Internationally accepted and widely recognized methods (AOAC/ ISO/ IS/ APHA/ BAM/FSSAI/IDA/IDF etc).
Validation	Validation is the confirmation by examination and provision of objective evidence that the particular requirements of the rapid test kit/equipment/method for a specific intended use are fulfilled. Method validation criteria may include: sensitivity, accuracy, trueness, reproducibility and robustness/ruggedness, precision.
Verification	Provision of objective evidence that a laboratory can adequately operate the rapid test kit/equipment/ method, achieving the performance requirements for the sample matrices to which they are being applied.
Verifying Laboratory	Those notified laboratories identified by the FSSAI for carrying out the work of verifying the test kits/equipment/method.

3. Summary of the process -

The process followed for approval of RAFT kit/equipment/method is shown in the flow diagram below:



Flow chart showing the steps involved in the approval process

4. Explanation of Process Step-wise:

Step 1: Receipt of application (Annexure 1) by RAFT Secretariat/Advisor Quality Assurance Division (QA), Food Safety and Standards Authority of India, FDA Bhawan, Kotla Road, New Delhi – 110002.

Step 2: Scrutiny of application by RAFT Secretariat (3-4 working days from the date of receipt) to ensure the following:

I. Requisite processing fee (non-refundable – Rs. 2000 + GST@18%) is enclosed
II. Duly filled and complete application in the prescribed format **(Annexure-I)**
III. Mandatory documents attached
IV. The minimum eligible criteria are met
V. Signature of relevant authority with official seal

Incomplete applications are returned to the applicant providing reasons/explanations for the same.

Step 3: Applications complete in all aspects are forwarded to the RAFT Committee electronically for further evaluation.

Step 4: Evaluation by RAFT Committee

I. Technical review of the applications and documents by an expert member of the committee
II. A scientific and regulatory review to evaluate the effectiveness of the rapid test kit/equipment/method in comparison with the conventional /standard method and documentation as detailed in the checklist **(Annexure-II)**
III. Estimated timeline for evaluation is 4-6 weeks

Step 5: Review and recommendation by RAFT Committee-

I. The review and recommendations discussed at the RAFT committee meeting
II. If required the committee may invite the applicant for presentation.
III. The decision of the committee for approval /rejection is based on stringent requirements of regulatory compliance and the intended use.
IV. The recommendation are of the following three categories:

1. Final approval without verification from any laboratory – This is given when the rapid kit/equipment/method meets the testing requirements of Food Safety and Standards Rules and Regulations (2011) and is validated against International Standards for food categories for the intended use. This is for a period of three years for both screening and surveillance purpose.

2. Provisional approval for one year – This approval is given when the rapid kit/equipment/ method needs verification in matrices of the Indian Food categories. Within the provisional approval period, the applicant is required to carry out the necessary verification and submit the verification results. The verification may be carried out in laboratories identified for the purpose (list of labs at **Annexure – III**). The fee for verification of rapid kit/equipment/method will be as prescribed by FSSAI from time to time. On receipt

of the verification results from the laboratory, the same is placed before the RAFT Committee for review and recommendation.

3. Not Approved – Any rapid kit/equipment/method that does not meet the criteria and does not have relevant documentation in place etc. is not approved/ rejected.

Step 6: Ratification by Scientific Panel on Methods of Sampling & Analysis (SPMSA) in subsequent meeting. If SPMSA raises some observation on any application, then it is returned to RAFT Committee at Step 5.

Step 7: Approval by the Competent Authority of FSSAI

All recommendations of RAFT Committee and SPMSA will be placed before the Competent Authority of FSSAI for final approval.

Step 8: Issue of Conformance Certificate (CC) and Provisional CC – The RAFT Secretariat after approval of Competent Authority will:

I. Seek requisite fee from applicants for issuance of Conformance Certificates (CC) to finally approved rapid kit/equipment/method (Rs. 25,000 + GST @18% per rapid kit/equipment).

II. Simultaneously, prepare certificates (both final & provisional approval).

III. The Conformance Certificate is issued to the applicant valid for 3 years.

IV. The Provisional Approval certificate for kit/equipment/method is valid for 1 year.

V. The applicant for rapid kit/equipment/method not approved is officially informed citing reasons for rejecting the same.

VI. Details of all approvals/rejections etc of kit/equipment/method will be made available at the FSSAI website (www.fssai.gov.in).

Step 9: Renewal/Withdrawal/Suspension of Certificate –

Renewal

I. Renewal applications and fees must be sent to FSSAI not less than 60 days prior to the expiration date on the certificate

II. A renewal fee of Rs. 10,000 + GST @18% is levied for subsequent renewal.

III. In case of 'no change' applicants are required to submit an undertaking to affirm that no changes have been made to the rapid kit/equipment/method since originally receiving FSSAI approval status and to confirm that the kit/equipment/method performs as originally evaluated (Annexure IV).

IV. In case of 'any modifications/changes to the rapid kit/equipment/method applicants are required to submit a list of all modifications to the test method components, instrumentation, intended use claims, or package insert with updated validation documents and additional data for further review

Withdrawal/Suspension

FSSAI may withdraw or suspend the CC or PCC issued to applicant on the basis of its own investigation or any complaint received thereof or voluntarily withdrawal request from the applicant.

FSSAI approval status shall be suspended if:

1) Serious adverse comments with supporting data have been received from rapid kit/method/device users indicating the rapid kit/method/device does not consistently perform as claimed, and applicant has not provided a satisfactory resolution.

2) Undisclosed modifications are discovered for which the applicant did not submit data, or the data submitted in support of modifications is determined to be insufficient to demonstrate equivalency to the original condition of FSSAI approval

3) The renewal application is more than 30 days past due. The rapid kit/method/device will be removed from the list of approved rapid kit/method/device maintained by FSSAI its website, and the applicant may not claim that the rapid kit/method/device is approved as a FSSAI approved rapid kit/method/device.

CHAPTER 3: GUIDELINES FOR THE VERIFICATION OF MICROBIOLOGICAL ANALYSIS

Scope: Verification is intended to demonstrate that a validated method performs, in the user's hands, according to the method's specifications determined in the validation study and is fit for its intended purpose. Hence, verification is applicable to methods that have been already validated using an interlaboratory study.

Applicable standard: ISO 16140-3: 2021- Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory

Requirements for conducting verification study

Before conducting verification of a rapid microbiological testing kit/method, the assigned laboratory must have the following documents/information:

1. Complete validation study report as per ISO 16140-2 or ISO 16140-5 or ISO 16140-6
2. Food categories in which validation study was conducted
3. LOD 50 value reported in the validation study
4. Inter-laboratory reproducibility standard deviation (SR) of the lowest mean value of the (food) item (Required for quantitative methods)

Types of Verification to be performed

- Implementation verification
- Food Item verification

A. VERIFICATION PROTOCOL FOR QUALITATIVE MICROBIOLOGICAL RAPID TEST KITS

For qualitative tests, determine eLOD50 for both implementation verification and food item verification.

I. Steps for implementation verification

- Select one food item that was tested during the validation. Also note the sample size that was used for validation.
- Artificially inoculate the test portion of the sample with overnight grown culture of the target organism at the following levels:
    - 9 × LOD50 level: 1 replicate
    - 3 × LOD50 level: 4 replicates
    - 1 × LOD50 level: 4 replicates
    - 1 Blank

      (subsequent 1:3 dilutions can also be made)

- Test all the replicates as per the instructions provided in the rapid testing kit
- Simultaneously determine the inoculation level of the target organism using a non-selective agar plating method or 3 dilutions × 3 tube Most Probable Number (MPN) approach
- Determine eLOD50 as per the following table:

Inoculation Levels				eLOD50 level cfu/test portion
9×LOD50 level/test portion (High)	3×LOD50 level/test portion (Medium)	1×LOD50 level/test portion (Low) LIL	Blank	
1/1	4/4	4/4	0/1	<1xLIL
1/1	4/4	3/4	0/1	=0.5x LIL
1/1	4/4	2/4	0/1	=0.7xLIL
1/1	4/4	1/4	0/1	=1.0xLIL
1/1	4/4	0/4	0/1	=1.5xLIL
1/1	3/4	4/4	0/1	=0.7xLIL
1/1	3/4	3/4	0/1	=1.0xLIL
1/1	3/4	2/4	0/1	=1.3xLIL
1/1	3/4	1/4	0/1	=1.7xLIL
1/1	3/4	0/4	0/1	=2.3xLIL
1/1	2/4	4/4	0/1	=1.1xLIL
1/1	2/4	3/4	0/1	=1.5xLIL
1/1	2/4	2/4	0/1	=1.9xLIL
1/1	2/4	1/4	0/1	=2.6xLIL
1/1	2/4	0/4	0/1	=3.7xLIL
1/1	1/4	4/4	0/1	Unreliable
1/1	1/4	3/4	0/1	=2.1xLIL
1/1	1/4	2/4	0/1	=2.8xLIL

1/1	1/4	1/4	0/1	=4.0xLIL
1/1	1/4	0/4	0/1	=6.3xLIL
1/1	0/4	4/4	0/1	Unreliable
1/1	0/4	3/4	0/1	=3.0xLIL
1/1	0/4	2/4	0/1	=4.3xLIL
1/1	0/4	1/4	0/1	=6.7xLIL
1/1	0/4	0/4	0/1	=14.0xLIL

- The rapid test kit is acceptable if the $eLOD50 \leq 4 \times LOD50$

II. Steps for food item verification

- Select the following food categories
    - 1 from already validated food category;
    - 4 from other categories not tested during validation
- Include the most challenging food item in the food category
- Estimate eLOD50 as above for each food item and apply the same acceptance criterion.
- When the result does not meet the acceptance criterion, perform a root cause analysis to provide an explanation for the observed results.

B. VERIFICATION PROTOCOL FOR QUANTITATIVE MICROBIOLOGICAL RAPID TEST KITS

For quantitative tests, determine "intra-laboratory reproducibility standard deviation (SIR)" for implementation verification and "estimated bias (eBias)" for food item verification.

I. Steps for implementation verification

- Select any (food) item that is within the scope of laboratory application
- Collect 10 samples of the same food item at contamination levels naturally observed in these food items
- Homogenize the samples individually and separate to 2 test portions
- If required, artificial contamination with the test organisms can be done to the initial suspension of test portions.
- Using the rapid test kit, analyze the two test portions of 10 food items individually by 2 different technicians.
- Calculate intra-laboratory standard deviation (S_{IR}) as ISO 19036 using the formula

$$S_{IR} = \sqrt{\frac{1}{2p}\sum(y_{iA-}y_{iB})^2}$$

where,
S_{IR} is the intra-laboratory reproducibility standard deviation;
i is the index of the laboratory sample, i = 1 to p (p ≥ 10);
p is the number of samples;
yiA, yiB are the log transformed data

- Compare calculated S_{IR} with S_R
  (Check for S_R value in the validation report submitted for the kit)
- The rapid test kit is acceptable if intra-laboratory reproducibility standard deviation (S_{IR}) is ≤ 2 × the interlaboratory reproducibility standard deviation (S_R) of the lowest mean value of the (food) item
- When the result does not meet the acceptance criterion, perform a root cause analysis to provide an explanation for the observed results

II. Steps for food item verification

- Select the following food categories
  - 1 from already validated food category;
  - 4 from other categories not tested during validation
- Include the most challenging food item in the food category
- Homogenise each food item separately and prepare suspension
- Artificially contaminate the initial suspension of food items at 3 inoculation levels within the range of the kit
- Run in parallel inoculum suspension (pure culture) at same dilution levels
- Using rapid test kit, analyse all inoculation levels (both artificially contaminated food item and pure inoculum suspension) in duplicates
- Also analyse negative control (uninoculated test portions) in duplicates
- Express the results in Log_{10} CFU/g or Log_{10} CFU/ml
- Compare the results, of the artificially contaminated (food) item to the results of the inoculum suspension
- The Rapid Test Kit is acceptable if at each level, the difference between the results of the artificially contaminated (food) item and that of the inoculum suspension is equal to or less than 0.5 log10 CFU/g (of CFU/ml)
- When the result does not meet the acceptance criterion, perform a root cause analysis to provide an explanation for the observed results

C. VERIFICATION PROTOCOL FOR VALIDATED ALTERNATIVE CONFIRMATION AND TYPING METHODS USING RAPID TEST KITS

It only requires implementation verification. Here the sample is isolated colony of the target organism on defined selective or non-selective agar plates.

Steps for Implementation Verification:

- Use the same selective or non-selective media used during validation (Refer the validation report submitted)
- Select 5 target and 5 non-target strains for conducting inclusivity and exclusivity study. The strains should be of food-origin.
- Perform the test for all 10 strains using the rapid alternative confirmation kit
- The rapid test kit is acceptable if there is 100% agreement
- When the result does not meet the acceptance criterion, perform a root cause analysis to provide an explanation for the observed result

A. Worked out example of verification of Qualitative microbiological rapid test kits

A rapid *Salmonella* Testing Kit validated as per ISO 16140- 2 is submitted for verification. The LOD_{50} value is 2.5 cfu/test portion (25 g). During validation, the kit was tested against raw beef meats, dairy products, Fruits and vegetables and environmental samples. The manufacturer wants to verify the suitability of the kit for other food matrices mentioned in FSSR.

I. Implementation verification

For implementation verification, 1 ml of 10^{-7} dilution of overnight culture of *Salmonella enterica* Typhimurium is inoculated to initial suspension of vegetable (cabbage). Sequential 1:3 dilutions of *Salmonella* were prepared from 10^{7} dilution and 1 ml of the corresponding dilutions were transferred to the initial suspension of the individual test portion (4 replicates each). Initial inoculum level was also determined. The Kit was used to find out presence of *Salmonella* from blank, low, medium and high inoculation levels.

The following values were reported: Initial Inoculum level: 6.0×10^{8} cfu/ml

Record of number of positive results obtained at each inoculum level

	High inoculation Level (20 cfu/test portion)	Intermediate inoculation level (6 cfu/test portion)	Low inoculation Level (2 cfu/test portion)	Blank
No. of tubes	1	4	4	1
No. of positive result obtained	1	4	1	0

In this example, lowest inoculation level is 2 cfu/test portion

As per the Table, eLOD50=1×LIL = 1× 2 cfu/ test portion = 2 cfu/ test portion

Acceptance criteria is that calculated eLOD50 should not be greater than 4 times LOD50

4 times LOD50 of this kit = 4×2.5 cfu/ test portion=10 cfu/test portion

As eLOD 50 (2 cfu/ test portion) is less than 4 times LOD50 of this kit (10 cfu/test portion), the kit passes implementation verification.

II. Food item verification

At this step verification was done for the following matrices:

(1 already validated matrix and 4 new matrices with challenging food item)

Sl.No.	Food Category	Type	Most Challenging Food
1.	Fresh produce and fruits	Leafy greens	Cabbage (already validated
2.	Ready- to- eat, ready to- reheat fishery products	Acidified and marinated fishery products	Sardine Pickle
3.	Raw milk and dairy products	Raw milk based products, with high fat content and/or high background microbiota	Butter
4.	Ready- to eat, Ready reheat meat products	Raw cured (smoked)	Ham
5.	Dried cereals, fruits, nuts, seeds and vegetables	Seasonings	Black pepper powder

eLOD50 values were calculated for the above 5 matrices and following observations were made:

Sl.No.	Food Item	eLOD50	Interpretation (should be less than 4*LOD50=10

1.	Cabbage	2.0	Satisfactory
2.	Sardine Pickle	3.0	Satisfactory
3.	Butter	3.0	Satisfactory
4.	Ham	3.4	Satisfactory
5.	Black pepper powder	7.4	Satisfactory

III. Conclusion: As the kit conforms to the acceptance criteria of both implementation verification and food item verification, the kit is accepted as a rapid testing kit for determination of Salmonella for the following matrices:

- Fresh produce and fruits
- Ready- to- eat, ready to- reheat fishery products
- Raw milk and dairy products
- Ready- to eat, ready to- reheat meat products
- Dried cereals, fruits, nuts, seeds and vegetables

B. Worked out example of verification of Quantitative Microbiological Rapid Test Kits

A rapid Staphylococcus aureus estimation kit validated as per ISO 16140- 2 is submitted for verification. During validation the kit was tested against heat- processed milk and dairy product, cooked shrimp,

cooked meat and pet food samples. The manufacturer wants to verify the suitability of the kit for other food matrices mentioned in FSSR.

I. Implementation verification

I. For implementation verification, the food item "cooked shrimp" was chosen and *Staphylococcus aureus* ATCC 25923 was chosen as the strain for the artificial inoculation.

II. Overnight grown culture of *Staphylococcus aureus* was diluted and kept ready for 10 different inoculum levels. Initial inoculum levels were enumerated prior to the experiment.

III. 10 different brands (independent samples) of cooked shrimp were collected and chosen for verification. Each brand of cooked shrimp was divided into 2 test portions (A &B).

IV. Each set of the ten laboratory samples were inoculated at different between 30 and 30 000 cfu/g. The culture were inoculated into the initial suspensions which have been prepared using 10 g test portions. Inoculum levels were same for A &B of each brand.

Using the rapid test kit, results were obtained for all the samples.

Laboratory sample number	Expected contamination level (cfu/g)	Result A (xiA) (cfu/g)	Result B (xiB) (cfu/g)	Log10 result A	Log10 result B	Absolute difference	Squared difference
1	30	35	28	1.54	1.45	0.10	0.009392
2	30	40	21	1.60	1.32	0.28	0.078311
3	300	110	182	2.04	2.26	-0.22	0.047820
4	300	410	620	2.61	2.79	-0.18	0.032259
5	300	350	510	2.54	2.71	-0.16	0.026733
6	600	780	640	2.89	2.81	0.09	0.007381
7	600	620	1300	2.79	3.11	-0.32	0.103395
8	6000	8600	6400	3.93	3.81	0.13	0.016466
9	6000	16000	5000	4.20	3.70	0.51	0.255177
10	30000	20000	32000	4.30	4.51	-0.20	0.041665
						SUM	0.618599
						SUM/(2x10)	0.030930
						$S_{IR}=\sqrt{(0.03930)}$	0.18

The calculated S_{IR} value of 0.18 is compared with the results of the validation study carried out for the rapid test kit as per ISO 16140- 2. The S_{IR} obtained is compared to the lowest mean value of S_R for any of the items tested in the validation study.

Summary of S_R values obtained during validation of rapid test kit for Staphylococcus aureus

Food Item	**S_R values from the validation study**			
	Inoculation level			
	Low	***Intermediate***	***High***	***Mean value***
Pasteurized milk	0.24	0.18	0.19	0.20
Cooked shrimp	0.19	0.22	0.23	0.21
Cooked meat	0.31	0.35	0.28	0.31
Pet food	0.23	0.2	0.25	0.23

Lowest mean value of S_R from validation study: 0.20

$2 \times S_R = 0.40$

Acceptability criteria is $S_{IR} < 2 \times S_R$

As 0.18 (S_{IR} obtained during verification) is less than 0.40 (i.e.2 times S_R), the rapid test complies with the acceptable criteria of implementation verification.

III.Food Item verification

The user laboratory wants to verify the rapid test kit for estimation of *Staphylococcus aureus* in

Smoked Tuna Meat "Masmin". For this eBias was determined as follows:

1. Three independent samples (manufactured in different batches) were collected.
2. From each Masmin samples, 2 test potions of 25 g each were collected and initial suspensions were made using phosphate buffer.
3. Overnight grown culture of Staphylococcus aureus were diluted to 3 different levels and inoculated in duplicates to initial suspensions. Corresponding inoculations were also made to phosphate buffer blanks (without food) and separate set of blanks (without inoculum) were also made.
4. Using rapid test kits, the count of *S. aureus* was determined. The counts were expressed in Log10 CFU/g

Sample	Artificially contaminated			Inoculum suspension [without			**eBIAS** Absolute difference in results between artificially contaminated (food) item and the inoculum suspension
	Test Portion 1	Test Portion 2	Mean (A)	Test Portion 1	Test Portion 2	Mean (B)	
1	1.45	1.9	1.675	1.98	2.12	2.05	0.375
2	3.26	2.97	3.115	3.45	3.33	3.39	0.275
3	3.96	4.32	4.14	4.54	4.32	4.43	0.29

Acceptable criteria: The absolute difference in results between artificially contaminated (food) item and the inoculum suspension at each level should be less than 0.5.

As the results indicate that at each level of contamination the absolute difference between the two results is less than 0.5 log10 CFU, so the method to be verified works correctly in the user laboratory.

III. Conclusion:

As the Kit conforms to the acceptance criteria of both implementation verification and food item verification, the kit is accepted as a rapid testing kit for estimation of *Staphylococcus aureus* for fish and fishery

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For implementation verification, 1 ml of 10^{-7} dilution of overnight culture of *Salmonella enterica* Typhimurium was inoculated to initial suspension of vegetable (cabbage). Sequential 1:3 dilutions of *Salmonella* were prepared from 10^{-7} dilution and 1 ml of the corresponding

dilutions were transferred to the initial suspension of the individual test portion (4 replicates each). Initial inoculum level was also determined. The Kit was used to find out presence of Salmonella from blank, low, medium and high inoculation levels.

The following values were reported: Initial Inoculum level: 6.0×10^8 cfu/ml

Record of number of positive results obtained at each inoculum level

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No. of tubes inoculated	1	4	4	1
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In this example, lowest inoculation level is 2 cfu/test portion

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Acceptance criteria is that calculated eLOD50 should not be greater than 4 times LOD50

4 times LOD50 of this kit = 4x2.5 cfu/ test portion=10 cfu/test portion

As eLOD 50 (2 cfu/ test portion) is less than 4 times LOD50 of this kit (10 cfu/test portion), the kit passes implementation verification.

II. Food Item verification

At this step verification was done for the following matrices:

(1 already validated matrix and 4 new matrices with challenging food item)

Sl.No.	**Food Category**	**Type**	**Most Challenging Food**
1.	Fresh produce and fruits	Leafy greens	Cabbage (already validated

2.	Ready- to- eat, Ready to- reheat fishery products	Acidified and marinated fishery products	Sardine Pickle
3.	Raw milk and dairy products	Raw milk based products, with high fat content	Butter
4.	Ready- to eat, ready to reheat meat products	- Raw cured (smoked)	Ham
5.	Dried cereals, fruits, nuts,	Seasonings	Black pepper powder

eLOD50 values were calculated for the above 5 matrices and following observations were made:

Sl.No.	**Food Item**	**eLOD50**	**Interpretation (should be less than 4*LOD50=10 cfu/test portion)**
1.	Cabbage	2.0	Satisfactory
2.	Sardine Pickle	3.0	Satisfactory
3.	Butter	3.0	Satisfactory
4.	Ham	3.4	Satisfactory
5.	Black pepper powder	7.4	Satisfactory

III. Conclusion: As the Kit conforms to the acceptance criteria of both implementation verification and food item verification, the kit is accepted as a rapid testing kit for determination of Salmonella for the following matrices:

- Fresh produce and fruits

- Ready- to- eat, ready to- reheat fishery products
- Raw milk and dairy products
- Ready- to eat, ready to- reheat meat products
- Dried cereals, fruits, nuts, seeds and vegetables

B. Worked out example of verification of Quantitative Microbiological Rapid Test Kits

A rapid Staphylococcus aureus estimation kit validated as per ISO 16140- 2 is submitted for verification. During validation the kit was tested against heat- processed milk and dairy product, cooked shrimp,

cooked meat and pet food samples. The manufacturer wants to verify the suitability of the kit for other food matrices mentioned in FSSR.

I. Implementation verification

For implementation verification, the food item "cooked shrimp" was chosen and Staphylococcus aureus ATCC 25923 was chosen as the strain for the artificial inoculation.

Overnight grown culture of Staphylococcus aureus was diluted and kept ready for 10 different inoculum levels. Initial inoculum levels were enumerated prior to the experiment.

10 different brands (independent samples) of cooked shrimp were collected and chosen for verification. Each brand of cooked shrimp was divided into 2 test portions (A &B).

Each set of the ten laboratory samples were inoculated at different between 30 and 30 000 cfu/g. The culture were inoculated into the initial suspensions which have been prepared using 10 g test portions. Inoculum levels were same for A &B of each brand.

Using the rapid test kit, results were obtained for all the samples.

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9	6000	16000	5000	4.20	3.70	0.51	0.255177
10	30000	20000	32000	4.30	4.51	-0.20	0.041665
						SUM	0.618599
						SUM/(2x10)	0.030930
						SIR=√(0.03930)	0.18

The calculated S_{IR} value of 0.18 is compared with the results of the validation study carried out for the rapid test kit as per ISO 16140- 2. The S_{IR} obtained is compared to the lowest mean value of S_R for any of the items tested in the validation study

Summary of S_R values obtained during validation of rapid test kit for *Staphylococcus aureus*

Food Item	**S_R values from the validation study**			
	Low inoculation	***Intermediate inoculation level***	***High inoculation***	***Mean value of***
Pasteurized milk	0.24	0.18	0.19	0.20
Cooked shrimp	0.19	0.22	0.23	0.21
Cooked meat	0.31	0.35	0.28	0.31
Pet food	0.23	0.2	0.25	0.23

Lowest mean value of S_R from validation study: 0.20

$2xS_R=0.40$

Acceptability criteria is $S_{IR}<2xS_R$

As 0.18 (SIR obtained during verification) is less than 0.40 (i.e.2 times SR), the rapid test complies with the acceptable criteria of implementation verification.

II. Food Item Verification

The user laboratory wants to verify the rapid test kit for estimation of *Staphylococcus aureus* in Smoked Tuna Meat "Masmin". For this eBias was determined as follows:

1. Three independent samples (manufactured in different batches) were collected.
2. From each Masmin samples, 2 test potions of 25g each were collected and initial suspensions were made using phosphate buffer.
3. Overnight grown culture of Staphylococcus aureus were diluted to 3 different levels and inoculated in duplicates to initial suspensions. Corresponding inoculations were also made to phosphate buffer blanks (without food) and separate set of blanks (without inoculum) were also made.
4. Using rapid test kits, the count of S. aureus was determined. The counts were expressed in Log_{10} CFU/g

Sample	Artificially contaminated			Inoculum suspension [without (food) item]			**eBIAS** Absolute difference in results between artificially contaminated (food) item and the inoculum suspension (A- B)
	Test Portion 1	Test Portion 2	Mean (A)	Test Portion 1	Test Portion 2	Mean (B)	
1	1.45	1.9	1.675	1.98	2.12	2.05	0.375
2	3.26	2.97	3.115	3.45	3.33	3.39	0.275
3	3.96	4.32	4.14	4.54	4.32	4.43	0.29

Acceptable criteria: The absolute difference in results between artificially contaminated (food) item and the inoculum suspension at each level should be less than 0.5.

As the results indicate that at each level of contamination the absolute difference between the two results is less than 0.5 log10 CFU, so the method to be verified works correctly in the user laboratory.

III. Conclusion:

As the Kit conforms to the acceptance criteria of both implementation verification and food item verification, the kit is accepted as a rapid testing kit for estimation of Staphylococcus aureus for fish and fishery products.

GLOSSARY

Verification

Demonstration that a validated method functions in the user's hands according to the method's specifications determined in the validation study and is fit for its purpose

Validation

Establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled

Test portion

Specified quantity of the sample that is taken for analysis, e.g. 10 g, 25 g, 375 g of samples, or sponges for environmental samples, or boot socks for primary production samples

Sample

Food, feed, environmental, or primary production specified item to be included in the validation as per the intended use of the method

Reproducibility standard deviation

Standard deviation of test results obtained under reproducibility conditions

Qualitative method

Method of analysis whose response is that the analyte is either detected or not detected, either directly or indirectly in a specified test portion

Quantitative method

Method of analysis whose response is the amount [count or mass] of the analyte measured either directly (e.g. Enumeration in a mass or a volume), or indirectly (e.g. Colour absorbance, impedance, etc.) in a specified test portion

LOD50

Level of detection for which 50 % of tests give a positive result

Estimated LOD50 (eLOD50)

Determination of the LOD50 (level of detection at 50 % probability of detection) based on the experimental design described in this document

Bias

Measurement bias estimate of a systematic measurement error, or the systematic difference between the quantitative assigned value and the average of measurement replicate results

Estimated bias (eBias)

Determination of the bias based on the experimental design described in this document

Inclusivity study

Study involving pure target strains to be detected or enumerated by the alternative method

Exclusivity study

Study involving pure non-target strains, which can be potentially cross-reactive, but are not expected to be detected or enumerated by the alternative method

CHAPTER 4: *GUIDELINES FOR THE VERIFICATION OF CHEMICAL ANALYSIS*

1. **Introduction**

ISO/IEC 17025:2017 section 7.2.1.5 states:

"…The laboratory shall verify that it can properly operate standard methods before introducing them by ensuring that it can achieve the required performance".

This Guide concentrates on the technical aspects of method verification to be done by a kit provider/method/equipment, with the acceptance criteria. Where a facility/laboratory uses a commercial test kit in which the methodology and reagents are unchanged from the manufacturer's instructions, the kit does not need to be independently revalidated in the testing facility. Essentially the facility only needs to verify that their operators using their equipment in their laboratory environment can apply the method obtaining the same outcomes as defined in the validation data provided in the standard method. Verification of methods by the facility must include statistical correlation with existing validated methods prior to use.

2. Purpose

The purpose of the guide is to define the activities that are required to fulfill method/kit/equipment verification based on analytical method performance characteristics. This guideline is applicable for the verification of kits/methods/equipment (Qualitative, Quantitative and Semi quantitative). The method/kit/equipment under verification shall be validated using an international protocol and shall be fit for purpose before taking it up for verification.

Method verification studies are typically less extensive than those required for method validation. Nevertheless, the kit/method/equipment should demonstrate the ability to achieve the claimed performance characteristics of the standard method/validated method under the laboratory's own test conditions. Essentially, the facility only needs to verify that their operators using their equipment in their laboratory environment can apply the method and obtain the same outcomes as defined in the validation data provided in the standard method/ in this guidelines. Verification of methods by the facility must include statistical correlation with existing validated methods prior to use.

3. Scope

Verification that a laboratory can adequately operate a standard method and provide objective evidence the performance characteristics specified in the test method have been met with the matrices to which the method is being applied. Most often, the critical requirements of method verification include evaluation of accuracy and the precision (generally accepted as repeatability and reproducibility), which are reflected in the measurement uncertainty. The objective evidence is the accuracy and precision obtained from actual lab data.

4. Method verification approaches

Chemical analytical methods fulfill many different purposes, from quantifying an analyte at a low concentration to qualitative (presence or absence). With such a variety of methods, it is logical that different test methods might require different verification approaches. For ease, the test methods can be divided into two different categories based on their purpose. The categories are listed below. For each of the categories of test methods, only relevant performance characteristics are required to be included in the method verification process. The approach of this guide is to list all performance characteristics needed for verification, and explain the reason for verifying the performance characteristic.

The two categories of chemical analytical methods are:

i. Quantitative analysis
ii. Qualitative analysis

Quantitative test requirements

The key parameters to be considered in the verification process for quantitative test will depend on the nature of the method and the range of sample types likely to be encountered. A statistically significant number of samples must be used in the evaluation process and these must cover the full range of results for the intended use. The measurement of bias and measurement of precision are the minimum requirements for methods that yield quantitative results. For trace analyses, the facility should also confirm that the achievable limit of detection (LOD) and limit of quantitation (LOQ) are fit for purpose. For qualitative methods, correlation studies with existing validated methods or comparisons with known outcomes are required. Method verification shall be carried out as per the Table 1 given below.

Table 1: Quantitative analysis requirements

Performance characteristics	Verification activities	Reason for verification	Matrix	Number of replicates	Work flow with example
Accuracy	Demonstrate accuracy for the concentration range at concentration levels (low, middle and high) by analyzing blank samples spiked at three levels considering the tolerance limits (MRL/ML/limits)	Over a wide concentration range, the accuracy and precision can vary, thus they need to be verified at the different concentration Levels.	Each matrix	6 replicates at each level	Refer point 6

Precision	Repeatability test must include low, middle and high concentrations by analyzing blank samples samples spiked at three levels considering the tolerance limits (MRL/ML/limits) and also market samples	Over a wide concentration range, the accuracy and precision can vary, thus they need to be verified at different concentration levels.	Each matrix	6 replicates at each level	Refer point 6
Specificity	**No**—if the kit validated samples are identical to those being verified and if any differences in instrumentation do not impact specificity. **Yes**- if the kit validated samples are not identical to those being verified and if any differences in instrumentation could impact specificity	Same as those required for validation	If different matrix	6 blank samples in quantitative analysis and qualitative method requirement	Refer point 6
LOD (if applicable)	Run a sample close to LOD	LOD is very likely to be matrix and	Each matrix	6 replicates	Refer point 6

		instrument specific			
LOQ	Run a sample close to LOQ	LOQ is very likely to be matrix and instrument specific	Each matrix	6 replicates	Refer point 6

Qualitative test requirements

Verification of a kit/method by its ability to properly operate a qualitative method can be demonstrated by analyzing populations of negative and positive fortified samples. If any matrix components are unique, the kit/method will need to demonstrate that there is no impact on specificity. The method precision of qualitative tests is generally expressed as false-positive/false-negative rates and is determined at several concentration levels.

Qualitative tests are used to identify a specific element or compound (analyte) based on the response of a material to the test. The most important characteristic of a qualitative test is its ability to reliably identify the analyte in the presence of other substances. This is referred to as the "specificity." The lack of cross reactivity demonstrates the specificity of the method. If samples are identical to those for which the method is intended, no verification of specificity is required.

For qualitative screening methods that have already been successfully validated through a collaborative laboratory trial, the method performance shall be verified. For this a minimum of 20 negative control and 20 positive control (at kit claimed capability) samples shall be analysed. Rates (false negative and false positive) comparable to those stated in the validated method demonstrate the labs ability to operate the method. In case this criterion is not met, re verification shall be carried out.

5. Criteria for method verification

The criteria of method verification for precision and accuracy are mentioned in Table 2 and 3 for quantitative method and in case of qualitative method it shall be based on the false positive and false negative rates mentioned in the method validation.

Table 2. Expected precision (repeatability) as a function of analyte concentration

Unit	Analyte (%)	RSD_r%
100%	100	1.3
10%	10	1.9
1%	1	2.7

0.1%	0.1	3.7
100mg/kg	0.01	5.3
10mg/kg	0.001	7.3
1mg/kg	0.0001	11
0.1mg/kg	0.00001	15
0.01mg/kg	0.000001	21
0.001mg/kg	0.0000001	30

Table 3. Expected recovery as a function of analyte concentration

Unit	Analyte (%)	Mean Recovery (%)
100%	100	98-102
10%	10	
1%	1	97-103
0.1%	0.1	95-105
100 mg/kg	0.01	90-107
10 mg/kg	0.001	80-110
1 mg/kg	0.0001	
0.1 mg/kg	0.00001	
0.01 mg/kg	0.000001	60-115
0.001mg/kg	0.0000001	40-120

6. Work flow for Method verification

- Identify the matrix for method verification
- Number of analyte spike level for at least one matrix source- **≥ 3 spike level (low, mid and high) + 1 matrix blank**
- Replicates required at each level tested per laboratory if only **one matrix source** used- **≥ 6 (Accuracy, Precision, LOD, LOQ)**

- 20 negative and 20 positive control in case of Qualitative analysis.

Example of Work flow

a. Matrix: Rice

b. Parameter: Aflatoxin

c. Levels of Verification: 3, 10, 15 µg/kg

d. Replicates at each level: 6

e. Blank: 6 replicates

f. Flow of analysis: Blank, 6 replicates at each level, naturally contaminated QC samples (if available), market samples: around 30 samples

g. In case of Qualitative analysis 20 negative and 20 positive (at kit claimed sensitivity) control samples

7. Glossary of terms

Accuracy - Closeness of agreement between a measured quantity value and a true quantity value of a measurand (JCGM200:2008)

Analyte - The component of a sample or test item which embodies a quantity or quality that is ultimately determined directly or indirectly. The term 'analyte' in this document is applied to any substance or material to be analysed (e.g. chemcical constituent, residue, contaminant etc.).

Bias- Estimate of a systematic measurement error (JCGM200:2008).

Blank - A blank value is obtained as a result of analysis of a matrix which does not, as far as possible, contain the analyte(s) in question. Use of various types of blanks (to which no analytes have been added) enables assessment of how much of the measured instrument response is attributable to the analyte and how much to other causes. Various types of blank are available to the user: **Reagent blanks**: Reagents used during the analytical process (including solvents used for extraction or dissolution) are analysed in isolation in order to see whether they contribute to the measurement signal. The measurement result arising from the analyte can then be corrected accordingly. **Sample blanks**. These are essentially matrices with no analyte. They may be difficult to obtain but such materials give the best estimate of the effects of interferences that would be encountered in the analysis of test samples (Eurachem, 1998).

False Negatives - A negative outcome of a test result when the true outcome is positive.

False Positives - A positive outcome of a test result when the true outcome is negative.

Fitness for Purpose - Degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose (IUPAC, 2000).

Limit of Detection - Measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a

probability α of falsely claiming its presence (JCGM200:2008). Note: IUPAC recommends default values for α and β equal to 0.05

Limit of Quantitation, Limit of Determination - Refers to the smallest analyte concentration or mass, which can be quantitatively analysed with a reasonable reliability by a given procedure

Intermediate Precision, Intermediate Measurement Precision - Measurement precision under a set of intermediate precision conditions (JCGM200:2008).

Intermediate Precision Condition of Measurement, Intermediate Precision Condition - Condition of measurement, out of a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, but may include other conditions involving changes (JCGM200:2008).

Matrix - The predominant material, component or substrate which contains the analyte of interest.

Measuring interval, working interval - Set of values of quantities of the same kind that can be measured by a given measuring instrument or measuring system with specified instrumental uncertainty, under defined conditions (JCGM200:2008). Note: The lower limit of a measuring interval should not be confused with detection limit.

Method Validation – The process of establishing the performance characteristics and limitations of a method and the identification of the influences which may change these characteristics and to what extent. Which analytes can it determine in which matrices in the presence of which interferences? Within these conditions what levels of precision and accuracy can be achieved? The process for verifying that a method is fit for purpose; i.e. for use of solving a particular analytical problem (Eurachem, 1998)

Precision, Measurement Precision - Closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions. Measurement precision is used to define measurement repeatability, intermediate measurement precision, and measurement reproducibility (JCGM200:2008).

Qualitative test results - Results of tests not numerically derived (e.g. visual examinations or binary classification tests such as absence/presence, positive/negative, reactive/non-reactive, etc). Qualitative test results based on a numerical outcome, e.g. based on thresholds, are often described as semiquantitative or semi-qualitative and it is expected that method validation or verification is in line with quantitative procedures.

Quantitative test results - Numerically derived test results.

Recovery - The extraction efficiency of an analytical process, reported as (a percentage of) the known amount of analyte carried through the sample extraction and processing steps of the method.

Repeatability, Measurement Repeatability - Measurement precision under a set of repeatability conditions of measurement (JCGM200:2008)

Reproducibility, Measurement Reproducibility - Measurement precision under reproducibility conditions of measurement (JCGM200:2008)

Ruggedness/Robustness - The degree of independence of the method of analysis from minor deviations in the experimental conditions of the method of analysis.

Verification - Confirmation by examination and provision of objective evidence that specified requirements have been fulfilled (ISO 9000:2005).

8. References

AOAC International (2007), *How to meet ISO/IEC 17025 requirements for method verification*, ALACC Guide, www.aoac.org/alacc_guide_2008.pdf.

Validation and verification of quantitative and qualitative test methods. NATA, January 2018

CHAPTER 5: *GUIDELINES FOR THE VERIFICATION OF NUCLEIC ACID BASED METHODS*

1. Introduction

The development of a number of advanced methods to ensure food safety and quality are more relevant now because of rapid changes in the quantity, diversity and mobility of food. The development of nucleic acid-based assays has represented one of the most significant advances in food diagnostics as it provides rapid, reliable and quantitative results. Technological advances in instrumentation have resulted in a wide range of PCR-based nucleic acid quantification approaches/instruments. Performing nucleic acid-based assays to a high standard of analytical quality can be challenging.

2. Purpose

Despite the fact that several guidelines and peer-reviewed papers on method validation for nucleic acid-based methods have been published, no specific guidelines are available for the verification of the methods. The aim of this document is to provide guidance and to harmonise the in-house verification of validated methods for the qualitative and quantitative nucleic acid-based FSSA(I) approved methods employed in food laboratories meet the highest analytical performance standards. The Rapid Analytical Food Testing (RAFT) committee establishes these criteria by which all analytical methods for targeted nucleic acid sequence-based analyses in food and feed, shall be evaluated and verified.

3. Scope

The scope of this document is to provide guidance on how to carry out the method verification of inter-laboratory validated methods for the qualitative and quantitative nucleic acid-based methods that have been approved for use by FSSA(I). These criteria apply to all analytical laboratories that propose to use the targeted nucleic acid sequence-based analytical methods for food and feed. It is the responsibility of the laboratory to demonstrate that the method is suitable for its intended purpose It is intended to be applicable to most fields of nucleic acid-based testing. This guideline does not cover sampling in connection with the performance of a method.

4. Verification of previously validated methods approved under RAFT scheme of FSSA(I)

Methods approved under the RAFT scheme have already been subject to validation (third party) by extensive collaborative studies and found to be fit-for-purpose as approved by FSSA(I). Therefore, the rigour of testing required to introduce such a method is less than that required to validate an in-house method. Where a laboratory uses a commercial test kit in which the methodology and reagents are unchanged from the manufacturer's instructions, the kit does not need to be independently revalidated in the testing facility. Essentially the laboratory only needs to verify that their operators using their equipment in their laboratory environment can apply the method obtaining the same outcomes as defined in the validation data provided in the approved method. Verification of the same must include statistical correlation with existing validated methods prior to use. The verification process must be documented. This includes a record of the

procedure used, the results obtained and a statement as to whether the method is fit for the intended use.

5. Definitions

Accuracy: Closeness of agreement between a measured quantity value and a true quantity value of a measurand.

Amplicon: DNA sequence produced by a DNA-amplification technology, such as PCR.

Analyte: Component of a system to be analyzed

Amplification efficiency: The rate of amplification calculated from the slope of the standard curve obtained after a decadic semi-logarithmic plot of Cq values over the quantity. The efficiency (in %) can be calculated by the following equation

Efficiency = $(10^{(-1/slope)}-1) \times 100$

Analytical sample: Sample prepared from the laboratory sample by grinding/ homogenization

Calibration: Operation that establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and uses this information to establish a relation for obtaining a measurement result from an indication.

Certified Reference Material (CRM): Reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability, using valid procedures.

Cross-Reactivity: Degree to which binding occurs between an antibody and antigenic determinants, or primers and a target sequence, which are not the analyte of primary interest.

Cycle Threshold (Ct) also known as quantification cycle is defined as the fractional cycle number at which the fluorescence generated by the amplification of a target DNA in a real time PCR experiment reaches a fixed threshold and so allows the quantification of the amount of target DNA.

Denaturation: Process of partial or total alteration of the native structure of a macromolecule resulting from the loss of tertiary and/or secondary structure that is a consequence of the disruption of stabilizing weak bonds of DNA: DNA that has been converted from double-stranded to a single-stranded form by a denaturation process such as heating

Deoxyribonuclease/Ribonuclease (DNase/RNase): Enzyme that catalyses the hydrolytic cleavage of deoxyribonucleic acid/ribonucleic acid that may produce a single nucleotide residue by cleavage at the end of the chain or a polynucleotide by cleavage at a position within the chain.

Deoxyribonuclease/Ribonuclease Inhibitor: Substance that either fully or partially blocks deoxyribonuclease/ribonuclease activity.

DNA extraction replicates (see Figure 1): DNA extracted from different test portions from the same analytical sample.

Deoxyribonucleotide Triphosphate (dNTP) Generic term referring to a deoxyribonucleotide that includes: deoxyadenosine nucleotide triphosphate (dATP), deoxycytidine nucleotide triphosphate (dCTP), deoxyguanosine nucleotide triphosphate (dGTP), deoxythymidine nucleotide triphosphate (dTTP) and deoxyuridine nucleotide triphosphate (dUTP).

DNA Extraction: Sample treatment for the liberation and separation of DNA from other cellular components.

DNA Polymerase: Enzyme that synthesizes DNA by catalysing the addition of deoxyribonucleotide residues to the free 3'-hydroxyl end of a DNA molecular chain, starting from a mixture of the appropriate triphosphorylated bases.

DNA Probe: Short sequence of DNA labelled isotopically or chemically that is used for the detection of a complementary nucleotide sequence.

Dynamic range: The range of concentrations over which the method provides a linear correlation between the measurement and the amount of the target, with an acceptable level of trueness and precision.

End-Point PCR Method: where the amplicons are detected at the end of the PCR reaction, typically by gel electrophoresis and the amplified product is visualized with a fluorescent dye.

False Negative Error of failing to reject a null hypothesis when it is in fact not true.

False Negative Rate: Probability that a known positive test sample has been classified as negative by the method. The false negative rate is the number of misclassified known positives divided by the total number of positive test samples.

$$\%\ \text{False negative} = \frac{\#\ \text{of misclassified positive samples}}{\#\ \text{of positive test resulst(inclusive of missclassified}} \times 100$$

False Positive Rate: Probability that a known negative test sample has been classified as positive by the method. The false positive rate is the number of misclassified known negatives divided by the total number of negative test samples.

$$\%\ \text{False positive} = \frac{\#\ \text{of misclassified negative samples}}{\#\ \text{of negative test resulst(inclusive of missclassified}} \times 100$$

Fitness for Purpose: Applicability of a prescribed method or the degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose

Fluorescence Resonance Energy Transfer/FRET: Distance dependent energy transfer from a donor molecule to an acceptor molecule resulting in enhanced fluorescence of the acceptor molecule after excitation with electromagnetic radiation of a defined wave length.

Fluorescent Probe Oligonucleotide: oligonucleotide analogue of defined sequence coupled with one or more fluorescent molecules emitting a fluorescent signal after specific hybridization to the target nucleic acid sequence which can be detected by the specific equipment.

Laboratory sample: Sample as received by the laboratory and intended for inspection or testing.

Limit of detection (LOD): LOD is the lowest amount or concentration of analyte in a sample, which can be reliably detected but not necessarily quantified. Experimentally, methods should detect the presence of the analyte for at least 95 % of the cases (samples) at the LOD, ensuring ≤5 % false negative results.

Limit of quantification (LOQ): LOQ is the lowest amount or concentration of analyte in a sample, which can be reliably quantified with an acceptable level of precision and trueness.

Linearity: Ability of a method of analysis, within a certain range, to provide an instrumental response or results proportional to the quantity of analyte to be determined in the laboratory sample.

Matrix: All relevant components of a sample inclusive of analyte.

Multiplex PCR: PCR technique that employs multiple pairs of primers combined within a single reaction mixture to produce multiple amplicons.

Melting Curve Analysis describing the dissociation characteristics of double-stranded DNA observed during heating. The information gathered can be used to infer the presence and identity of single-nucleotide polymorphisms.

Melting Temperature (Tm): Temperature at which 50 % of double-stranded DNA helices are dissociated since a DNA helix melts in a temperature range rather than at one very specific temperature

Probability of detection (POD): The probability of a positive (i.e., presence detected) analytical outcome for a qualitative method for a given matrix at a given concentration. It is estimated by the expected ratio of positive to negative results for the given matrix at the given analyte concentration.

Precision (Relative repeatability standard deviation (RSD_r): The relative standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. RSDr is calculated by dividing the repeatability standard deviation by the mean of results.

Passive Reference Dye: Fluorescent molecules present in the reaction mix used to normalize the signal and may be coupled with nucleic acid sequences or other molecules not taking part in the reaction.

PCR Target Sequence Specific: region of DNA that becomes selectively amplified during PCR-based detection, identification and/or quantification. The PCR target sequence is characterized by being located between the primers, and in the case of real-time PCR, may include the probe hybridization site.

Percent Error: Relative error expressed as a percentage.

Polymerase Chain Reaction (PCR) In vitro enzymatic technique to increase the

number of copies of a specific DNA fragment by several orders of magnitude.

Primer: Strand of nucleic acid sequence that serves as a starting point for DNA synthesis.

Qualitative Method: Method of analysis that yields a binary result.

Quality Assurance: Planned and systematic actions necessary to provide adequate confidence that analytical results will satisfy given requirements for quality.

Quantitative Analysis: Analyses in which the amount or concentration of an analyte may be determined and expressed as a numerical value in appropriate units.

Repeatability standard deviation (SDr): Standard deviation of test results obtained under repeatability conditions.

R^2coefficient: R^2 is the coefficient of determination, which is calculated as the square of the correlation coefficient (between the measured Ct-value and the decadic logarithm of the concentration) of a standard curve obtained by linear regression analysis.

Robustness: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Specificity: The property of the method to respond exclusively to the characteristic or the analyte of interest.

Test portion: Sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time (Figure 1)).

Test result: A test result is a Ct value or copy number concentration originating from a PCR replicate.

Trueness: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Validation of method: Validation is the confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled. Method validation criteria may include: sensitivity, accuracy, trueness, reproducibility and robustness/ruggedness, precision

Verification of method: Provision of objective evidence that a laboratory can adequately operate a method, achieving the performance requirements for the sample matrices to which the method is being applied.

Working DNA concentration: The highest DNA concentration intended to be used in PCR analysis.

5.1. Definitions for verification/validation tools

The following general tools should be used to generate method performance characteristics for verification. Note: Some of these items are not applicable to all of the method types covered in this document.

Extraction Blank: This type of blank incorporates all the reagents and steps of the nucleic acid extraction and is processed simultaneously with the samples. Extraction controls are used to verify that the extraction reagents are free of contamination. Additionally, these controls are used to demonstrate that no cross-contamination between samples has occurred.

Matrix Blank: This type of blank is a substance that closely matches the samples being analyzed with regard to matrix components. Matrix blanks are used to verify that sample matrix and equipment used does not interfere with or affect the analytical signal.

Positive Control*:* Any reliable source of well characterized positive sample material, containing intact target nucleic acid sequences for PCR. Reference DNA or DNA extracted from a certified reference material/reference material is generally used to demonstrate that PCR reagents are working as intended

Negative DNA Target Control Well-characterized DNA preparation material that does not contain target nucleic acid

Internal Amplification Control: Internal amplification controls should be included in the PCR assays design to ensure that PCR inhibitors are not present. Internal controls are amplified using different primer and probe sets from those used to amplify assay targets and may be based on exogenous DNA or endogenous DNA.

Matrix Spikes: Matrix effects can be assessed by spiking known amounts of analyte into a matrix of interest. Accuracy or bias and precision are calculated from these results. The data can also be used to evaluate robustness/ruggedness of the method resulting from changes in the sample matrix.

No Template Control (for PCR*):* This type of blank incorporates all reagents used in the PCR except the template DNA but including the internal control. It serves to verify that reagents are analyte-free, and the equipment used does not interfere with or affect the analytical signal. Instead of the template DNA, for example, a corresponding volume of nucleic acid free water is added to the reaction.

Reference Materials and Certified Reference Materials: The use of known reference materials (when available and applicable) should be incorporated to assess the accuracy or bias of the method, as well as for obtaining information on interferences.

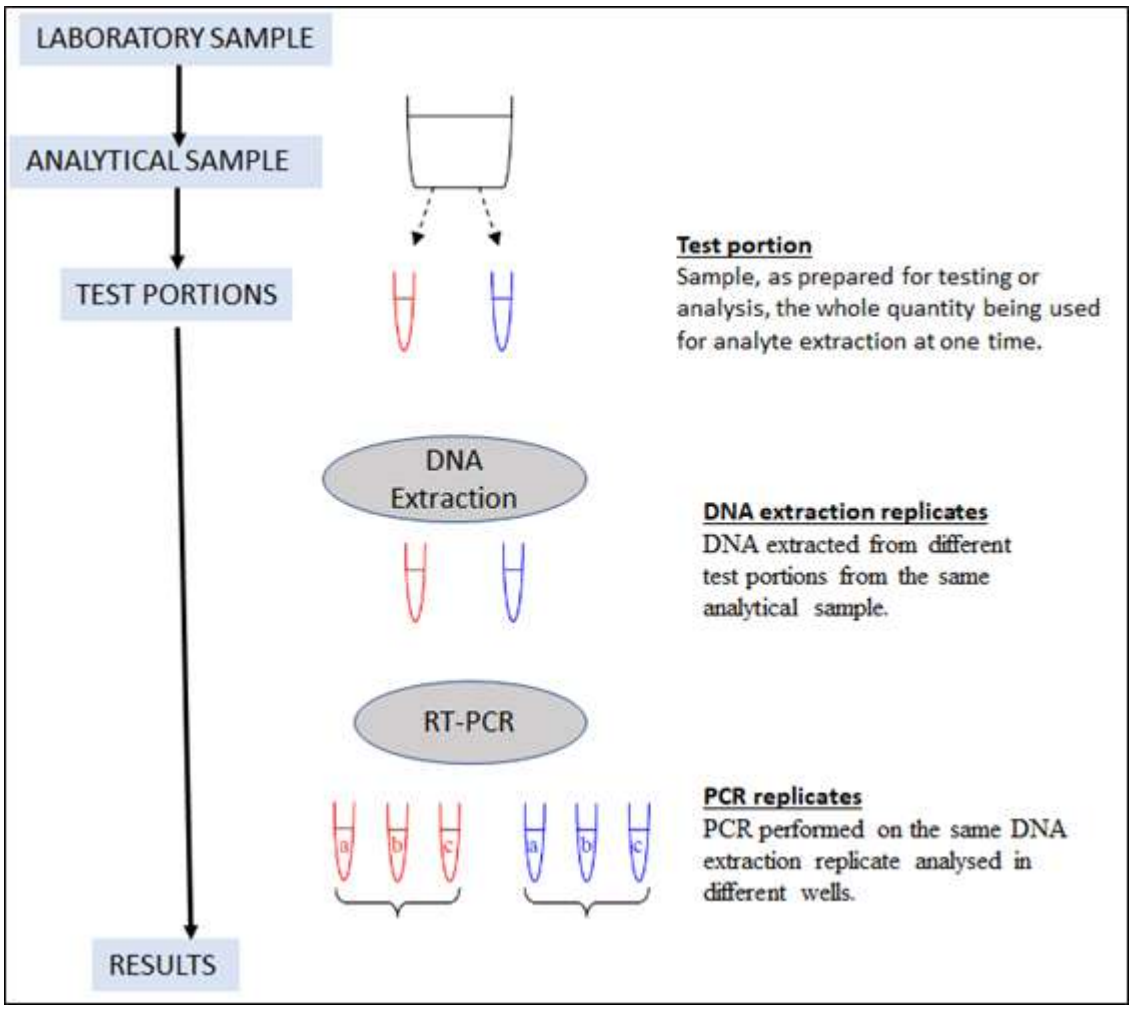

Figure 1: Illustration of replicates terminology. Adapted from 'Verification of analytical methods for GMO testing when implementing interlaboratory validated methods Guidance document from the European Network of GMO laboratories (ENGL). EUR 24790 EN – 2011'

Replicate Analyses: The precision of the analytical process can be evaluated using replicate analyses. The originating laboratory should assure that adequate sample replicates are performed and that results from replicate measurements of each analyte are compared. PCR performed on the same DNA extraction replicate analysed in different reaction wells (see Figure 1)

Statistics: Statistical techniques are employed to evaluate accuracy, trueness (or bias) precision, linear range, limits of detection and quantitation, and measurement uncertainty.

6. Required information for assembled kits from manufacturer

The PCR based assembled kits must include the following information:

1. A protocol describing the DNA extraction method which is applicable to a relevant matrix;
2. Aprotocol describing the conditions, including the apparatus used, under which PCR can be used to detect the target DNA sequence;

3. A description of the oligonucleotide primer sequences which uniquely amplify the target DNA sequence;
4. If applicable, a description of the fluorescent oligonucleotide probe sequence which uniquely identifies the target DNA sequence.
5. A description of oligonucleotide primer sequences, which amplify a housekeeping DNA sequence that should be present in the conventional food matrix irrespective of the presence of the specific analyte, in order to differentiate a negative result from failed extraction/amplification processes, and to quantify the amount of target DNA relative to the taxon-specific DNA.
6. If applicable, a description of the fluorescent oligonucleotide probe sequence which uniquely identifies the taxon-specific DNA sequence.
7. A description of the method used to detect the DNA appropriate control samples and standards.
8. descriptions of calculations used to derive the result

7. Independent laboratory verification (ILV)

The purpose of an ILV is to determine if a method/kit can be successfully used by a regulatory laboratory for screening and compliance. An ILV study may be required when the matrices do not cover many of the foods listed in the food categories. An ILV is required for methods extensions of fully validated methods/kits where the sample preparation procedure has been changed for a particular matrix or set of matrices.

8. Practical evaluation of parameters and acceptance criteria

To evaluate the fitness for purpose of a method and its performance, several parameters need to be tested. Only when they comply with the predetermined criteria, a method can be adopted for routine analysis and can be considered. There are various acceptability ranges for performance criteria that may be appropriate depending on the application or intended use of the methodology. For nucleic acid-based methodologies, there are several quality parameters and acceptance criteria, which vary and are listed under each parameter.

8. Qualitative PCR methods

Nucleic acid-based methods that are used for the detection of a specific DNA/RNA sequence which could be part of a mixture of related targets should allow for the unequivocal detection of a nucleic acid sequence that is specific to the target organism, group or sub-set of organisms (family, genus, pathogenic strain, etc.), or transformation event in the case of genetically altered organisms. For instance, target-specific methods that are used for detection of a single transformation event should allow for unequivocal detection, identification and/or confirmation of a nucleic acid sequence that is unique or specific to that transformation event. For food authentication, the specific target sequence/s should uniquely define the target as required. For qualitative PCR methods, the basic performance characteristics are:

- Extraction Efficiency
- Sensitivity (Limit of Detection-LOD)
- Specificity (Selectivity)

- False Negative and Positive Rates
- Robustness/Ruggedness

8.2 Quantitative PCR methods

The analysis of nucleic acid, especially in processed foods, requires the detection of very small amounts of target-specific DNA/RNA. The result of a quantitative PCR analysis is often expressed in % as the amount of target nucleic acid relative to an endogenous control. For quantitative PCR methods, the basic performance characteristics are:

- Extraction Efficiency-
- PCR (amplification) Efficiency
- Linear Dynamic Range (Range of quantification)
- Sensitivity (Limit of Detection-LOD)
- Sensitivity (Limit of Quantification-LOQ)
- Specificity (Selectivity)-
- Precision-Repeatability and Reproducibility Standard Deviations
- Robustness/Ruggedness
- Trueness

The parameters that need to be evaluated and the acceptance criteria for all qualitative/quantitative PCR methods during method validation and verification are listed in Table 1. All verification documents must contain the Applicability statement

Table 1 Parameters to be evaluated during verification of quantitative and qualitative qPCR methods. The acceptance criteria are given between brackets.

Parameter	Quantitative qPCR	Qualitative qPCR
Method acceptance parameters		
Specificity	√	X
Sensitivity (LOD)	X	√
Sensitivity (LOQ)	√	X
PCR efficiency	√ (90%-110%)	√ (only for multiplex)
Linearity (R^2)	√ ($R^2 \geq 0.98$)	√ (only for multiplex)
Accuracy	√	X
Trueness	√ (±25%)	X
Precision (Repeatability)	√ ($RSD_R \leq 25\%$)	X
Robustness	√ (≤30%)	√ (correct +ve/-ve classification)
Method performance parameters		
False Positive/Negative rate	X	√
Precision (repeatability)	√ ($RSD_R \leq 25\%$)	X

9. Method acceptance/performance parameters

9.1. Applicability

The applicability statement should contain complete information on the scope of the method i.e., which target, which matrix and DNA amount have been tested by the manufacturer. It can be evaluated using different matrices (raw/processed material, food/feed, genomic (gDNA). Some methods that can be applied to a single raw matrix may not be necessarily applicable to complex matrices and/or processed food, since the DNA may be altered. Additionally, warnings on the interference with other analytes and its inapplicability to certain matrices and conditions should be included when identified. Applicability of the methods could be determined by confirming whether the methods may be used in the intended foods with the required performance and it should be clearly stated. In principle the method/kit should be applicable to the matrix of concern. In the case of "general purpose" methods to identify and quantify DNA sequences in a range of food matrices, at least one extraction method applicable to each food matrix should be available. The analytes, matrices and concentrations for which a method of analysis may be used should be stated clearly.

9.2. DNA extraction

9.2.1. Extraction efficiency- Empirical results from testing the DNA extraction method for its efficiency should be provided for each matrix being validated; this is necessary to demonstrate the extraction is sufficient and reproducible. Extraction efficiency for a given matrix can be determined by spiking known amounts of the target analyte into that matrix prior to extraction. Extraction blanks will be included to ensure that cross contamination does not occur during the extraction protocol.

9.2.2. DNA extraction- The evaluation of DNA extraction methods is a crucial step for PCR-based kits, as the quality and quantity of DNA extracted may significantly affect the final result. The DNA isolation method to be used with the kit should provide DNA of suitable quality and quantity for subsequent analysis. The DNA isolation method should be assessed on a range of representative food matrices and provide DNA of suitable quality and quantity for subsequent analysis. A number of DNA isolation methods are available. The choice of the extraction method should be based on the required molecular weight of the target DNA, required quantity, purity, extraction time

Procedure: If the DNA extraction method is provided with the kit, the DNA extraction is carried out at least two times (three times recommended) each time on 2 test portions (Figure 1), if possible, on different days and with different operators (see Figure 2).

DNA extraction methods applied to one food matrix may not be suitable for other matrices. This procedure may need to be carried out on different food matrices that approval is required for. The number of matrices to be used for verification are: minimum one each from each food category applied and preferably from the high-risk category.

Acceptance criteria: The DNA extractions must meet the acceptance criteria for DNA concentration, purity and quality (e.g., for controlling amplification efficiency and absence of inhibitors by real-time PCR).

9.2.3. DNA concentration

The concentration of the DNA extracts should be measured by the method applied to the routine samples.

Procedure: The DNA concentration can be determined by using fluorimetry or UV-spectrophotometry by taking absorbance at 260 nm and 280 nm.

It is recommended to use the same technique in the verification study as foreseen for the analyses of samples since the quantification of DNA could be affected by the method used.

Acceptance criterion: The method should provide DNA in an appropriate yield for the intended analysis (at least enough to meet the desired practical LOD*). Where applicable, the yield should be comparable to the results obtained in the validation study.

If a DNA extraction method does not give an appropriate yield for the intended analysis on a particular matrix, the practical LOD will be affected

*Practical limit of detection (practical LOD) is the lowest quantity of analyte, expressed as mass fraction or absence/presence, that can be reliably detected in a food sample.

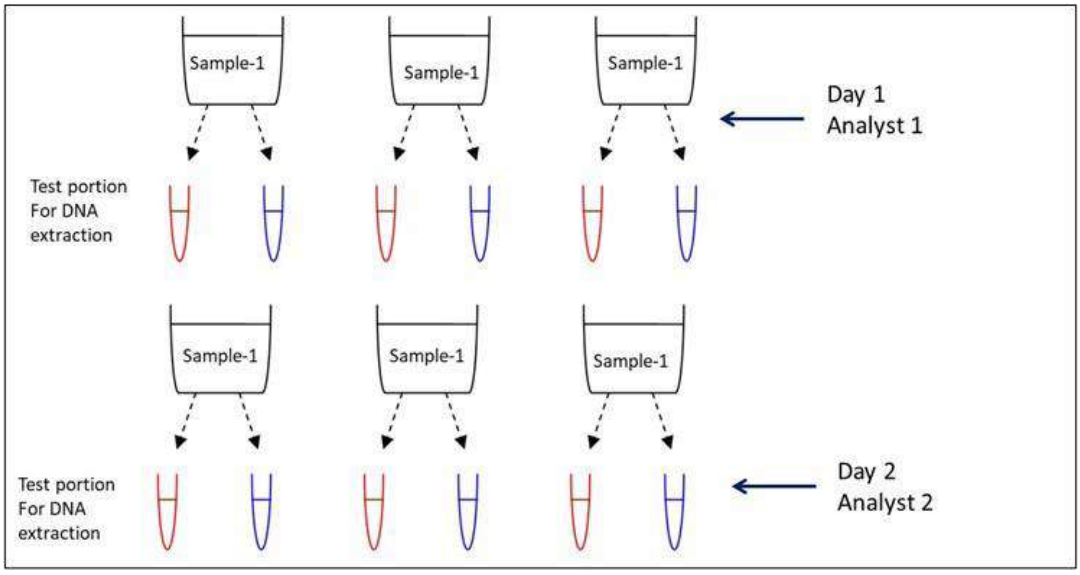

Figure 2 Schematic for DNA extraction from two independent test portions on different days and with different operators

9.2.4. DNA purity- The concentration and purity of isolated DNA should be estimated by measuring the absorbance at 260 and 280 nm using a calibrated UV-Vis spectrophotometer or alternately by using fluorimetry. The ratio of absorbance at A260/A280 nm is used to assess the

purity of DNA (a ratio of ~1.8 is generally accepted as "pure" for DNA; and a range of 1.65 to 1;85 is acceptable for further analysis).

The purity of DNA extract mainly refers to the absence of PCR inhibitors in a DNA sample. The extracted DNA has to meet the acceptance criteria for DNA concentration and quality/purity, which is detailed in below

Acceptance criteria:

- Purity: A260/A280: 1.65 -1.85
- Agarose gel electrophoresis: Intact band of DNA

DNA extraction methods applied to one matrix may not be suitable for other matrices. This procedure may need to be carried out on different matrices. For the verification of a DNA extraction method the tested matrix must be identified from different food categories applied for.

9.2.5. Absence of PCR inhibitors

The isolation of the DNA may lead to the co-extraction of substances that inhibit the RT-PCR reaction resulting in the absence or a lower rate of amplification often leading to false negative results or, underestimation of the analyte. Therefore, the laboratory needs to verify that the DNA extraction procedure guarantees the removal of such inhibitors.

***Procedure*:** The presence or absence of PCR inhibitors can be verified by testing different dilutions prepared from a DNA solution. The more the DNA solution is diluted, the less is the concentration of inhibitors.

DNA quality (relative absence of PCR inhibitors) can be demonstrated by analysing two PCR replicates using four points of four-fold serial dilutions (1:4, 1:16, 1:64 and 1:256) of each DNA extraction replicate.

a. The DNA extract is first brought to a level corresponding to the highest DNA concentration intended to be used called 'undiluted' sample (working dilution e.g., 25-40 ng/µL).
b. Four-fold dilution series is prepared (from 1:4. 1:16, 1:64, and 1:256).
c. RT-PCR for target gene carried out
d. The Ct values of the four serially diluted samples are plotted against the logarithm of the dilution factor.
e. The Ct value of the 'undiluted' sample extrapolated from the linear regression equation is compared with the Ct measured from the same sample.

Acceptance criteria:

1. slope of the regression line must be between -3.6 and -3.1;
2. coefficient of regression (R2) is equal to or above 0.98;
3. The difference between the measured ΔCt value and the theoretical ΔCt (2.0) value of the sample should be <0.5.

If the extracted DNA contains inhibitors the DNA has to be further purified or diluted to the level where no inhibition of PCR reaction is observed, before it is used for RT- PCR.

9.3. SPECIFICITY

The method should be tested with DNA/RNA from closely related or potentially co-occurring non-target species/varieties and DNA/RNA from the reference species/variety material. Verification of the specificity of a novel assay can be accomplished in several phases.

Theoretical test for specificity- Carry out a computer-aided ("in-silico") test, examining the oligonucleotide sequences (primer, probe) as well as the amplicon.

Sequence for similarities to other sequences by searching suitable databases (e.g. BLASTn).

Experimental test for specificity- The method must be tested with DNA from non-target species/varieties (exclusivity) and DNA from the reference species/variety (inclusivity) material. This testing should include closely related materials and cases where the limits of the sensitivity are truly tested.

The specificity of a validated method has already been investigated, hence it not required to be re-verified if the conditions of the assay (e.g., primers/probe concentration; annealing temperature; fluorescent dye) are unchanged. Data regarding specificity can be retrieved from the validation report submitted.

The number of species used for inclusivity/exclusivity testing will vary with the analysis being conducted and the target organism/s. Samples for inclusivity assessment should be chosen to reflect the genetic diversity of species on which the assay will be used; samples for exclusivity testing should be chosen to reflect related and potentially cross-reactive organisms and species, as well as those likely to co-occur in food products. Both inclusivity and exclusivity testing should be performed on purified samples and amounts of DNA should be equal between inclusivity samples and exclusivity samples. Samples used in specificity testing should be traceable to the source.

Acceptance Criteria*:* Only the target gene of interest should be detected with the method. No false positive & negative results**.**

9.4. Linear dynamic range, PCR (Amplification) EFFICIENCY AND R^2

Linear dynamic range is defined as the range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision. This desired concentration range defines the standard curves which will be used for quantification.

Amplification efficiency is defined as the rate of PCR amplification that leads to a theoretical slope of -3.32 with an efficiency of 100 % in each cycle. If the amplification efficiency is 100%, a two-fold reduction in template DNA should result in an increase in the C_t value of one cycle. If DNA is diluted 10-fold, the theoretical difference in C_t values between the two concentrations of template nucleic acid should be approximately 3.32 cycles. The efficiency (in %) can be calculated by the following equation:

Efficiency (%) = $-1+10^{(-1/\text{slope})}$

***Procedure*:** All the three (linear dynamic range, amplification efficiency and R^2) are verified simultaneously from standard curves of the template DNA and determining the C_t-value for each dilution. The average values of at least two standard curves should be taken.

Example 1: Two calibration curves minimum requirements 5 calibration points with 3 PCR replicates each (triplicates)

All slopes shall be in the range of -3.6 ≤slope ≤-3.1 and all R2 values should be ≥0.98. (30 PCR reactions)

Example 2: Four calibration curves-5 calibration points with 2 PCR replicates each (duplicates) average of 4 slopes and R2 are used to verify the acceptance. (40 PCR reactions)

Example 3: Two calibration curves;8 calibration points in 5 PCR replicates (pentaplicates) also covering the low concentrations for LOD and LOQ. Average of the part above LOQ for slope and R^2 are used to verify the acceptance. (80 PCR reactions)

***Acceptance criterion for dynamic range*:** The dynamic range must cover the values corresponding to the expected use. This can be expressed as % (m/m).

Acceptance criterion for amplification efficiency: For both, qualitative and quantitative methods, the average value of the slope of the standard curve shall be in the range of -3.6 ≤slope ≤-3.1 corresponding to an amplification efficiency of 90 - 110 %.

Acceptance criterion for R^2 coefficient: the average value of R^2 shall be ≥0.98

9.5. False positive/false negative rate

False Positive Rate: This is the probability that a known negative test sample has been classified as positive by the method. For convenience this rate can be expressed as percentage:

$$\%\ \text{false positive results} = \frac{100 \text{ x number of misclassified known negative samples}}{\text{total number of known negative samples}}$$

False Negative Rate: This is the probability that a known positive test sample has been classified as negative by the method. For convenience this rate can be expressed as percentage

$$\%\ \text{false negative results} = \frac{100 \text{ x number of misclassified known positive samples}}{\text{total number of known positive samples}}$$

In order to demonstrate the false negative rate for qualitative assay, a series of samples with a constant, known concentration of positive material in a pool of negative material have to be analysed and the results evaluated. It is important to note that the concept of confidence intervals and statistical uncertainty needs to be applied to the risk of false positive and/or false negative results as well. The desired level of confidence determines the size and number of pools that need to be tested.

9.5.1. Statistical approach to confirm false negative and false positive rates as <5%

The minimum number of samples that must be tested depends on the criteria for the defect rate and the level of statistical confidence is calculated using the formula

$$n = \frac{\text{Log}(\propto)}{\text{Log}\,(1-\rho)}$$

where 1-α is the confidence level and p is the maximum acceptable FN or FP rate. Sample sizes to assess selected criteria for FN or FP rates with varying levels of confidence are listed in Table 2.

Table 2 Sample sizes recommended for assessing FN or FP rates

False Positive/ Negative rate	Confidence level			
	80%	90%	95%	99%
< 1%	161	230	299	459
<2%	80	114	149	228
<5%	32	45	59	59
<10%	16	22	29	44

For example, if the goal is to have 95% confidence that the FN rate is <5% then test 59 samples with the nucleic acid target present at the concentration of interest, typically the LOD or a relevant level of concern, in a range of matrices. The criteria are satisfied if all 59 test results are positive for the target.

This sample size formula is related to the Clopper-Pearson confidence interval for Binomial proportions and frequently used for zero defect acceptance sampling plans for commodity lots. The rationale for the sample size is that when the probability of a false positive/negative response is p for each sample then (1 - p) n is the probability that n samples will have the correct response.

9.6. Sensitivity

9.6.1. Limit of Detection (LOD)

By their very nature, qualitative test results refer to the identification above/below a detection limit. Like the limit of detection for quantitative methods, the limit of detection for a qualitative method can be defined as the concentration at which a positive sample yields a positive result at least 95% of the time. This results in a rate of false negative results of 5% or less.

The LOD is usually understood as the concentration of the target DNA at which an amplification product is detected with a confidence of 95% (LOD $_{95\%}$). This approach allows an approximate estimation of the LOD or relative LOD. Data obtained from testing the method at different concentrations of the target sequence in order to determine the sensitivity of the method should be provided. LOD should be defined using samples comprised of single ingredients only.

To estimate the LOD of a method with 95 % confidence it is necessary to analyse at least 60 PCR replicates for each matrix. As this may not be feasible, a pragmatic approach based on a lower number of replicates could be followed for the verification of the LOD. The LOD should be

determined by means of a dilution series of the target DNA. After the LOD of the assay is determined using a dilution series of the target DNA, an estimate the LOD of the assay in various food matrices is performed.

Procedure for Relative LOD (LOD): A reference material or spiked sample with low concentration of target (spiked food material of low target content, e.g., 0.1% or 10 cfu) can be measured in e.g. The dilution level for which all 12 replicates are positive is considered to be an approximate value for LOD.This data may be represented as DNA weight/reaction (ng or pg etc.) or the target copy number/reaction. If needed spiked material at different specific levels could also be prepared.

A real-time PCR method may employ a Ct cut off value above which a result is considered negative. It is the responsibility of the originating laboratory to determine if a cut off value should be established and if so, what cut off value should be used. The decision shall be based upon verification data and if available, results of testing naturally incurred material

Acceptance criteria: If all replicates are positive, this infers that the LOD_{rel} is below or equal to the dilution of the positive control material level.

9.6.2. Limit of Quantification (LOQ)

The limit of quantification is the lowest amount of analyte in a sample that can be reliably quantified. There are multiple experimental approaches to determine the LOQ; such as assaying spiked samples that have a known amount of analyte, or by analyzing a number of samples that contain known amounts of analyte.

The quantification should be determined by spiking the target organism into a relevant food matrix prior to sample preparation and DNA/RNA extraction. Quantification should be expressed in units which are relevant to the intended purpose of the method, for example as mg/kg, parts per million, or percentage in a food matrix

Procedure

a. A dilution series of a known amount of a positive spiked food material of e.g., 1 % shall be measured in 12 PCR replicates (e.g.,0. 08, 0.06, 0.04, 0.02, 0.01, 0.005 and 0.0025 %).

b. The LOQ can be estimated as the last dilution in a series where the RSD of the measurements is below 25 %. The LOQ can also be expressed as is the minimum nucleic acid concentration for which all 12 replicates give a positive result with a C_t coefficient of variability (CV) of no more than 0.5 C_t.

***Acceptance criteria*:** The RSD of the LOQ should be <25 %

9.7. Relative Repeatability Standard Deviation (RSD_R)

RSD_R is the relative standard deviation of results obtained with the same method, by the same analyst, in the same laboratory, with the same equipment, on the same samples (repeatability conditions).

Procedure: It is calculated from results obtained on PCR replicates run under repeatability conditions (see Terminology). Repeatability should be available for all tested food matrices.

The analytical procedure used should be the same as during routine testing of samples. At least 16 single test results should be evaluated. Examples for possible test designs are shown Figures 3 and 4.

***Acceptance criterion*:** The RSDR should be ≤ 25 %, over the dynamic range of the method.

9.8. Trueness

Trueness compares the obtained value from a series of samples to the actual or reference value. Trueness should be with ± 25% of the accepted reference value across the whole dynamic range of the assay.

9.9. Robustness/Ruggedness

Robustness is determined by measuring the capacity of an analytical procedure to remain unaffected by small but deliberate deviations/variations in method procedures and provides an indication of its reliability during normal usage. The method should provide the expected results. For qualitative analyses, all replicates should give positive results. Optimally, the target amount/concentration to be tested should be at the LOD. Robustness/ruggedness testing: different thermal cycler (brands and models), master mix (e.g., final concentrations of salts, dNTPs, changing reaction volume, probe and primer concentrations and thermal cycling parameters

10. Qualitative and Quantitative Multiplex Assays

For multiplex assays, all method verification must be carried out in multiplex and performance metrics described above must be reported for each individual target as it performed under multiplex conditions. For probe-based assays the signals from the fluorophores on different targets must not interfere with each other. Multiplex intercalating dye-based assays will not be considered quantitative because intercalating dyes do not distinguish between different targets in a multiplex assay.

11. Food matrix and sample selection

Food matrix and sample source selection should be based on the types of foods most likely to be used in the analysis or based on risk of contamination. A PCR method intended for use in processed foods should be tested on samples subjected to similar processing. Processing conditions such high-temperature and high-pressure treatments (e.g., canning) and low pH (e.g. tomato-based products) that have adverse effects on DNA such as degradation should not be chosen. The number of food categories to be used will depends on the intended use of the method.

The number of different food categories to be verified depends on the applicability and intended use of the method. Depending on how many categories will be verified, a minimum of 1 – 3 representative matrices from each category listed below should be selected,

A list of foods that can be used based on the applicability are:

a. Meats: Fresh meat, Frozen meat, Raw marinated/minced/comminute meat, Semi-cooked /Smoked Meat, partially heat treated and/ or smoked meat and meat product, Canned/Retorted

meat product, Chilled meat, Cooked Meat/meat product, Cured/pickled meat products, Dried/Dehydrated meat/meat products, Fermented meat products sausage, lunch meat, meat substitutes etc ("meat" means all edible parts (including edible offal) of any food animal slaughtered in an abattoir that are intended for or have been judged as safe and suitable for, human consumption; "meat food products" means any product prepared from meat and other ingredients through various processing methods in which meat should be the major ingredient of all the essential ingredients

b. Seafood: Chilled/Frozen Finfish, crustaceans, cephalopods, molluscs, bivalves, dried or Salted and dried fish products, thermally processed, fermented, smoked, canned fish products. Fish sticks, surimi, raw fish filet, raw oysters, raw mussels, raw clams, cooked crawfish, crabmeat (fresh or pasteurized), battered and breaded fish products,

c. Fruits, Vegetables, and Nuts: Fresh / frozen /dehydrated or dried fruits and vegetables, fresh fruit juice, apple cider, tomato juice, fruit cubes, berries, peanut butter, coconut, fruit powders almonds, minimally processed lettuce, spinach, kale, collard greens, cabbage, bean sprouts, seed sprouts, peas, mushroom, green beans and other minimally processed fruit and vegetable products

d. Dairy: Dahi, Yogurt, Paneer, Khoa, Channa, hard and soft cheeses, raw or pasteurized liquid milk, infant formula, coffee creamer, ice cream, milk powders, casein, whey, non-fat dry milk/dry whole milk,

e. Chocolate / bakery: Frosting and topping mixes, candy and candy coating, milk, chocolate, cake mixes,

f. Egg and egg products: Shell eggs, liquid whole eggs, dried whole egg or dried egg yolk, dried egg whites,

g. Herbs and spices: Oregano, pepper, paprika, black pepper, white pepper, celery seed or flakes, chili powder, cumin, parsley flakes, rosemary, sesame seed, thyme, vegetable flakes, onion flakes, onion powder, garlic flakes, allspice,

h. Processed grains and legumes: Flours, grits, rice corn meal, soy flour, dried yeast, cereal based complementary food, Uncooked noodles, macaroni, spaghetti, soygurt, tofu, soy beverage.

12. Information to be provided with method verification documents

A listing of information that should be provided from the verifying laboratory when the results for a laboratory verification study are prepared for review.

This list of information is in addition to the results obtained for the validation criteria/insert accompanying the kit

For Qualitative and Quantitative PCR-Based Assays:

A. Assay Design

- Type of assay: oligonucleotide probe-based or double stranded DNA dye-based
- Name of target gene or region
- Internal control/amplification control type: exogenous or endogenous
- Exact oligonucleotide sequences for all primers and probe(s)
- Length of PCR product (amplicon)

- Dye and probe characteristics. For probe-based assays, provide a brief description of the probe chemistry and the identities and locations of fluorophores and quenchers, including internal quenchers. For dye-based assays, state which dye is being used.
- Any other reporter molecules.

B. Sample Preparation and Nucleic Acid Extraction

- Form and quantity of sample required. Include information on subsampling or sample compositing as well as relevant aspects of handling and storage.
- Method or kit used for DNA extraction. Include any relevant modifications as well as information on RNAse treatment. Independent assessments of DNA quality and quantity are not required as long as the method is shown to yield acceptable/reliable PCR results. For quantification of targets in complex food matrices, normalization of total DNA/RNA amounts prior to PCR may result in higher quality data.

C. PCR Conditions

- Reaction: reaction volume; identities and concentrations of all reaction components, including buffer or master mix, all primers, all probes and/or dyes, template DNA/RNA, $Mg^{2+,}$ and additives (e.g., BSA, DMSO, or glycerol).
- Platform: State make and model of real-time PCR platform as well as name and version of accompanying software. Include brief descriptions of physical format (e.g., 96 well thermal block or other) and optical system.
- Thermal cycling conditions. Include PCR cycling conditions for both dye-and probe-based assays; also include melt conditions for dye-based assays. Optimal cycling conditions should be determined empirically and not through software-based calculations of primer or probe annealing temperature, as annealing temperatures can be significantly affected by specific reaction conditions.

D. Data Analysis

- Specify which software program and version was used for data analysis.
- Report and explain any adjustments made to baseline and threshold determination, or other software default analysis parameters.
- For dsDNA dye-based assays (e.g., SYBRgreen), analysis of melt curves must be performed to confirm the presence of a single, sharp melting peak optimally with a melting temperature (Tm) of approximately 80-90°C in all samples and standards.

13. Conclusion

Based on verification of test method for the above defined parameters meeting the acceptance criteria as per the procedure defined above, the method will be declared as fit for intended use.

14. Reference

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Broeders S. et al. 2014. Guidelines for validation of qualitative real-time PCR methods. Trends in Food Science & Technology 37:115-126.

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US-FDA OFVM 2015. Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds.

CHAPTER 6: *GUIDELINES FOR THE VERIFICATION OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)-BASED METHODS*

1. Introduction

Enzyme Linked Immunosorbent Assay (ELISA) is a unique biomolecular detection procedure characterized by recognition and binding of specific antigens by antibodies. This document provides the requisites for carrying out verification of ELISA based methods. Immunoassays are used for qualitative detection and quantitation of:

- Antibiotics (tetracycline, sulfonamide, gentamicin etc.) and other veterinary drug residues in animal derived foods.
- Food pathogens such as *Salmonella enterica*, *Bacillus cereus*, *E. coli* etc.
- Contaminants such as Aflatoxins, DON, Fumonisin etc in cereals
- Food Allergens such as peanut, egg, milk, gluten, soybean, fish, tree nuts etc.

2. Purpose

Several documents for guidelines to validate the performance characteristics for qualitative and quantitative immunoassays have been published. The aim of this document is to provide guidance on how to perform verification of qualitative and quantitative ELISA-based methods that have already been validated by following published international guidelines. FSSAI approved rapid methods/kits etc employed in food laboratories are required to meet the highest analytical performance standards. The Rapid Analytical Food Testing (RAFT) committee establishes these guidelines and criteria by which ELISA-based analytical methods for food shall be evaluated and verified.

3. Scope

The scope of this document is to provide guidance on how to carry out the method verification of validated qualitative and quantitative ELISA-based methods/kits that have been approved for use by FSSA(I). These criteria apply to all analytical FSSAI approved laboratories involved in verification. It is the responsibility of the laboratory to demonstrate that the method is suitable for its intended purpose. It is intended to be applicable to most fields of ELISA-based testing. This guideline does not cover sampling in connection with the performance of a method

4. Verification of previously validated methods approved under RAFT scheme of FSSA(I)

Methods/kits approved by FSSAI have already been subject to validation (third party) by extensive collaborative studies and found to be fit-for-purpose. Therefore, the rigour of testing required to introduce such a method is less than that required to validate an in-house or newly developed method. Where a laboratory uses a commercial test kit in which the methodology and reagents are unchanged from the manufacturer's instructions, the kit does not need to be independently revalidated in the testing facility. Essentially the laboratory only needs to verify that their operators

using the equipment in their laboratory environment can apply the method obtaining the same outcomes as defined in the validation data provided in the approved method. Verification of the same must include statistical correlation with existing validated methods prior to use. The verification process must be documented. The verification document must include:

1. Design and planning of the verification;
2. Details of the procedure
3. Matrix used
4. Acceptance criteria and performance requirements
5. Test data and records;
6. Assessment of the method: a statement as to whether the method is fit for the intended use

The verification process is required to performed on food matrices for which approval is being sought and which do not form a part of the validation data.

5. Verification parameters and acceptance criteria

5.1. Applicability

The applicability statement should contain complete information on the scope of the method i.e., which target matrix and allergen/ analyte have been tested by the manufacturer. It can be evaluated using different matrices (raw/processed material, food/feed). Some methods that can be applied to a single raw matrix may not be necessarily applicable to complex matrices and/or processed food due to different protein profile.

Additionally, warnings on the interference with other analytes and its inapplicability to certain matrices and conditions should be included when identified. Applicability of the methods could be determined by confirming whether the methods may be used in the intended foods with the required performance and it should be clearly stated. In principle the method/kit should be applicable to the matrix of concern. The analytes, matrices and concentrations for which a method of analysis may be used should be stated clearly.

5.2. Implementation of verification

- For qualitative ELISA, select the food item as indicated in *Annexure 1*. Analyse 3 concentration levels of the same food matrix, (1) **positive,** (2) **negative,** (3) **weak positive** (if claimed in validation studies)) using the required number of kits. Analyse the samples strictly as per the manufacturer's instructions provided with the kit. Conduct and record all performance parameters as described in Table 1.

- For semi - quantitative and quantitative ELISA, select an item from the food category as elaborated in Annexure 1. Analyse at least 4 concentration levels of the same food matrix: (1) **blank,** (2) one of the remaining concentration levels must be less than or equal to two times the LLA or LOD stated for the kit, (3) one mid value and (4) one value equal to or more than Maximum Residue Level (MRL, wherever applicable), using the required number of replicates. Analyse the

samples strictly as per the manufacturer's instructions provided with the kit. Record performance parameters as described in the table.

5.3. Selecting the food matrix for verification

I. Select the following food categories:

- **a.** One from already validated food category;
- **b.** Four from other categories not tested during validation

II. Include the most challenging food item in the food category

III. Include spiked/contaminated samples and reference/pure standards for target analytes (at same dilutions) and a negative control.

IV. Analyse all samples in duplicate or triplicate as instructed in the rapid kit.

V. Estimate performance characteristics as described in the Table I, for each food item.

Table 1 Parameters to be evaluated during verification of quantitative and qualitative ELISA methods.

Verification characteristics	**Semiquantitative and Quantitative ELISA method**	**Qualitative ELISA including Lateral Flow Strips (LFS)**
Selectivity	+	+
Sensitivity (LOD and LOQ)	+	-
Linearity (R^2)	+	-
Linearity-of-dilution	+	-
Accuracy	+	-
Trueness	+	-
Precision (Repeatability)	+	+
Ruggedness/ Robustness	+	+
False Positive/Negative rate	-	+

6. Performance characteristics and acceptance criteria to be considered while verifying qualitative and quantitative rapid ELISA based kits:

6.1. Acceptance criteria

Acceptance criteria are defined as numerical limits, ranges, or other suitable measures for acceptance of the analytical results to which a method should conform to be considered acceptable for its intended use. Acceptability of method performance is generally based on a number of factors, including percent recovery for spiked or incurred samples.

Ideal percent recovery levels would range from 80 to 120%. Recovery levels are affected by both the efficiency of the extraction step and the ELISA procedure. With ELISA methods for food allergens, this level of recovery is not always possible, particularly when certain difficult matrixes are analysed. In addition, the recovery from incurred samples can be substantially different from those obtained using spiked samples. For this reason, recoveries between 50 and 150% will be considered acceptable so long as they can be shown to be consistent.

6.2. False positive/false negative rate

For a binary classification test (e.g., Lateral flow strip (LFS), qualitative ELISA) sensitivity is defined as the ability of a test to correctly identify the true positive rate, whereas test specificity is defined as the ability of the test to correctly identify the true negative rate.

False Positive (FP) Rate: This is the probability that a known negative test sample has been classified as positive by the method. For convenience this rate can be expressed as percentage:

$$\%\ \text{false positive results} = \frac{100\ \text{x number of misclassified known negative samples}}{\text{total number of known negative samples}}$$

*False Negative (FN) Rate***:** This is the probability that a known positive test sample has been classified as negative by the method. For convenience this rate can be expressed as percentage:

$$\%\ \text{false negative results} = \frac{100\ \text{x number of misclassified known positive samples}}{\text{total number of known positive samples}}$$

In order to demonstrate the false negative rate for qualitative assay, a series of samples with a constant, known concentration of positive material in a pool of negative material have to be analysed and the results evaluated. It is important to note that the concept of confidence intervals and statistical uncertainty needs to be applied to the risk of false positive and/or false negative results as well. The desired level of confidence determines the size and number of pools that need to be tested. Statistical approach to confirm false negative and false positive rates as <5%

The minimum number of samples that must be tested depends on the criteria for the defect rate and the level of statistical confidence is calculated using the formula

$$n = \frac{\text{Log}(\propto)}{\text{Log}\,(1-\rho)}$$

where 1-α is the confidence level and ρ is the maximum acceptable FN or FP rate. Sample sizes to assess selected criteria for FN or FP rates with varying levels of confidence are listed in Table 2.

Table 2 Sample sizes recommended for assessing FN or FP rates				
False Positive/ Negative rate	**Confidence level**			
	80%	**90%**	**95%**	**99%**
< 1%	161	230	299	459
<2%	80	114	149	228
<5%	32	45	59	59
<10%	16	22	29	44

Acceptance criteria: For example, if the goal is to have 95% confidence that the FN rate is <5% then test 59 samples with the protein present at the concentration of interest, typically the LOD or a relevant level of concern, in a range of matrices. The criteria are satisfied if all 59 test results are positive for the target.

This sample size formula is related to the Clopper-Pearson confidence interval for Binomial proportions and frequently used for zero defect acceptance sampling plans for commodity lots. The rationale for the sample size is that when the probability of a false positive/negative response is ρfor each sample then (1 - ρ) n is the probability that n samples will have the correct response.

6.3. Selectivity

Selectivity demonstrates that the method does not detect non-target compounds, and at the same time demonstrates method's ability to detect the related compounds. Organize a "selectivity" test panel of related compounds that are expected to give a positive result (e.g., if the kit is for Aflatoxins the panel will be Aflatoxin M1, M2. B1. B2. G1, G2.).

Organize a panel of non-target compounds that might be expected to be encountered when the method is used; or to be erroneously detected by virtue of chemical or other similarities. Prepare at least one replicate of each target compound from the selectivity test panel at the 95% POD concentration. Prepare at least one replicate of each non-target compound from the selectivity panel at an appropriate concentration.

Blind code and randomly mix the selectivity and non-target compounds. An analyst (or analysts) not involved in the preparation of the test panel shall evaluate the compounds using the candidate method and record the results.

If an individual test panel compound yields an incorrect result (a negative in the case of a target compound; a positive in the case of a non-target compound) then the compound may be retested with a number of replicates to be determined by subject matter experts.

6.4. Sensitivity

6.4.1. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Qualitative tests: For exclusively qualitative analysis, the LOD can be determined by applying the procedure to items containing progressively smaller levels of the characteristic until the likelihood of producing false results reaches a pre-established criterion.

Quantitative ELISA: The LOD should be estimated by a statistical analysis of the calibration data according to the ISO standard ISO 11843-2. for linear data, or ISO 11843-5 for linear and nonlinear data, using as default probabilities $\alpha = \beta = 0.05$, where α and β represent the probability of a false positive and false negative, respectively. LOD for ELISA is defined as the lowest analyte concentration that can be distinguished from the assay background, while the LOQ is the lowest concentration at which the analyte can be quantitated at defined levels for precision and accuracy. LOD is determined from standard deviation of the sample blank and the slope of the linear curve LOD = 3.3 (SD (b) / k (where k is slope of the linear curve and SD (b)—standard deviation of the blank)

LOQ is the lowest level of analyte in a test sample that can be reasonably quantified at a specified level of precision. The most common recommendation is to determine the LOQ as the blank value plus 10 times the repeatability standard deviation, or 3 times the LOD (which gives largely the same figure). The working range for a method is defined by the lower and upper limits of quantification (LLOQ and ULOQ, respectively).

6.4.2. Probability of Detection Limit (POD)

POD is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level. POD from qualitative single-laboratory data is calculated as the number of positive results divided by the total number of tests at each level of added analyte (AOAC 2013). POD is concentration dependent. Analyses to obtain POD is performed by 3 different analysts in different days and 6 batches of the test are included in the study.

6.5. Linearity

Linearity is the ability of the analytical method to produce results by calculating a direct proportion, within the working range. Linearity is described by range and detection limits. Linearity for quantitative ELISA is determined by selecting a minimum of 5 different concentrations of the analyte (multi-point calibration at low, medium and high levels) of standards. The lowest level should fall at approximately the limit of detection, the medium and high levels one and two levels higher respectively (additional intermediate levels may be added to improve precision). The collected data is then statistically analyzed, by performing regression analysis using the method of the least squares, in order to mathematically determine the line that best fits a set of data. Blank and zero samples should not be included in the determination of the regression equation for the calibration curve. Each calibration standard may be analysed in replicate, in which case data from all acceptable replicates should be used in the regression analysis.

Acceptance criteria: $R2 \geq 0.98$

6.6. Linearity-of-Dilution

Linearity-of-dilution experiments provide information about the precision of the assay results for different diluted samples in the chosen sample diluent. These experiments are performed to demonstrate that highly concentrated samples can be accurately measured by diluting into the assay's quantitative range and the concentration can be calculated by multiplying the measured concentration by the dilution factor. Thereby dilution of samples should not affect the accuracy and precision.

Linearity-of-dilution assays also measure the accuracy of the ELISA assay and its compatibility with a sample matrix. This experiment involves serially diluting a sample, preferably with a high endogenous concentration of analyte. If no sample with high endogenous concentration is available, a known concentration of analyte can be added as a spike to the sample and then diluted. The antigen concentration is then measured against the standard curve. Once adjusted for the dilution factor, the analyte concentration at each dilution should be between 80-120% of the concentration measured at the previous dilution, which then demonstrates dilutional linearity. The dilution factor where the change in concentration from previous dilution starts to be linear or constant and is between 80-120% of expected sample recovery becomes the Minimum Required Dilution (MRD) for that particular sample. A serially diluted sample that is not affected by matrix effects should be parallel with the expected calibration curve.

Example: Make serial dilutions of the sample 1:2, 1:4, 1:8, 1:16 …..

$$\%\ \text{change in concentration from previous dilution}\ (1:8) = \frac{c(\textit{analyte concentration from}\ 1:8\ \textit{dilution})}{c(\textit{analyte concentration from}\ 1:4\ \textit{dilution})} \times 100$$

$$\%\ \text{change in concentration from previous dilution}\ (1:16) = \frac{c(\textit{analyte concentration from}\ 1:16\ \textit{dilution})}{c(\textit{analyte concentration from}\ 1:8\ \textit{dilution})} \times 100$$

Calculate in a similar manner for all further dilutions

Note: Analyte concentrations are calculated and adjusted for the sample dilution factor.

Acceptance criteria: The analyte concentration at each dilution should be between 80-120% of the concentration measured at the previous dilution, which then demonstrates dilutional linearity.

6.7. Accuracy

Accuracy, referring to both trueness and precision, is "the closeness of agreement between a test result and the accepted reference value. Trueness refers to the "closeness of agreement between the arithmetic mean of a large number of test results and the true or accepted reference value." Precision, alternatively, deals with the "closeness of agreement between test results."

6.7.1. Precision

Precision for qualitative tests: For a qualitative test (yes/no, binary classification test) it is defined as the proportion of the true positives against all the positive results (both true positives and false

positives). An accuracy of 100% means that the measured values are exactly the same as the given values.

$$Precision = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + false\ positives}$$

Precision for quantitative tests: Repeatability and reproducibility of measured values describe precision for quantitative analysis. Repeatability is the degree of agreement between measurements taken under certain conditions by the same observer in the same laboratory. On the other hand, reproducibility is defined as the degree of agreement between measurements using the same method previously described but independently by a different observer. In ELISA, reproducibility is significant to reported results because it fundamentally describes the precision of ELISA's reagents/components working together to facilitate the antibodies binding to the target of interest and producing signal accurately to quantify its presence within a heterogeneous sample matrix from a population.

Repeatability and reproducibility are usually reported as the standard deviation (σ) or as the coefficient of variation (CV or %CV) which are both measures of variability that are evaluated to define precision. The repeatability standard deviation (RSD), must be determined with at least 6 degrees of freedom. Repeatability should be assessed using a minimum of 6 determinations covering the specified range for the procedure (e.g., 3 concentrations, 2 replicates each) or a minimum of 6 determinations at 100% of the test concentration. This may be done by using incurred material or by fortifying material (blank or incurred) with the required amount of the analyte(s). Replicate extracts are prepared of each of these samples and analysed by one analyst on the same day. Calculate mean, standard deviation and percent relative standard deviation. The average of the individual CVs is reported.

***Acceptance criteria*:** CV of 10% or less is considered satisfactory. Repeatability should be available for all tested food matrices.

7. Trueness

Measurement trueness (or accuracy) describes the closeness of agreement between the average of an infinite number of replicates measured quantity values and an accepted reference value. Lack of trueness indicates systematic error. Ideally, the reference value is derived directly from a certified reference material (CRM) or from materials that can be traced to the CRM. The quantity in which the trueness is measured is called bias, which is the systematic difference between the test result and the accepted reference value.

This is assessed on samples spiked with known amounts of the analyte, the QC samples. The level of accuracy must be determined for the whole range of the analytical procedure. Minimal requirements for this are three concentrations one close to ULOQ, one close to LLOQ and one in the middle of the range, each in six replicates. The accuracy can be expressed as the difference between the obtained experimental value and the nominal value (which is accurate), using the absolute or even better the relative error.

Acceptance criteria: Trueness should be with ± 20% of the accepted reference value across the whole dynamic range of the assay.

7.1. Recovery

Recovery assays involve adding ('spiking') a known concentration of exogenous protein analyte to a diluted sample and testing this sample in the ELISA against an identical concentration of spike added to the sample diluent (Blank). The sample should also be assayed without spike to allow quantification of endogenous analyte. The spiked sample, spiked blank and sample without spike are each measured in the ELISA and the concentrations calculated against the standard curve. Identical recovery responses for both the blank and spiked sample (100% recovery) are generally expected.

The recovery (*R*) is calculated from the difference between the results obtained before and after spiking as a fraction of the added amount. The recovery (*R*) is calculated from the difference between the results obtained before and after spiking as a fraction of the added amount.

$$\% \text{ Recovery} = \frac{c1 - c2}{c3} \times 100$$

Where:

*c*1 = measured concentration in spiked sample

*c*2 = measured concentration in unspiked sample

*c*3 = concentration of spiking

***Acceptance criteria*:** A range between 80-120% recovery is considered acceptable and indicates any matrix affect has been overcome. Recoveries outside this range indicate interference from sample components possibly due to incorrect choice of sample diluent.

7.2. Ruggedness (Robustness)

Robustness or ruggedness is the ability of a method to remain unaffected by small variations in method parameters The ruggedness of the method should be investigated by introducing changes in the procedure and evaluating the effects on the results.

Procedure

Identify critical parameters in the procedure such as deviations in incubation times and temperature (±5% or more), reagent volumes (±5% or more), extraction conditions (time and temperature (±5% or more), variations on assay time and volume of sample dilution buffer

Perform the assay with systematic changes in these parameters, one at the time, using the same set samples (in duplicate) at each occasion. Optimally, the target amount/concentration to be tested should be at the LOD.

Acceptance criteria: The method should provide the expected results irrespective of these minor alterations. For qualitative analyses, all replicates should give positive results

8. Food matrix and sample selection

Food matrix and sample source selection should be based on the types of foods most likely to be used in the analysis or based on risk of contamination. An ELISA/LFS method intended for use in processed foods should be tested on samples subjected to similar processing. The number of food categories to be used will depends on the intended use of the method.

The number of different food categories to be verified depends on the applicability and intended use of the method. Depending on how many categories will be verified, a minimum of 1 – 3 representative matrices from each category listed below should be selected,

A list of foods that can be used based on the applicability are:

i. Meats: Fresh meat, Frozen meat, Raw marinated/minced/comminuted meat, Semi-cooked /Smoked Meat, partially heat treated and/ or smoked meat and meat product, Canned/Retorted meat product, Chilled meat, Cooked Meat/meat product, Cured/pickled meat products, Dried/Dehydrated meat/meat products, Fermented meat products sausage, lunch meat, meat substitutes etc ("meat" means all edible parts (including edible offal) of any food animal slaughtered in an abattoir that are intended for or have been judged as safe and suitable for, human consumption; "meat food products" means any product prepared from meat and other ingredients through various processing methods in which meat should be the major ingredient of all the essential ingredients

j. Seafood: Chilled/Frozen Finfish, crustaceans, cephalopods, molluscs, bivalves, dried or Salted and dried fish products, thermally processed, fermented, smoked, canned fish products. Fish sticks, surimi, raw fish filet, raw oysters, raw mussels, raw clams, cooked crawfish, crabmeat (fresh or pasteurized), battered and breaded fish products,

k. Fruits, Vegetables, and Nuts: Fresh / frozen /dehydrated or dried fruits and vegetables, fresh fruit juice, apple cider, tomato juice, fruit cubes, berries, peanut butter, coconut, fruit powders almonds, minimally processed lettuce, spinach, kale, collard greens, cabbage, bean sprouts, seed sprouts, peas, mushroom, green beans and other minimally processed fruit and vegetable products

l. Dairy: Dahi, Yogurt, Paneer, Khoa, Channa, hard and soft cheeses, raw or pasteurized liquid milk, infant formula, coffee creamer, ice cream, milk powders, casein, whey, non-fat dry milk/dry whole milk,

m. Chocolate / bakery: Frosting and topping mixes, candy and candy coating, milk, chocolate, cake mixes,

n. Egg and egg products: Shell eggs, liquid whole eggs, dried whole egg or dried egg yolk, dried egg whites, salad dressing.

o. Nuts: All nuts such as cashew nut, walnut, peanut, almond, pistachios

p. Herbs and spices: Oregano, pepper, paprika, black pepper, white pepper, celery seed or flakes, chili powder, cumin, parsley flakes, rosemary, sesame seed, thyme, vegetable flakes, onion flakes, onion powder, garlic flakes, allspice,

q. Processed grains and legumes: Flours, grits, rice corn meal, soy flour, dried yeast, cereal based complementary food, Uncooked noodles, macaroni, spaghetti, soygurt, tofu, soy beverage

9. Information to be provided with method verification documents

A listing of information that should be provided from the verifying laboratory when the results for a laboratory verification study are prepared for review.

This list of information is in addition to all the results obtained for the validation criteria/insert accompanying the kit. For Qualitative and Quantitative Assays:

A. Assay Design

- Type of assay: ELISA/Sandwich ELISA/Competitive ELISA/LFS
- Name of analyte (Protein)
- Nature of antibody (Monoclonal/polyclonal)
- Reporter molecule: Enzyme/substrate characteristics
- Detection method: UV/Vis/Fluorescence etc.
- Any other reporter molecules.

B. Sample Preparation and Protein Extraction

- Form and quantity of sample required. Include information on subsampling or sample compositing as well as relevant aspects of handling and storage.

C. Method or kit used for extraction. Include any relevant modifications

D. ELISA Conditions

- Reaction: reaction volume; identities and concentrations of all reaction components, including buffer/diluent/additives.
- Platform: State make and model of instrument used for detection as well as name and version of accompanying software. Include brief descriptions of physical format (e.g., 96/384 well or other) and optical system.
- Assay conditions and detailed methodology. Include washing and incubation steps

E. Data Analysis

- Specify which software program and version was used for data analysis.
- Report and explain any adjustments or other software default analysis parameters.
- Include all data: tables, calibration curves, recovery data etc

10. Conclusion

Based on verification of test method for the above defined parameters meeting the acceptance criteria as per the procedure defined above, the method will be declared as fit for intended use.

11. Definitions

Accuracy: Closeness of agreement between a quantity value obtained by measurement and the true value of the measurand.

Analytical sample: Sample prepared from the laboratory sample by grinding, if necessary, and homogenization.

Certified reference material: Use of known materials can be used to assess the accuracy of the method, as well as obtaining information on interferences.

Cross-Reactivity: Degree to which binding occurs between an antibody and antigenic determinants, or primers and a target sequence, which are not the analyte of primary interest.

***ELISA*-** For the purposes of this document, ELISA is defined as "an analytical procedure characterized by the recognition and binding of specific antigens by antibodies". This definition is not meant to be restrictive and encompasses other related binding-based technologies.

False Positive and False Negative*:* The false positive and false negative are the probability that respectively a negative sample would be classified positive or a positive one would be regarded as negative.

Lateral flow Immunoassays- The lateral flow immunoassay (LFIA) is a paper/membrane-based platform for the detection and quantification of analytes in complex mixtures, where the sample is placed on a test device and the results are displayed within 5–30 min.

Limit of Application *(LLA):* Manufacturers or method developers are free to define an LLA at whatever level of confidence they choose. This value may be higher than the LOQ and represents a level below which the method developer does not support or recommend use of the method.

Limit of Detection (LOD): LOD is the lowest amount or concentration of analyte in a sample, which can be reliably detected but not necessarily quantified. Experimentally, methods should detect the presence of the analyte at least 95% of the times at the LOD, ensuring ≤5% false negative results.

Limit of Quantification (LOQ): LOQ is the lowest amount or concentration of analyte in a sample, which can be reliably quantified with an acceptable level of precision and trueness.

Matrix: Totality of components of a material system except the analyte.

Precision: Closeness of agreement between quantity values obtained by replicate measurements of a quantity, under specified conditions. Precision is usually expressed as the standard deviation or relative standard deviation.

Relative Repeatability Standard Deviation (RSDr): The relative standard deviation of test results obtained under repeatability conditions.

Probability of detection (POD): The probability of a positive (i.e., presence detected) analytical outcome for a qualitative method for a given matrix at a given concentration. It is estimated by the expected ratio of positive to negative results for the given matrix at the given analyte concentration.

Qualitative ELISA: can be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen.

Quantitative ELISA: data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples.

Recovery*:* The fraction or percentage of analyte that is recovered when the test sample is analysed using the entire method.

Repeatability*:* Variation arising when all efforts are made to keep conditions constant by using the same instrument and operator (in the same laboratory) and repeating during a short time period. Expressed as the repeatability standard deviation (SDr); or % repeatability relative standard deviation (%RSDr)

Reproducibility*:* Variation arising when identical test materials are analyzed in different laboratory by different operators on different instruments. The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility standard deviation (SDR); or % reproducibility relative standard deviation (%RSDR).

Robustness*:* The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviation from the experimental conditions described in the procedure.

Semi-Quantitative ELISA: can be used to compare the relative levels of antigen in assay samples, since the intensity of signal will vary directly with antigen concentration

Specificity: The property of the method to respond exclusively to the characteristic or the analyte of interest.

Verification: Verification is the confirmation, through the provision of objective evidence, that specified test characteristics have been fulfilled.

12. REFERENCES

AOAC (2013): Appendix N: ISPAM Guidelines for Validation of Qualitative Binary Chemistry Methods

AOAC International (2007), *How to meet ISO/IEC 17025 requirements for method verification*, ALACC Guide, www.aoac.org/alacc_guide_2008.pdf.

Guidance for Industry. Bioanalytical method validation. *Fed Regist* (2001) 66:28526–7. [Google Scholar]

ISO/IEC 17025. *General Requirements for the Competence of Testing and Calibration Laboratories* (2017).

ISO 5725-2. Accuracy (Trueness and Precision) of Measurement Methods and Results – Part 2: Basic Method for the Determination of Repeatability and Reproducibility of a Standard Measurement Method (2019).

Minic, R. and Zivkovic, I. Optimization, Validation and Standardization of ELISA DOI: 10.5772/intechopen.94338

Tate, J. and Ward, G. Interferences in Immunoassay. Clin. Biochem. Rev. 2004 May 25(2): 105-120

Validation and verification of quantitative and qualitative test methods. NATA, January 2018

Annexure I : *APPLICATION FORM FOR APPROVAL OF RAPID ANALYTICAL FOOD TESTING (RAFT) KIT/EQUIPMENT/ METHOD BY FSSAI*

A. Application for (tick whichever is appropriate) ☐ Rapid food testing kit/media ☐ Rapid Equipment ☐ Rapid Method ☐ Rapid food testing kit with equipment ☐ Any other, please specify
B. Details of the kit
(a) Name of the Rapid test kit/media/device/method
(b) Proposed regulatory use (specific product testing/analytical method)
C. General Information
1. Details of Applicant
(a) Name of Principal manufacturer/ OEM
(b) Name of authorized person/ dealer in India (attach Authorization letter from principal manufacturer)
(c) Mobile No/Phone No
(d) Email (all communication will be through provided email/phone number)
(e) Name of the organization/manufacturer
(f) Address of the organization/registered office

(g) Manufacturing License number in India if any
D. Technical Information - Contents to be submitted with the Dossier for pre-evaluation by FSSA(I) ***NOTE: The applicant should mark any proprietary information***
1. Product Information
(a) Market name, product name and product code
(b) Names and corporate addresses of manufacturers
(c) Country of Origin
(d) Address(es) of manufacturing site(s)
(e) Whether approved/verified by regulatory bodies/ organizations
(f) If yes, name of regulatory bodies/organizations and validity of approval
(g) If validated by international bodies (e.g. ISO/AOAC etc.)
(h) If yes, attach documents/certificates/approvals etc.
(i) Bar code scanner, power source, data storage capacity (if applicable)
(j) Evidence that manufacturers have a certified quality management system or Good Manufacturing Practice (GMP) certification; if applicable

2. Provide details of the conventional method/equipment/test kit with which the said product should be compared with
3. Technical Specifications on rapid testing kits/device/method (*this list is only indicative all necessary information to support and strengthen the application must be submitted)*
(a)The principle and detailed methodology
(b) Specify Food Category/Matrix as per FSSR for which approval is sought
(c) Test procedure, including the time needed to run the test
(d) Qualitative/ Semi Quantitative/Quantitative
(e) Range and Reporting Units (if applicable)
(f) LOD/LOQ/Detection capability
(g) Sensitivity (wherever applicable)
(h) Specificity (including where the studies where performed to generate these values and 95% confidence intervals with supporting documents)
(i) Reproducibility across multiple test kit lots (e.g. including number of samples, type of food, number of different lots/devices)
(j) Inclusivity/ exclusivity (applicable for microbiology kits/methods/device etc.)
(k) Robustness of the kit/method
(l) Details of inter-laboratory validation of method/multiple users of device

(m) Demonstration of stability throughout the shelf life of the product under recommended storage conditions(not applicable to devices and methods)
(n) If device, warranty period, availability of maintenance service/ spare parts etc
(o) Evidence of satisfactory test performance for kits from users (minimum three) within India
4. Operational characteristics for kits/devices
(a) Number of steps (from starting to results)
(b) Total run time (sample preparation to final result)
(c) Ease of data interpretation
(d) Overall ease of use
(e) Training requirements
(f) Recommended storage conditions
(g) Shelf life of kit
(h) Kit size/Device (hand-held/table top/portable/non-portable)
(i) Image/flow-chart of rapid kit/equipment/method
(i) Minimum quantity of sample required for one analysis

(j) Number of Individual tests/package
(k) Required accessories necessary for operation that are not provided. (If the accessories/equipment are proprietary, then provide the validation data).
(l) Availability of Certified Reference Material/Standard Reference Material/Quality Control material provided
(m) Advantages and disadvantages over the conventional technique/method/device
(n) Amount and type of waste generated (e.g. chemical/biological hazard)
(o) Cost/Kit and Cost/Test, cost/device
5. Fee details: Amount paid (Rs. ……..) Mode of payment (Online or Offline*) Transaction id/UTR No. with date and bank name & account no. (In case of Online payment) Name of Bank, IFSC Code, DD No. & date (In case of Offline payment) Name of the payee (both in case of Online or Offline payment) **payment through cheque mode and cash will not be accepted.* The application processing fee of Rs. 2000/- (Rupees two thousand) + GST @18% can be paid through online mode, in the bank account mentioned below- Name: Senior Accounts Officer, FSSAI, New Delhi Bank: Bank of Baroda, NirmanBhawan

Account No:	26030100008653
IFSC Code:	BARB0(Zero)NIRDEL
The GST No. of FSSAI is 07AAAGF0023K1ZV (0 is Zero).	
6. Any additional specific information	

I/ We understand that incomplete submissions, submission not conforming to the prescribed format, and applications containing excessive errors will be summarily rejected. I/ We undertake that requisite material/ content will be submitted to FSSAI as desired in case the pre-evaluation document is approved by FSSAI and FSSAI will provide the applicant with instructions for further action. If the documentation is not approved, FSSAI will notify the applicant with reasons.

Name of the authorized personnel …….

Signature and Seal…….

Contact details……..

To

Advisor, Quality Assurance Division

Annexure II: CHECKLIST FOLLOWED FOR SCRUTINIZING RAFT KIT/EQUIPMENT/METHOD APPLICATIONS

Name of Rapid Kit:

RAFT COMMITTEE REVIEWER'S COMMENTS

A. Application for (tick whichever is appropriate) **√Rapid food testing kit** □ Rapid Equipment □ Rapid Method □ Any other, please specify	**REMARKS**
B. General Information	
1. Details of Applicant	
(a) Name of Principal manufacturer/ OEM	
(b) Name of authorized person/ dealer in India (attach Authorization letter from principal manufacturer)	
(c) Mobile No/Phone No	
(d) Email (all communication will be through provided email/phone number)	
(e) Name of the organization/manufacturer	
(f) Address of the organization/registered office	
(g) Manufacturing License number in India if any	
(h) Name of the Rapid test kit	

(i) Proposed regulatory use (specific product testing/analytical method)	
C. Technical Information	
1. Product Information	
(a) Market name, product name and product code	
(b) Names and corporate addresses of manufacturers	
(c) Country of Origin	
(d) Address(es) of manufacturing site(s)	
(e) Whether approved/verified by regulatory bodies/ organizations	
(f) If yes, name of regulatory bodies/organizations and validity of approval	
(g) If validated by international bodies (e.g. ISO/AOAC etc.)	
(h) If yes, attach documents/certificates/approvals etc.	
(i) Bar code scanner, power source, data storage capacity (if applicable)	
(j) Evidence that manufacturers have a certified quality management systemor Good Manufacturing Practice (GMP) certification; if applicable	
2. Provide details of the conventional method/equipment/test kit with which the said product should be compared with	
3. Technical Specifications on rapid testing kits/device/method (*this list is only indicative all necessary information to support and strengthen the application must be submitted)*	

(a)The principle and detailed methodology	
(b) Specify Food Category/Matrix as per FSSR for which approval is sought	
(c) Test procedure, including the time needed to run the test	
(d) Qualitative/ Semi Quantitative/Quantitative	
(e) Range and Reporting Units (if applicable)	
(f) LOD/LOQ/Detection capability	
(g) Sensitivity (wherever applicable)	
(h) Specificity (including where the studies where performed to generate these values and 95% confidence intervals with supporting documents)	
(i) Reproducibility across multiple test kit lots (e.g. including number of samples, type of food, number of different lots/devices)	
(j) Inclusivity/ exclusivity (applicable for microbiology kits/methods/device etc.)	
(k) Robustness of the kit/method	
(l) Details of inter-laboratory validation of method/multiple users of device	
(m) Demonstration of stability throughout the shelf life of the product under recommended storage conditions(not applicable to devices and methods)	

(n) If device, warranty period, availability of maintenance service/ spare parts etc	
(o) Evidence of satisfactory test performance for kits from users (minimum three) within India	
4. Operational characteristics for kits/devices	
(a) Number of steps (from starting to results)	
(b) Total run time (sample preparation to final result)	
(c) Ease of data interpretation	
(d) Overall ease of use	
(e) Training requirements	
(f) Recommended storage conditions	
(g) Shelf life of kit	
(h) Kit size/Device (hand-held/table top/portable/non-portable)	
(i) Image/flow-chart of rapid kit/equipment/method	
(i) Minimum quantity of sample required for one analysis	
(j) Number of Individual tests/package	
(k) Required accessories necessary for operation that are not provided	

(l) Availability of Certified Reference Material/Standard Reference Material/Quality Control material provided	
(m) Advantages and disadvantages over the conventional technique/method/device	
(n) Amount and type of waste generated (e.g. chemical/biological hazard)	
(o) Cost/Kit and Cost/Test, cost/device	
5. Any additional specific information	
6. Recommendation by Reviewer (with justification)	
7. Labs identified for verification (if applicable)	

NAME OF THE REVIEWER: -

Signature of Reviewer:

Signature of Chairman, RAFT Committee:

Annexure-III LIST OF LABORATORIES FOR VERIFICATION OF PROVISIONALLY APPROVED RAPID KIT/EQUIPMENT/METHOD

S. No.	Name of Laboratories*
Referral Food Laboratory	
1.	National Food Laboratory, 3 Kyd Street, Kolkata- 700016
2.	Food Safety & Analytical Quality Control Laboratory, C/o Central Food Technological Research Institute, Mysore-570013
3.	State Public Health Laboratory, Stavely Road, Cantonment Water Works Compound, Pune-411001
4.	National Food Laboratory, Ahinsa Khand-II, Indirapuram Ghaziabad-201014
5.	Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore-560089
6.	Quality Evaluation Laboratory, Spices Board, Palarivattom P.O. Kochi-682025
7.	Quality Evaluation Laboratory, Spices Board, Chuttugunta Center, GT Road, Guntur-522004
8.	Quality Evaluation Laboratory, Spices Board, Plot No. R-11, Sipcot Industrial Complex, Gummidipoondi, Thiruvallur Dt., Chennai-601201
9.	Quality Evaluation Laboratory, Spices Board, First Floor, Banking complex II, Sector 19A, Vashi, Navi Mumbai-400703
10.	Centre for Analysis and Learning in Livestock in Food (CALF), National Dairy Development Board (NDDB), Anand-388001, Gujarat
11.	CSIR-Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad - 500007
12.	National Research Centre on Meat, Chengicherla, Buduppal, Hyderabad – 500092
13.	Indian Institute of Food Processing Technology, Food Safety and Quality Testing Laboratory, Pudukkottai Road, Thanjavur – 613005, Tamil Nadu

14.	ICAR- Central Institute of Fisheries Technology, Willingdon Island, CIFT Junction, Matsyapuri P.O., Cochin – 682029, Kerala
15.	ICAR-National Research Centre for Grapes, P.O. Manjiri Farm, Solapur Road, Pune - 412307
16.	Pesticide Formulation and Residue Analytical Centre, National Institute of Plant health Management, Rajendranagar, Hyderabad – 500030
17.	Punjab Biotechnology Incubator, Mohali SCO7 & 8, Phase-5, SAS Nagar, Mohali -160059, Punjab
18.	CSIR-Indian Institute of Toxicology Research, Vishvigyan Bhawan, 31, Mahatma Gandhi Marg, Lucknow - 226 001, Uttar Pradesh, India
19.	Centre for Food Research and Analysis (CFRA), NIFTEM, Plot No. 97, Sector-56, HSIIDC, Industrial Estate, Kundli, Sonipat, Haryana-131028
National Reference Laboratory	
20.	Centre for Analysis and Learning in Livestock in Food (CALF), National Dairy Development Board (NDDB), Anand-388001, Gujarat
21.	CSIR-Indian Institute of Toxicology Research, Vishvigyan Bhawan, 31, Mahatma Gandhi Marg, Lucknow - 226 001, Uttar Pradesh
22.	Edward Food Research & Analysis Centre Limited, Subhas Nagar, Barasat P.O., Nilgunj Bazar, Kolkata – 700121, West Bengal
23.	Export Inspection Agency, 27/1767 A, Shipyard Quarters Road, Panampilly Nagar (South), Kochi, Kerala – 682036
24.	Fare Labs Pvt. Ltd., L-17/3, DLF, Ph – II, IFFCO Chowk, M.G. Road, Gurugram – 122002, Haryana
25.	Food Safety & Analytical Quality Control Laboratory, C/o Central Food Technological Research Institute, Mysore-570020, Karnataka
26.	ICAR-National Research Centre for Grapes, P.O. Manjiri Farm, Solapur Road, Pune – 412307, Maharashtra
27.	ICAR- Central Institute of Fisheries Technology, Willingdon Island, CIFT Junction, Matsyapuri P.O., Cochin – 682029, Kerala

28.	Neogen Food & Animal Security (India) Private Limited, Uchikkal Lane, Poonithura P.O., Kochi – 682038, Kerala
29.	Punjab Biotechnology Incubator, C-134, Phase 8, Industrial Area, Sector 73, Sahibzada Ajit Singh Nagar, Punjab 160071
30.	Trilogy Analytical Laboratory Pvt. Ltd., Plot No. 7, C.F. Area, Phase-II, IDA Cherlapally, Hyderabad – 500051, Telangana
31.	Vimta Labs Limited, Life Sciences Campus, 5, MN Park, Genome Valley, Shameerpet, Hyderabad – 500101, Telangana
Ancillary facility of NRLs (ANRLs)	
32.	Export Inspection Agency EIA, Chennai, 6th Floor, CMIDA Tower-II, 1, Gandhi Irwin Road, Egmore, Chennai – 600008, Tamil Nadu
33.	Export Inspection Agency EIA, Kolkata, 101, Southend Conclave, 1582, Rajdanga Main Road, Kolkata – 700107, West Bengal

**Note: Other than the laboratories mentioned in the table above, FSSAI may identify more laboratories for verification. This list may be updated by FSSAI from time to time.*

Annexure-IV Order-Using FSSAI Logo on rapid kits approved by FSSAI

File No. 11014/09/2020-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 31st March, 2021

ORDER

FSSAI grants final approval to the rapid analytical food testing kits/equipment for use in both screening and surveillance purpose. A Conformance Certificate is subsequently issued to the applicant which is valid for a period of three years from the date of issue.

2. In view of above, based on representations received at FSSAI, a decision has been taken to allow the applicant to use "FSSAI Logo" alongwith "certificate number" and "validity period" on the approved kits/equipment.

3. The applicants should ensure that FSSAI Logo and Certificate number must be as mentioned on the certificate. Further, the validity period of finally approved kits is mentioned in the document titled 'Status of RAFT Applications' which is available on the website of FSSAI at link and will be updated from time to time:

https://fssai.gov.in/cms/raft.php

4. Further, the applicants shall submit a self-declaration form (annexed) to FSSAI within 2 days of initiating the process to print aforesaid information on the rapid kit/equipment.

This issues with the approval of Competent Authority.

Sd/

Encl: As above

(Dr. Dinesh Kumar)
Assistant Director (QA)

To:

(i) IT Division, FSSAI – for uploading on FSSAI website

Copy To:

(i) PPS to Chairperson, FSSAI
(ii) PS to CEO, FSSAI
(iii) PS to Advisor (QA), FSSAI
(iv) PS to Advisor (S&S), FSSAI

SELF-DECLARATION

…….. (Name of company) has developed a Food Safety Kit/Equipment to assess the safety parameters of …….. (Name of Food Product/Category).

2. FSSAI has assessed the Food Safety Kit/Equipment and found the same to be useful for …….. (as per certificate issued)

3. …….. (Name of company) is allowed to use the logo of FSSAI alongwith "certificate number" and "validity period" together.

4. I …….. (Name of authorized signatory) hereby declare that all the information i.e. FSSAI logo, certificate number and validity period is as per instructions by FSSAI only. FSSAI has the right to withdraw the approval and permission to use FSSAI logo etc. if information provided is found to be misleading/ incorrect; without any prior notice.

Name of the authorized personnel ……..

Signature and Stamp……..

Contact details……..

Annexure-V format for undertaking in case there is no change in the kit for renewal

(Company letter head)

Self-declaration

I, Shri ……. resident …… as Director/Authorized signatory for M/S……. and ……. (Company address), manufacturers of ……. (name of kit/method/equipment name), do here by solemnly affirm and declare that no change (s) /modification (s) has/have been made to the rapid kit/method/equipment since originally receiving FSSAI approval *vide* Certificate number…… dated……... and that the rapid kit/method/equipment performs as originally evaluated for the intended use and claims.

Date:

Place:

Authorized Signatory with seal for ……..(Company name)

Food Safety and Standards Authority of India
(Ministry of Health and Family Welfare)
FDA Bhawan, Kotla Road,
New Delh i-110002
https://www.fssai.gov.in/cms/raft.php
For any query contact: raft-approval@fssai.gov.in

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
Inspiring Trust, Assuring Safe & Nutritious Food
Ministry of Health and Family Welfare, Government of India
FDA Bhawan, Kotla Marg, Near Bal Bhavan,
New Delhi-110002, Tel: 011 23220993