ICMR-CDSCO/IVD/GD/PROTOCOLS/11/2025

Indian Council of Medical Research and Central Drugs Standard Control Organization
Department of Health Research and Drugs Controller General of India
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Licensure of In-Vitro Diagnostics (IVDs) under Medical Devices Rules 2017 requires a detailed evaluation protocol for the performance evaluation of IVDs to evaluate their quality and performance. To facilitate this process, the Indian Council of Medical Research (ICMR) and CDSCO have come together to draft standard evaluation protocols for use by IVD manufacturers testing labs in India. Currently, the following IVD evaluation protocols have been developed by ICMR and CDSCO:

1. *PerformanceevaluationprotocolforInfluenzavirusmoleculardetectionand/or differentiation assay (single plex/multiplex format)*
2. *PerformanceevaluationprotocolforSARS-CoV-2moleculardetectionassay(single plex/multiplex format)*
3. *Performance evaluation protocol for Respiratory Syncytial Virus molecular detection assay (single plex/multiplex format)*
4. *Performance evaluation protocol for Influenza virus and SARS-CoV-2 molecular detection differentiation assay (multiplex format)*
5. *PerformanceevaluationprotocolforInfluenzavirus,SARS-CoV-2andRSVmoleculardetection and differentiation assay (multiplex format)*
6. *Performance evaluation protocol for Malaria rapid diagnostic test (RDT) for P falciparum and/or P vivax*
7. *Performance evaluation protocol for Malaria ELISA assay*
8. *Performance evaluation protocol for Malaria real time PCR assay*
9. *Field evaluation protocol for combo Malaria Rapid Diagnostic Test (RDT) kits (detecting P vivax and P falciparum)*
10. *Performance evaluation protocol for Nipah virus Real Time PCR*
11. *Performance evaluation protocol for Chandipura virus Real Time PCR*
12. *Performanceevaluationprotocolformultiplexrespiratoryvirus(expandedpanel)Real Time PCR*
13. *Performance evaluation protocol for Dengue IgG RDT*
14. *Performance evaluation protocol for Dengue IgM/IgG Combo RDT*
15. *Performance evaluation protocol for Dengue IgG ELISA*

These protocols are now being placed in the public domain for comments from relevant stakeholders. This window of opportunity will close on 25th August 2025, and, once finalized, there will be minimal scope for change in these documents. Therefore, all interested stakeholders are requested to provide their comments before 25th August 2025, at ivdevaluation@gmail.com as per the enclosed format. Once the public

Consultation period concludes, all comments will be reviewed and considered in finalizing the draft protocols before final clearance by ICMR and CDSCO.

Dated: 11th August2025
Place: New Delhi

STANDARDIVDPERFORMANCEEVALUATIONPROTOCOL

STAKEHOLDERFEEDBACKFORM

S.N.	Name of the Protocol	Document No.	Page No.	Line No.	Current Text	Proposed Text	Explanation/Reference

Name: _____

Designation and Affiliation:_____

STANDARD PERFORMANCE EVALUATION PROTOCOLS

DRAFT FOR STAKEHOLDER COMMENTS

Influenza virus, SARS-CoV-2, Respiratory Syncytial Virus In-Vitro Diagnostics

ICMR-CDSCO/IVD/GD/PROTOCOLS/05/2025

AUGUST, 2025

New Delhi, India

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GENERAL GUIDELINES

Protocols for performance evaluation of in vitro molecular diagnostic kits for detection and differentiation of Influenza virus and/or SARS-CoV-2 and/or RSV

1. Introduction:

This document provides a framework for evaluating the performance characteristics of *in vitro* diagnostic (IVD) kits used in identifying and distinguishing various strains of Influenza viruses and/or SARS-CoV-2 and/or RSV, aligning with international standards to ensure reliability and accuracy in diagnosis. The coronavirus diseases 2019 (COVID-19) pandemic, caused by the SARS-CoV-2 virus, has necessitated the rapid development and validation of in vitro molecular diagnostic kits. These kits are crucial for the timely detection and differentiation of major respiratory viruses (influenza/SARS-CoV-2/RSV) to control their spread. This protocol outlines a systematic approach for validating these diagnostic kits to ensure their accuracy, sensitivity, specificity, and reliability.

Although SARS-CoV -2 is no longer a public health emergency globally, it is prudent to implement integrated surveillance for Influenza, SARS-CoV-2 and other respiratory viruses, making differential diagnosis for these viruses essential. Additionally, timely diagnosis of other respiratory viruses, particularly Respiratory syncytial virus (RSV), is crucial for providing effective clinical management to pediatric cases.

This document provides guidance for single plex or multiplex assays for the differential diagnosis of Influenza and/or SARS-CoV-2 and/or RSV. It outlines the evaluation of IVD devices/kits intended for the detection and differentiation of influenza virus strains and/or detection of SARS-CoV-2 and/or detection and differentiation of RSV using nucleic acid detection methods as outlined in the scope below. This includes IVD devices/kits that detect and differentiate between influenza virus types (Influenza A or B), subtypes (A (H1N1) pdm09 or A (H3N2)), and/or multiple influenza virus types/subtypes; kits that identify only SARS-CoV-2, as well as kits that only detect and/or differentiate RSV. Additionally, this protocol may be used for multiplex IVD devices/kits designed to simultaneously detect Influenza A & B (with or without subtyping), and/or SARS-CoV-2, and/or RSV. This document outlines the following aspects of performance evaluation of IVD devices/kits as per the scope outlined in the document:

1.1 The procedure for validating entities to determine operational parameters of IVD devices/kits that detect influenza virus gene segment(s).

1.2 T procedure for validating entities to determine operational parameters of IVD devices/kits that detect SARS-CoV-2 gene segment(s).

1.3 The procedure for validating entities to determine operational parameters of IVD devices/kits that detect RSV gene segment(s).

1.4 The techniques for identifying influenza virus/SARS-CoV-2/RSV nucleic acid targets in single-plex or multiplex formats (using appropriate protocols listed in the document).

1.5 This document is not useful for performance evaluation of serological assays for detection of antigen and antibody for influenza viruses/SARS-CoV-2/RSV. The IVD device/kit to be validated is henceforth known as the "Kit under Evaluation."

2. Objective:

This document aims to offer a comprehensive set of instructions for evaluating the performance of molecular IVD assays mentioned in the scope below for detecting Influenza A and Influenza B viruses with/without subtyping, and other common respiratory viruses such as SARS-CoV-2 and RSV. This evaluation will focus on measuring the analytical sensitivity and specificity, cross-reactivity, repeatability, and reproducibility as compared against a reference assay using clinical sample panel.

In brief, the objectives are as follows:

2.1 To validate the performance characteristics of in vitro molecular diagnostic kits for detecting Influenza A & B (with/without subtyping)/ SARS-CoV-2/ RSV.

2.2 To ensure the kits under evaluation meet the necessary standards for sensitivity, specificity, repeatability, and reproducibility.

2.3 To evaluate the cross-reactivity of the kits with other respiratory viruses.

3. Scope:

This guideline is solely for the evaluation and establishment of the performance characteristics of IVD kits and devices designed for the detection and subtyping of commonly circulating seasonal Influenza viruses (Influenza A(H1N1) pdm09, Influenza A(H3N2), Influenza B(Yamagata) and Influenza B(Victoria) subtypes) and/or other common respiratory viruses such as SARS-CoV-2 and RSV, using single or multiplex molecular assays (as outlined in the scope below) intended for human clinical samples. This document is a guide to assess:

3.1 The analytical assay performance characteristics with clinical specimens for the detection and/or differentiation of influenza viruses. (Protocol A)

3.2 The analytical assay performance characteristics with clinical specimens for the detection of SARS-CoV-2 (Protocol B)

3.3 The analytical assay performance characteristics with clinical specimens for the detection of RSV (Protocol C)

3.4 The analytical performance characteristics of multiplex assay for detection of two or more of these viruses by combining Protocols A, B & C as per the kit format.

3.5 Analytical performance characteristics which should include sensitivity, specificity, cross-reactivity, and lot-to-lot variation including functionality of devices that identify and/or differentiate influenza viruses, SARS-CoV-2 and/or RSV depending on the kit format.

3.6 The performance of the kit, only if the kit includes an internal control (**preferably** endogenous, or exogenous).

3.7 This document may also apply to forthcoming influenza, SARS-CoV-2 and RSV molecular diagnostic devices that do not fit within these current classifications.

3.8 The document will serve as a reference for assessing kits based on Nucleic Acid Amplification Test (single plex or multiplex assays) as listed below:

3.8.1 Real-time Reverse Transcription Polymerase Chain Reaction format (rRT-PCR): including Real-time PCR probe-based assays or non-probe based assays

3.8.2 Other NAT testing platforms such as LAMP/RPA, and other closed system platforms such as TrueNat /cartridge-based assays

Note: *This protocol is not suitable for the kits where amplicons are handled outside the amplification system.*

4. Requirements:

4.1 Supply of kits under evaluation (Along with batch/lot No. Expiry & required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment and consumables.

4.2 Evaluation sites/laboratories (With required equipment)

4.3 Reference test kits

4.4 Characterized samples for evaluation panel

4.5 Laboratory supplies

5. Ethical approvals:

Laboratory validation of IVDs using irreversibly de-identified samples is exempted from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024. A self-declaration form as provided in ICMR guidelines to be submitted by the investigators to the institutional authorities and ethics committee for information (https://ethics.ncdirindia.org/asset/pdf/Guidance_on_Ethical_Requirements_for_Laboratory_Validation_Testing.pdf)

6. Procedure:

6.1 Study design/type: Diagnostic accuracy study using leftover irreversibly de-identified archived clinical samples.

6.2 Evaluation site/laboratory considerations: Identified IVD kit evaluation laboratories should establish their proficiency through

6.2.1 Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

6.2.2 Have sufficient numbers of archived as well as contemporary clinical specimens positive for respiratory viruses targeted by the kit under evaluation (Influenza A(H1N1)pdm09, A(H3N2), B(Yamagata), B(Victoria), and/or SARS-CoV-2 and/or RSV A & B), with aliquots stored at -80 ℃ deep freezers or in lyophilized form.

6.2.3 Virus strains should be well-characterized by ICMR approved or US FDA/ ATAGI Australia/PMDA Japan approved/WHO Pre-Qualified reference assay and/or by influenza virus HA gene/segment or gene-specific sequencing (for SARS-CoV-2 and RSV) or Next-Generation Sequencing.

6.2.4 Have a minimum BSL-2 level facility with trained manpower and at least two different Real Time platforms to perform molecular diagnostic assays for Influenza virus and other respiratory viruses.

6.2.5 Have a good record of External Quality Assurance programs for influenza, SARS-CoV-2, and other respiratory viruses.

6.2.6 Staff training: All the staff involved in IVD kit evaluation should undergo hands-on training and competency testing on the following:

6.2.6.1 Preparation & characterization of kit evaluation panel

6.2.6.2 Handling of respiratory virus PCR kits received for performance evaluation (Verification/Storage/Unpacking etc).

6.2.6.3 Testing, interpreting, recording of results & reporting

6.2.6.4 Data handling, data safety & confidentiality

6.3 Performance characteristics: To be assessed for all assay targets of influenza A/B, SARS-CoV-2 and RSV (single plex or multi-plex assays)

6.3.1 Analytical Sensitivity and specificity

6.3.2 Cross-reactivity

6.4.3 Repeatability

6.4.4 Reproducibility

Protocol A

Evaluation of performance characteristics of Molecular Kit detecting influenza A & B viruses, and subtyping into A (H1N1) pdm 09, A(H3N2), B(Yamagata) & B(Victoria) in single plex or multiplex format

1. **Objective:**

1.1 To evaluate the performance of molecular IVD device /KIT for detection and differentiation of Influenza viruses as per the scope outlined in this document.

1.2 To ensure the kits under evaluation meet the necessary standards for sensitivity, specificity, repeatability, and reproducibility.

1.3 To evaluate the cross-reactivity of the kits with other respiratory viruses.

2. **Evaluation of performance characteristics should be done for the following parameters:**

2.1 Sensitivity and specificity

2.2 Cross-reactivity

2.3 Repeatability

2.4 Reproducibility

3. **Panel development: Clinical sample (archived/contemporary) panel for testing:**

3.1 Contemporary leftover irreversibly de-identified clinical/archived respiratory samples (in VTM) for the panel should be irreversibly de-identified.

3.2 Samples to be used for panel preparation shall be stored properly at – 80 °C or lyophilized.

3.3 Unless the manufacturer has specific requirement of nucleic acid extraction kit, the validation laboratory can use WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved/ICMR validated total RNA / viral RNA extraction kits for the evaluation.

3.4 Clinical samples for evaluation should be characterized by a reference kit / Sequencing/NGS.

3.5 All positive samples should be confirmed positive for the target pathogens by the reference assay.

3.6 All negative samples should be confirmed negative for the target pathogens by the reference assay.

4. Sample size and sample panel composition for evaluation of performance characteristics:

Sample sizes of positive and negative samples of the analyte/pathogen targeted by the kit against different values of sensitivity and specificity are provided in Table 1. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the number of positive samples.*
- *n (sp) is the number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Sample sizes for positive samples and their composition for evaluating subtyping are provided in Table 2.

Table 1. Sample sizes per target pathogen for different values of sensitivity/ specificity claimed by the manufacturer.

Sensitivity/ Specificity	*Sample size: Minimum number of positive samples*[¥]	*Composition of positive samples*[#]	*Sample size: Minimum number of negative samples (rounded)* [¥]	*Minimum number of cross reactive* samples among the negative samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07	20	5
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28	80	20
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55	150	38
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76	210	53
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91	260	65
[#]Strong positive: (Ct value <25) Moderate positive: (Ct value between 25-30) Weak positive: (Ct value >30 and and ≤ 34)				
[¥] Equal distribution of positive nasopharyngeal and/or oropharyngeal swabs in virus transport medium (VTM) to be used				
* Samples positive for common respiratory viruses (such as SARS-CoV-2, Parainfluenza viruses, Adenoviruses, Rhinoviruses, Respiratory Syncytial Virus (including its types and subtypes), common human coronaviruses), other than the ones targeted by the kit under evaluation. Equal distribution of cross-reactive viruses is desirable.				

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 2. Sample sizes for positive samples and their composition for evaluating subtyping

	Sample size* (per target pathogen)			
	Influenza A (H1N1) pdm09	Influenza A/H3N2	Influenza B	
Sensitivity	Minimum number of nasopharyngeal swabs/ oropharyngeal swabs (rounded figures)	Minimum number of nasopharyngeal swabs/ oropharyngeal swabs (rounded figures)	Minimum number of nasopharyngeal swabs/ oropharyngeal swabs (rounded figures)	Minimum total number of positive samples (rounded figures)
99%	20	20	20	60
95%	80	80	80	240

90%	150	150	150	450
85%	210	210	210	630
80%	260	260	260	780
*Combination of strong, moderate and weak positive samples should be considered as per the information provided in Table 1.				

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

4.1 Repeatability testing will be performed on 3 positive (strong, moderate and weak positive) and 3 Negative samples (within the selected positive and negative samples) per target pathogen 5 times (replicates of 5).

5. Methodology:

5.1 Samples should be tested in parallel with the Kit Under Evaluation and the reference assay. The ICMR-NIV RT-qPCR assay for Influenza/SARS-CoV-2 or WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved kit will be considered as the reference assay for these parameters.

5.2 The validation laboratory can use the established total RNA / viral RNA extraction protocol for the evaluation.

5.3 The instruction for the assay setup and the interpretation of the results will be as per the protocol outlined by the manufacturer of the reference test and the kit under evaluation.

5.4 The results shall be compared with the reference assay for sensitivity and specificity calculations.

5.5 If there is a discrepancy observed in the results with the index test, this discrepancy should be taken as discordant. Repetition of the assay may introduce bias. If the reference kit itself has failed, then these samples with discrepancies should be discarded, and new well-characterized samples should be used instead.

True positive samples: These are samples positive by both reference assay and index test.

True negative samples: These are samples negative by both reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

5.6 The interpretation for internal control (**preferably** endogenous, or exogenous) will be as per manufacturer's instruction.

5.7 PCR should be performed using IVD-approved machines. If any equipment(s) is specified in the IFU of the index test, it should be used for the evaluation, and it should be provided by the manufacturer if not available within the lab's IVD evaluation scope.

Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.
The details on the Real-time Equipment used for validation should be recorded, including calibration status.

6. Cross-reactivity Analysis:

6.1 Objective:

To assess the primer-probe set for true detection of influenza viruses and assess its cross-reactivity with other respiratory viruses.

6.2 Methodology:

6.2.1 Potential cross-reactivity of the kit shall be ruled out by testing other respiratory pathogen positive samples (N=30), with equal representation (n=5 each) of samples positive for SARS-CoV-2, Parainfluenza viruses, Adenoviruses, Rhinoviruses, Respiratory Syncytial Virus, common human coronaviruses).*

6.2.2 Cross-reactivity will be assessed by comparing the results of these samples using kit under evaluation and reference kit.

6.2.3 The kit targets should not show any amplification with other respiratory viruses (ORVs). If amplification is observed for ORV then the kit will fail validation and the same needs to be mentioned in the report.

** For multiplex assays targeting influenza, SARS-CoV-2, and RSV, samples positive for these viruses may be suitably interchanged for assessing cross-reactivity, apart from the ORV panel. (i.e. Influenza A positive samples may be used for detecting cross-reactivity against Influenza B)*

7. Acceptance criteria for the kit:

Sensitivity for each pathogen/ type/ subtype: ≥95%

Specificity for each pathogen/ type/ subtype: ≥99%

Cross-reactivity: Nil

Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥80 positive samples and ≥20 negative samples should be tested for evaluation for each pathogen/ type/ subtype.

8. Repeatability Assessment:

8.1 Objectives:

To assess the repeatability of the detection of Influenza virus and its subtypes using the kit under evaluation

8.2 Sample size:

3 positive samples (strong, moderate and weak positive-as per the Ct values outlined in the document) and 3 negative samples for each target pathogen should be tested 5 times.

8.3 Result: Concordance should be 100% based on positive and negative test result (qualitative).

9. Precision (Reproducibility):

Lot to Lot Reproducibility

9.1 Objectives: To assess Precision (Reproducibility) among 3 different lots of the kit under evaluation.

9.2 Sample size: Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

9.3 Result: Concordance should be 100% based on positive and negative test result (qualitative)

10. Internal Control Analysis:

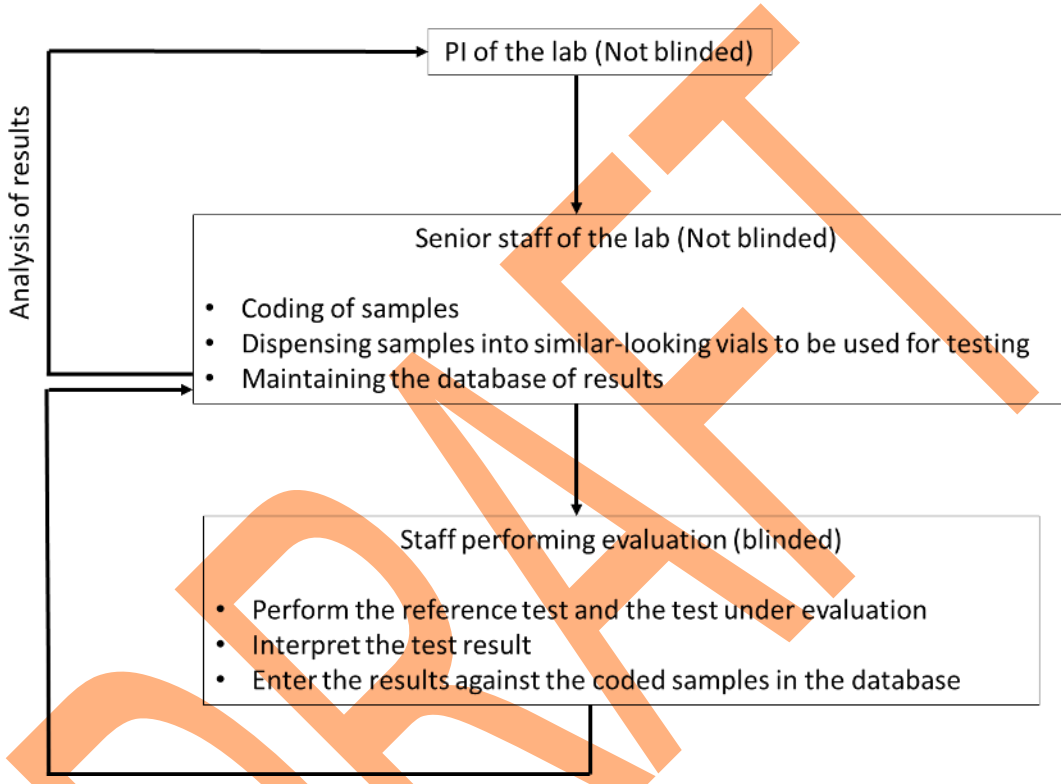
10.1 Monitor the internal control (preferably RNaseP or other housekeeping gene) to ensure consistent extraction and amplification efficiency across samples and runs.
10.2 Ct-values of internal controls should be within the manufacturer's prescribed limit.
10.3 Tests will be marked invalid if Ct-values are outside the prescribed limit.

11. Blinding of Laboratory Staff:

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff

selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 1.

Fig.1: Blinding in evaluation exercise



12. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

13. Conclusion:

Based on the comprehensive evaluation conducted, the [Kit & Manufacturer's Name] Influenza Virus RT-PCR Assay has been found **[Satisfactory/Not Satisfactory]** for its intended *in vitro* diagnostic (IVD) use.

The assay demonstrates [Strengths/Concerns] in terms of sensitivity, specificity, and performance characteristics compared to established reference IVD approved RT-PCR kits.

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

14. Performance evaluation report format

The performance evaluation report format (page 34) is designed for multiplex assays with several targets. It should be modified and used accordingly for single plex assays/multiplex assays with fewer targets.

Protocol B

Evaluation of performance characteristics of Molecular Kit detecting SARS-CoV-2 in single plex or multiplex format

1. Objective:

1.1. To validate the performance characteristics of in vitro molecular diagnostic kits for detecting SARS-CoV-2 as per the scope outlined in this document.

1.2. To ensure the kits under evaluation meet the necessary standards for sensitivity, specificity, repeatability, and reproducibility.

1.3. To evaluate the cross-reactivity of the kits with other respiratory viruses.

2. Evaluation of Performance characteristic should be done for the following:

2.1 Sensitivity and specificity

2.2 Cross-reactivity

2.3 Repeatability

2.4 Reproducibility

3. Panel development: Clinical sample (archived/ contemporary) panel for testing:

3.1 Contemporary leftover irreversibly de-identified clinical/archived respiratory samples in VTM for the panel should be irreversibly de-identified.

3.2 Samples to be used for panel preparation shall be stored properly at – 80 ℃ or lyophilized.

3.3 Unless the manufacturer has specific requirement of nucleic acid extraction kit, the MDTLs/ validation laboratory can use WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved/ICMR validated an established total RNA / viral RNA extraction kits for the evaluation.

3.4 Clinical samples for evaluation should be characterized by a reference kit / Sequencing/NGS.

3.5 All positive samples should be confirmed positive for the target pathogens by the reference assay.

3.6 All negative samples should be confirmed negative for the target pathogens by the reference assay.

4. Sample size and sample panel composition for evaluation of performance characteristics:

Sample sizes of positive and negative samples of SARS-CoV-2 against different values of sensitivity and specificity are provided in Table 3. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size that is outlined for 85% specificity. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the number of positive samples.*
- *n (sp) is the number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Table 3. Sample sizes for different values of sensitivity/ specificity claimed by the manufacturer.

Sensitivity/ Specificity	*Sample size: Minimum number of positive samples*[¥]	*Composition of positive samples*[#]	*Sample size: Minimum number of negative samples (rounded)*[¥]	*Minimum number of cross reactive* samples among the negative samples*

99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07	20	5
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28	80	20
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55	150	38
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76	210	53
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91	260	65
#Strong positive: (Ct value <25) Moderate positive: (Ct value between 25-30) Weak positive: (Ct value >30 and ≤ 34)				
¥ Nasopharyngeal/ oropharyngeal swabs in virus transport medium (VTM) to be used				
*Samples positive for common respiratory viruses (such as Influenza (including its types and subtypes), Parainfluenza viruses, Adenoviruses, Rhinoviruses, Respiratory Syncytial Virus (including its types and subtypes), common human coronaviruses), other than the ones targeted by the kit under evaluation. Equal distribution of cross-reactive viruses is desirable.				

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

4.1 Repeatability testing will be performed on 3 positive (strong, moderate and weak positive) and 3 negative samples (within the selected positive and negative samples) per target pathogen 5 times (replicates of 5).

5. Methodology:

5.1 Samples should be tested in parallel with the Kit Under Evaluation and the reference assay. The ICMR-NIV RT-qPCR assay for Influenza/SARS-CoV-2 or WHO Pre-Qualified/ US FDA/ PMDA Japan/ ATAGI Australia approved kit will be considered as the reference assay for these parameters.

5.2 The validation laboratory can use established total RNA / viral RNA extraction protocol for the evaluation.

5.3 The instruction for the assay setup and the interpretation of the results will be as per the protocol outlined by the manufacturer of the reference test and the kit under evaluation. The results shall be compared with the reference assay for sensitivity and specificity calculations.

5.4 If there is a discrepancy observed in the results with the index test, this discrepancy should be taken as discordant. Repetition of the assay may introduce bias. If the reference kit itself has failed, then these samples with discrepancies should be discarded, and new well-characterized samples should be used instead.

True positive samples: These are samples positive by both reference assay and index test.

True negative samples: These are samples negative by both reference assay and index test.
False positive samples: These are samples negative by reference assay and positive by index test.
False negative samples: These are samples positive by reference assay and negative by index test.

5.5 The interpretation for internal control (**preferably** endogenous, or exogenous) will be as per manufacturer's instruction.

5.6 PCR should be performed using IVD-approved machines. If any equipment(s) is specified in the IFU of the index test, it should be used for the evaluation, and it should be provided by the manufacturer if not available within the lab's IVD evaluation scope.

Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.

The details on the Real-time Equipment used for validation should be recorded, including calibration status.

The details on the Real-time Equipment used for validation should be recorded including calibration status.

6. Cross-reactivity Analysis:

6.1 Objective:

To assess the primer-probe set for true detection of SARS-CoV-2 and assess its cross-reactivity with other respiratory viruses.

6.2 Methodology:

6.1.1 Potential cross-reactivity of the kit shall be ruled out by testing other respiratory pathogen positive samples (N=30), with equal representation (n=5 each) of samples positive for Influenza, Parainfluenza viruses, Adenoviruses, Rhinoviruses, Respiratory Syncytial Virus, common human coronaviruses).*

6.1.2 Cross-reactivity will be assessed by comparing the results of these samples using kit under evaluation and reference kit.

6.1.3 The kit targets should not show any amplification with other respiratory viruses (ORVs). If amplification is observed for ORV then the kit will fail validation and the same needs to be mentioned in the report.

7. Acceptance criteria for the kit:

Sensitivity: ≥95%
Specificity: ≥99%
Cross-reactivity: Nil

Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥80 positive samples and ≥20 negative samples should be tested for evaluation for each pathogen/ type/ subtype.

8. Repeatability Assessment:

8.1 Objectives: To assess the repeatability of the detection of SARS-CoV-2 using the kit under evaluation

8.2 Sample size: Five replicates of 3 positive samples (strong, moderate and weak positive- as per the Ct values outlined in the document), and five replicates of 3 negative samples for SARS-CoV-2 should be tested. For multiplex panels, these sample numbers shall be used per target pathogen for repeatability assessment.

8.3 Result: Concordance should be 100% based on positive and negative test result (qualitative).

9. Precision (Reproducibility):

Lot to Lot Reproducibility

9.1 Objectives: To assess precision (reproducibility) among 3 different lots of the kit under evaluation.

9.2 Sample size: Lot to lot variation testing: Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

9.3 Result: Concordance should be 100% based on positive and negative test result (qualitative).

10. Internal Control Analysis:

10.1 Monitor the internal control (preferably RNaseP or other housekeeping gene) to ensure consistent extraction and amplification efficiency across samples and runs.

10.2 Ct-values of internal controls should be within the manufacturer's prescribed limit.

10.3 Tests will be marked invalid if Ct-values are outside the prescribed limit.

11. Blinding of Laboratory Staff:

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 1 in Protocol A.

12. Conclusion:

Based on the comprehensive evaluation conducted, the [Kit & Manufacturer's Name] SARS-CoV-2 RT-PCR Assay has been found **[Satisfactory/Not Satisfactory]** for its intended *in vitro* diagnostic (IVD) use.

The assay demonstrates [Strengths/Concerns] in terms of sensitivity, specificity, and performance characteristics compared to established reference IVD approved RT-PCR kits.

13. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

14. Performance evaluation report format:

The performance evaluation report format (page 34) is designed for multiplex assays with several targets. It should be modified and used accordingly for single plex assays/multiplex assays with fewer targets.

Protocol C

Evaluation of performance characteristics of Molecular Kit detecting Respiratory Syncytial Virus (RSV) in single plex or multiplex format

1. Objective:

1.1. To validate the performance characteristics of in vitro molecular diagnostic kits for detecting and/or differentiating RSV A/B as per the scope outlined in this document.

1.2. To ensure the kits under evaluation meet the necessary standards for sensitivity, specificity, repeatability, and reproducibility.

1.3. To evaluate the cross-reactivity of the kits with other respiratory viruses.

2. Evaluation of Performance characteristic should be done for the following:

2.1 Sensitivity and specificity

2.2 Cross-reactivity

2.3 Repeatability

2.4 Reproducibility

3. Panel development: Clinical sample (archived/ contemporary) panel for testing:

3.1 Contemporary leftover irreversibly de-identified clinical/archived respiratory samples in VTM for the panel should be irreversibly de-identified.

3.2 Samples to be used for panel preparation shall be stored properly at – 80 ℃ or lyophilized.

3.3 Unless the manufacturer has specific requirement of nucleic acid extraction kit, the MDTLs/ validation laboratory can use WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved/ ICMR validated an established total RNA / viral RNA extraction kits for the evaluation.

3.4 Clinical samples for evaluation should be characterized by a reference kit / Sequencing/NGS.

3.5 All positive samples should be confirmed positive for the target pathogens by the reference assay.

3.6 All negative samples should be confirmed negative for the target pathogens by the reference assay.

4. Sample size and sample panel composition for evaluation of performance characteristics:

Sample sizes of positive and negative samples of the RSV A/B against different values of sensitivity and specificity are provided in Table 4. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes for positive samples and their composition for evaluating subtyping (RSV A/B) are provided in Table 5. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the number of positive samples.*
- *n (sp) is the number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Table 4. Sample sizes per target pathogen (RSV A/B) for different values of sensitivity/ specificity claimed by the manufacturer.

Sensitivity/ Specificity	*Sample size: Minimum number of positive samples*[¥]	*Composition of positive samples*[#]	*Sample size: Minimum number of negative*	*Minimum number of cross reactive* samples*

			samples (rounded)[¥]	*among the negative samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07	20	5
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28	80	20
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55	150	38
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76	210	53
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91	260	65
[#]Strong positive: (Ct value <25) Moderate positive: (Ct value between 25-30) Weak positive: (Ct value >30 and and ≤ 34)				
[¥] Nasopharyngeal/ oropharyngeal swabs in virus transport medium (VTM) to be used				
*Samples positive for common respiratory viruses (such as Influenza (including its types and subtypes), SARS-CoV-2, Parainfluenza viruses, Adenoviruses, Rhinoviruses, common human coronaviruses), other than the ones targeted by the kit under evaluation. Equal distribution of cross-reactive viruses is desirable.				

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 5. Sample sizes for positive samples and their composition for evaluating subtyping

		RSV A	RSV B	
Sensitivity	Sample size* (per target pathogen)	Minimum number of nasopharyngeal swabs/ oropharyngeal swabs	Minimum number of nasopharyngeal swabs/ oropharyngeal swabs	Minimum total positive samples
99%	20	20	20	40
95%	80	80	80	160
90%	150	150	150	300
85%	210	210	210	420
80%	260	260	260	520
*Combination of strong, moderate and weak positive samples should be considered as per the information provided in Table 4.				

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

4.1 Repeatability testing will be performed on 3 positive (strong, moderate and weak positive) and 3 negative samples (within the selected positive and negative samples) per target pathogen 5 times (replicates of 5).

5. Methodology:

5.1 Samples should be tested in parallel with the Kit Under Evaluation and the reference assay. The ICMR-NIV RT-qPCR assay for RSV or WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved kit will be considered as the reference assay for these parameters.

5.2 The validation laboratory can use established total RNA / viral RNA extraction protocol for the evaluation.

5.3 The instruction for the assay setup and the interpretation of the results will be as per the protocol outlined by the manufacturer of the Kit Under Evaluation.

5.4 The results shall be compared with the reference assay for sensitivity and specificity calculations.

5.5 If there is a discrepancy observed in the results with the index test, this discrepancy should be taken as discordant. Repetition of the assay may introduce bias. If the reference kit itself has failed, then these samples with discrepancies should be discarded, and new well-characterized samples should be used instead.

True positive samples: These are samples positive by both reference assay and index test.
True negative samples: These are samples negative by both reference assay and index test.
False positive samples: These are samples negative by reference assay and positive by index test.
False negative samples: These are samples positive by reference assay and negative by index test.

5.6 The interpretation for internal control (**preferably** endogenous, or exogenous) will be as per manufacturer's instruction.

5.7 PCR should be performed using IVD-approved machines. If any equipment(s) is specified in the IFU of the index test, it should be used for the evaluation, and it should be provided by the manufacturer if not available within the lab's IVD evaluation scope.
Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.
The details on the Real-time Equipment used for validation should be recorded, including calibration status.
The details on the Real-time Equipment used for validation should be recorded including calibration status.

6. Cross-reactivity Analysis:

6.1 Objective:

To assess the primer-probe set for true detection of RSV and assess its cross-reactivity with other respiratory viruses.

6.2 Methodology:

6.1.1 Potential cross-reactivity of the kit shall be ruled out by testing other respiratory pathogen positive samples (N=30), with equal representation (n=5 each) of samples positive for Influenza, SARS-CoV-2, Parainfluenza viruses, Adenoviruses, Rhinoviruses, common human coronaviruses.*

6.1.2 Cross-reactivity will be assessed by comparing the results of these samples using kit under evaluation and reference kit.

6.1.3 The kit targets should not show any amplification with other respiratory viruses (ORVs). If amplification is observed for ORV then the kit will fail validation and the same needs to be mentioned in the report.

** For multiplex assays targeting influenza, SARS-CoV-2, and RSV detection, samples positive for these viruses may be suitably interchanged for assessing cross-reactivity*

7. Acceptance criteria for the kit:

Sensitivity for each pathogen/ type/ subtype: ≥95%
Specificity for each pathogen/ type/ subtype: ≥99%
Cross-reactivity: Nil
Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥80 positive samples and ≥20 negative samples should be tested for evaluation for each pathogen/ type/ subtype.

8. Repeatability Assessment:

8.1 Objectives: To assess the repeatability of the detection of SARS-CoV-2 using the kit under evaluation

8.2 Sample size: Five replicate of 3 positive samples per target pathogen (strong, moderate and weak positive) and five replicates of 3 negative samples per target pathogen should be tested.

8.3 Result: Concordance should be 100% based on positive and negative test result (qualitative).

9. Precision (Reproducibility):

Lot to Lot Reproducibility

1.1 Objectives: To assess precision (reproducibility) among 3 different lots of the kit under evaluation.

9.2 Sample size: Lot to lot variation testing: Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

9.3 Result: Concordance should be 100% based on positive and negative test result (qualitative).

10. Internal Control Analysis:

10.1 Monitor the internal control (RNaseP or other endogenous housekeeping gene) to ensure consistent extraction and amplification efficiency across samples and runs.

10.2 Ct-values of internal controls should be within the manufacturer's prescribed limit.

10.3 Tests will be marked invalid if Ct-values are outside the prescribed limit.

11. Blinding of Laboratory Staff:

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 1 in Protocol A.

12. Conclusion:

Based on the comprehensive evaluation conducted, the [Kit & Manufacturer's Name] SARS-CoV-2 RT-PCR Assay has been found **[Satisfactory/Not Satisfactory]** for its intended *in vitro* diagnostic (IVD) use.

The assay demonstrates [Strengths/Concerns] in terms of sensitivity, specificity, and performance characteristics compared to established reference IVD approved RT-PCR kits.

13. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

14. Performance evaluation report format:

The performance evaluation report format (page 34) is designed for multiplex assays with several targets. It should be modified and used accordingly for single plex assays/multiplex assays with fewer targets.

Protocol D

Evaluation of performance characteristics of Molecular Kit detecting Influenza virus and SARS-CoV-2 in multiplex format

To assess the performance of multiplex assays, Protocols A and B can be used as per kit format to check the performance of each virus for its sensitivity and specificity assessment, including cross reactivity, repeatability, reproducibility and Lot to lot variation.

A comprehensive report can be generated which will include sensitivity and specificity for all targets.

Sample size for multiplex molecular assay (as per the scope outlined in the document) detecting Influenza virus and SARS-CoV-2 in multiplex format is given below. All other parameters/conditions outlined in the single plex protocols (Protocols A and B) are to be essentially followed.

1. **Sample size and sample panel composition for evaluation of performance characteristics**:

Sample sizes of positive and negative samples against different values of sensitivity and specificity are provided in Table 6. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the number of positive samples.*
- *n (sp) is the number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*

- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Table 6. Sample sizes for different values of sensitivity/ specificity claimed by the manufacturer.

Sensitivity/ Specificity	*Sample size for each of the 04 target pathogens[a]: Minimum number of positive samples[¥]*	*Composition of positive samples for each pathogen[#]*	*Total number of positive samples (including all 04 pathogens)*	*Sample size: Minimum number of negative samples[¥]*	*Minimum number of cross reactive* samples among the negative samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07	80	20	5
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28	320	80	20
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55	620	150	38
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76	860	210	53
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91	1040	260	65

[a]Influenza A: (H1N1) pdm09, Influenza A/H3N2, Influenza B, and SARS CoV-2

[#]Strong positive: (Ct value <25)
Moderate positive: (Ct value between 25-30)
Weak positive: (Ct value >30 and and ≤ 34)

[¥] Nasopharyngeal or oropharyngeal swabs in virus transport medium (VTM) to be used

*Samples positive for common respiratory viruses (such as Parainfluenza viruses, Adenoviruses, Rhinoviruses, common human coronaviruses, RSV), other than the ones targeted by the kit under evaluation. Equal distribution of cross-reactive viruses is desirable.

For multiplex assays targeting influenza and SARS-CoV-2, samples positive for these viruses may be suitably interchanged for assessing cross-reactivity

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

2. **Acceptance Criteria for the kit:**

Sensitivity for each pathogen/ type/ subtype: ≥95%
Specificity for each pathogen/ type/ subtype: ≥99%
Cross-reactivity: Nil
Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥80 positive samples and ≥20 negative samples should be tested for evaluation for each pathogen/ type/ subtype.

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

Protocol E

Evaluation of performance characteristics of Molecular Kit detecting Influenza virus, SARS-CoV-2 and Respiratory Syncytial Virus (RSV) in multiplex format

To assess the performance of multiplex assays, Protocols A, B or C can be used as per kit format to check the performance of each virus for its sensitivity and specificity assessment, including cross reactivity, repeatability, reproducibility and Lot to lot variation.

A comprehensive report can be generated which will include sensitivity and specificity for all targets.

Sample size for multiplex molecular assay (as per the scope outlined in the document) detecting Influenza virus, SARS-CoV-2 and Respiratory Syncytial Virus (RSV) in multiplex format is given below. All other parameters/conditions outlined in the single plex protocols (Protocol A, B and C) are to be essentially followed.

1. **Sample size and sample panel composition for evaluation of performance characteristics**:

Sample sizes of positive and negative samples against different values of sensitivity and specificity are provided in Table 7. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the number of positive samples.*
- *n (sp) is the number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*

- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Table 7. Sample sizes for different values of sensitivity/ specificity claimed by the manufacturer.

Sensitivity/ Specificity	*Sample size for each of the 06 target pathogens[a]: Minimum number of positive samples[¥]*	*Composition of positive samples for each pathogen[#]*	*Total number of positive samples (including all 06 pathogens)*	*Sample size: Minimum number of negative samples[¥]*	*Minimum number of cross reactive * samples among the negative samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07	120	20	5
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28	480	80	20
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55	930	150	38
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76	1290	210	53
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91	1560	260	65

[a]Influenza A: (H1N1) pdm09, Influenza A/H3N2, Influenza B, SARS CoV-2, RSV A, and RSV B

[#]Strong positive: (Ct value <25)
Moderate positive: (Ct value between 25-30)
Weak positive: (Ct value >30 and and ≤ 34)

[¥] Nasopharyngeal/ oropharyngeal swabs in virus transport medium (VTM) to be used

* Samples positive for common respiratory viruses (such as Parainfluenza viruses, Adenoviruses, Rhinoviruses, common human coronaviruses), other than the ones targeted by the kit under evaluation. Equal distribution of cross-reactive viruses is desirable.

For multiplex assays targeting influenza, SARS-CoV-2, and RSV, samples positive for these viruses may be suitably interchanged for assessing cross-reactivity

It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

2. **Acceptance Criteria for the kit:**

Sensitivity for each pathogen/ type/ subtype: ≥95%
Specificity for each pathogen/ type/ subtype: ≥99%
Cross-reactivity: Nil
Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥80 positive samples and ≥20 negative samples should be tested for evaluation for each pathogen/ type/ subtype.

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

Performance evaluation report for Respiratory Virus in-vitro molecular diagnostic kit

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	
	Negative samples (provide details, including cross reactivity panel)	

i. **Analytes/Pathogens targeted by the kit under evaluation:**
……
ii. ……
iii. ……
iv. ……
v. ……
vi. ……
vii. ……

RESULTS INTERPRETATION

SENSITIVITY AND SPECIFICITY FOR INDIVIDUAL VIRUS TARGETS

1. Sensitivity and specificity for Influenza A (H1N1) pdm09

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			
	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

2. Sensitivity and specificity for Influenza A (H3N2)

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			
	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

3. Sensitivity and specificity for Influenza B (Victoria)

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			
	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

4. Sensitivity and specificity for Influenza B (Yamagata)

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			
	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

5. Sensitivity and specificity for SARS-CoV-2

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			
	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

6. Sensitivity and specificity for RSV A

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			
	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

7. Sensitivity and specificity for RSV B

Name of the Kit Under Evaluation		Reference assay		
		Positive	**Negative**	**Total**
	Positive			
	Negative			

	Total			

	Estimate (%)	**CI 95%**
Sensitivity		
Specificity		

a. Cross-reactivity Analysis:

b. Repeatability Assessment:

c. Precision (Reproducibility):

- **Lot to Lot**

Details of lots tested (3 lots to be tested):

1. Lot No.: Lot No: Tested By:
2. Lot No.: Lot No: Tested By:
3. Lot No.: Lot No: Tested By:

- **Lot-to-lot variation was <u>observed / not observed.</u>**

d. <u>Internal Control Analysis:</u>

<u>Conclusion:</u> Satisfactory / Not satisfactory

<u>RECOMMENDATIONS:</u>

Suggestions for improvements or modifications (if applicable):

_____

- **ICMR-CDSCO guidelines were followed for kit performance evaluation.**

This evaluation report is exclusively for_____ In Vitro Molecular Diagnostic Kit manufactured by _____.

Sensitivity and specificity have been assessed in controlled lab settings using the kits of the Lot number:

i. **Lot No._____,**
ii. **Lot No._____,**
iii. **Lot No._____,**

Provided by the manufacturer, using ……. samples. Results should not be extrapolated to other sample types.

DISCLAIMER:

1. **This validation process does not approve/disapprove the Kit design.**

2. **This validation process does not certify user friendliness of the Kit.**

3. **Influenza and SARS-CoV-2 are continuously evolving viruses and therefore primer probe sequences of the assay may require periodic updates, which will amount to a changed version of the assay. Re-validation is required for changed version of the assay, and needs to be considered while issuing license**

Signature of the Lab Manager **Signature of the Lab Director**

Signature of Head of the Institute

Seal of Head of the Institute

**********End of the Report**********

Annexure-1: Information on Operational and Test Performance Characteristics Required from Manufacturers

The manufacturer should provide the following details about the IVD:

1. Instructions for Use
2. Scope of the IVD: to diagnose influenza and/or SARS-CoV-/RSV.
3. Intended Use Statement
4. Principle of the assay
5. Intended testing population (cases of ARI/ILI/SARI)
6. Intended user (laboratory professional and/or health care worker at point-of-care)
7. Lot/batch No.
8. Date of manufacture
9. Date of Expiry
10. Information on operational Characteristics
    - i. Configuration of the kit/device
    - ii. Requirement of any additional equipment, device
    - iii. Requirement of any additional reagents
    - iv. Operation conditions
    - v. Storage and stability before and after opening
    - vi. Internal control provided or not
    - vii. Quality control and batch testing data
    - viii. Biosafety aspects- waste disposal requirements
11. Information on Test Performance Characteristics
    - i. Type of sample-NP/OP swab, other respiratory specimen
    - ii. Volume of sample
    - iii. Any specific sample NOT to be tested
    - iv. Any additional sample processing required
    - v. Any additional device/consumable like sample transfer device, pipette, tube, etc required

vi. Name of analyte to be detected

vii. Pathogens targeted by the kit

viii. Time taken for testing

ix. Time for result reading and interpretation

x. Manual or automated(equipment)reading

xi. Limit of detection

xii. Diagnostic sensitivity

xiii. Diagnostic specificity

xiv. Stability and reproducibility

xv. Training required for testing

xvi. If yes, duration

xvii. Details of Cut-off and /or Equivocal Zone for interpretation of test

xviii. Interpretation of invalid and indeterminate results to be provided

xix. It is recommended to provide data demonstrating the precision

xx. Limit of detection

STANDARD PERFORMANCE EVALUATION PROTOCOLS

DRAFT FOR STAKEHOLDER COMMENTS

MALARIA IN-VITRO DIAGNOSTICS

ICMR-CDSCO/IVD/GD/PROTOCOLS/06/2025

AUGUST, 2025

New Delhi, India

Table of Contents

Performance evaluation protocol for Malaria Rapid diagnostic test (RDT) kits

I. Background:

CDSCO/ICMR, New Delhi have aimed to facilitate the evaluation and supply of Quality-Assured in vitro Diagnostics (IVD) kits suitable for use in India. Hence, the following guidelines shall establish the uniformity during the performance evaluation of IVD kits The objective of performance evaluation is to independently validate the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

II. Purpose:

To evaluate the performance characteristics of rapid diagnostic test kit for the diagnosis of malaria parasite using irreversibly de-identified leftover archived/ spiked clinical samples.

III. Requirements:

a) Instructions for use (IFU)

b) Supply of RDT kits under evaluation (with batch no.; lot no.; manufacturing and expiry date and other required details).

c) Evaluation sites/laboratories (With required equipment)

d) Reference test kits

e) Characterised Evaluation panel

f) Laboratory supplies

IV. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. **Study design/type**: Diagnostic accuracy study using irreversibly de-identified leftover clinical/spiked samples.
2. **Preparation of Evaluation sites/laboratories:**
   Identified IVD kit evaluation laboratories should establish their proficiency through:

   a) Laboratory accreditation: Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO: 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

   b) It is recommended that malaria Medical Device Testing Labs (MDTLs) participate in Quality Control exercises such as EQAP (External Quality Assurance Programme).

c) **Staff training:** All the staff involved in IVD kit evaluation should undergo hands on training and competency testing on the following at referral level malaria labs before initiation of MDTL activity:

- Preparation and characterization of evaluation panel for the respective IVD kit.
- Management of RDT kits (specific for *Plasmodium falciparum / Plasmodium vivax*) received for performance evaluation (Verification/Storage/Unpacking etc.).
- Perform tests interpretation and documentation of results, and reporting.
- Data management and safety and confidentiality.

3. Preparation of QC panel members for Malaria RDT kit evaluation

To evaluate the performance of IVD kit, a well characterized species specific malaria antigen sample panel is required. Statistically significant number of blood samples as defined in this protocol should be collected from malaria confirmed cases in health facilities, (as mentioned in Table 1). The panel should comprise positive and negative samples as described in section 7.

The reference sample panel should be stored in appropriate storage conditions, and the quality of the panel should be checked periodically through appropriate testing.

4. Reference assay:

WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved RDT should be used as reference standard.

All positive samples should be confirmed positive by the reference assay.

All negative samples should be confirmed negative by the reference assay.

5. Sample size and sample panel composition for performance evaluation:

Sample sizes of positive and negative samples of each species targeted by the kit against different values of sensitivity and specificity are provided in Tables 1 and 2, with recommended composition. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate of 5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity.

Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\ (1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\ (1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Table 1. Positive sample sizes (per species) and composition for different values of sensitivity claimed by the manufacturer for evaluation of Pf (single/combo RDT) or Pv (single/combo RDT)

Sensitivity	*Sample size: Minimum number of positive samples #*	*Composition of positive samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91
75%	305 (rounded to 310 for better distribution of samples)	Strong positive = 92 Moderate positive = 109 Weak positive = 109

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 2. Negative sample sizes and composition for different values of specificity claimed by the manufacturer for evaluation of Pf (single/combo RDT) or Pv (single/combo RDT)

Specificity	*Sample size: Minimum number of negative samples #*	*Composition of negative samples#*
99%	16 (rounded to 20)	Dengue NS1/IgM positive samples: 03 Chikungunya IgM positive samples:03 Serum reactive for RA factor – low positive and high positive:02 Serum reactive for TPHA/other specific test for syphilis:02 Healthy controls from endemic regions: 10
95%	77 (rounded to 80)	Dengue NS1/IgM positive samples: 10 Chikungunya IgM positive samples:10 Serum reactive for RA factor – low positive and high positive:10 Serum reactive for TPHA/other specific test for syphilis:10 Healthy controls from endemic regions: 40
90%	146 (rounded to 150)	Dengue NS1/IgM positive samples: 18 Chikungunya IgM positive samples:18 Serum reactive for RA factor – low positive and high positive:18 Serum reactive for TPHA/other specific test for syphilis:18 Healthy controls from endemic regions: 78
85%	207 (rounded to 210)	Dengue NS1/IgM positive samples: 26 Chikungunya IgM positive samples:26 Serum reactive for RA factor – low positive and high positive:26 Serum reactive for TPHA/other specific test for syphilis:26 Healthy controls from endemic regions: 106
80%	259 (rounded to 260)	Dengue NS1/IgM positive samples: 35 Chikungunya IgM positive samples:35 Serum reactive for RA factor – low positive and high positive:30 Serum reactive for TPHA/other specific test for syphilis:30 Healthy controls from endemic regions: 130

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Sample panel composition:

Positive samples: Malaria positive samples should be obtained from health facilities (*tertiary care centers and their linked hospitals, private clinics, field practice areas etc.*) and confirmed using PCR (Snounou protocol/FDA approved assay).

Malaria samples confirmed positive by PCR should be characterized for parasite load on in-house calibrated equipment using blood smear microscopy and ELISA. Samples with analyte values satisfying the range of acceptance criteria (as mentioned in this document) should be included in the positive sample panel for the evaluation of malaria RDT kits.

For the RDT kits which have other antigen/antibody as target analyte (for which limits of detection have not been established), characterization of samples should be performed on calibrated equipment, leading to their classification as low and high parasitemic samples, which should then be used for performance evaluation of the assay.

Range of Parasitemia: Panel members should have low (≤200 parasites per microliter) to high (≥2000 parasites per microliter) range of *Plasmodium falciparum, P. vivax* and/or other *Plasmodium* species, as obtained from ELISA results. Characterized panels **must** contain equal number of samples of both low and high parasitemia.

Consistent ELISA quantification results should be obtained in ≥3 runs of ELISA experiments performed for each of the three antigens (PfHRP2, LDH and aldolase) with the results obtained at the 200 p/µL and the 2,000 p/µL being consistent with each other as well (factor of roughly 10 between results). The limit of detection of Pfhrp2 is 5-10 ng/ µL, and PvLDH is 15-45 ng/ µL.

** *It should be noted that no such limit of detection is defined for aldolase. Where values/standard reference assay not available, standard procedure on calibrated equipment will be followed for obtaining results.*

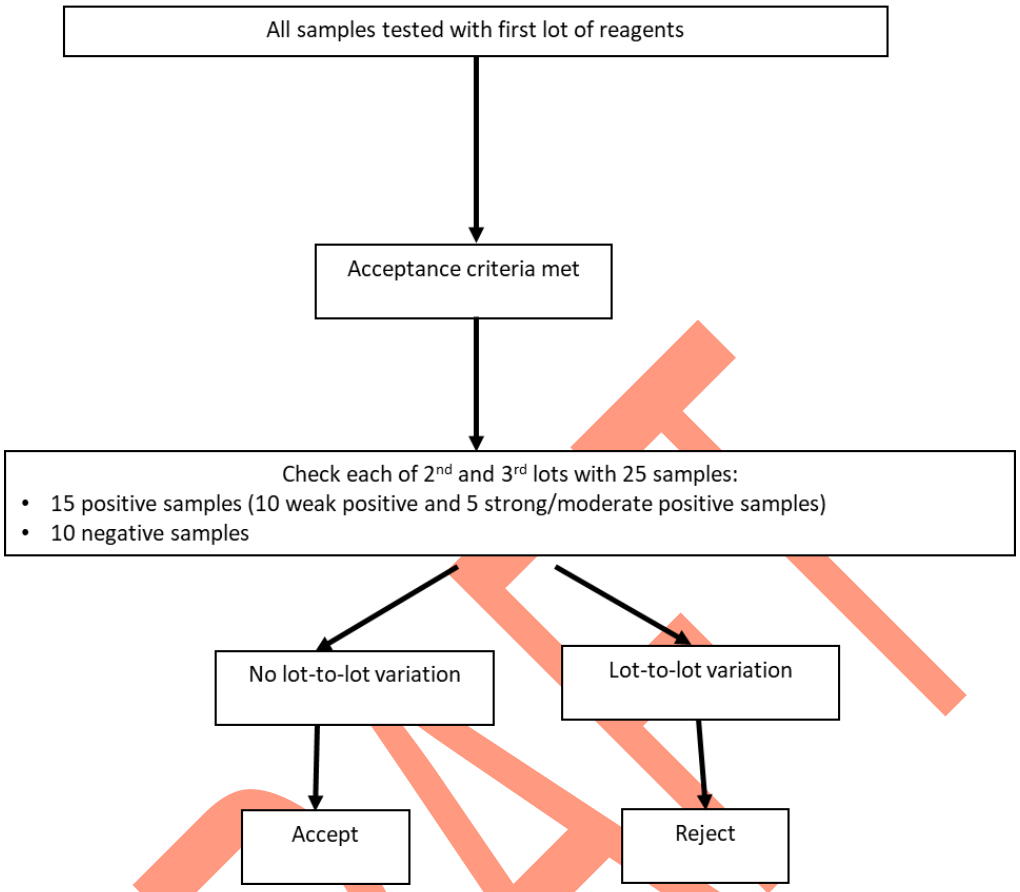
6. Test reproducibility:

A. Sample size for lot-to-lot reproducibility

Three lots of an assay shall be evaluated. The first lot shall be evaluated on the entire panel of samples (statistically significant sample size). For the subsequent two lots, 25 samples should be used for evaluation (15 positive samples including 10 weak positive samples and 5 moderate/strong positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

Fig.1: Lot-to-lot reproducibility



B. Reader-to-reader reproducibility: 25 samples (15 positive samples including 10 weak positive samples and 5 strong/moderate positive samples, and 10 negative samples) need to be tested by at least 2 trained personnel. Agreement should be 100%.

Note: Testing Methodology

Read the instructions for use (IFU) thoroughly. Take out the required number of RDTs kits from the recommended storage conditions. Bring RDTs to room temperature (20°C - 30°C) and thaw the required number of QC/sample panel aliquots for a minimum of 20 minutes to maximum 60 minutes before performing the test. Note that more than one aliquot may be needed for the testing of each sample. Record the results of the performance evaluation on the recommended report format (Annexure 1).

7. Evaluation method:

The reference assay and the index test should be run on the sample panel in parallel.

8. Interpretation of results:

Results should be interpreted as per the IFU of the reference assay and the index test.

9. Resolution of discrepant results:

True positive samples: These are samples positive by both reference assay and index test.

True negative samples: These are samples negative by both reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

10. Acceptance criteria[1]:

Expected sensitivity: ≥75% for P. vivax and ≥95% for P. falciparum

Expected specificity: ≥90% for P. vivax and ≥95% for P. falciparum

Cross-reactivity: Nil

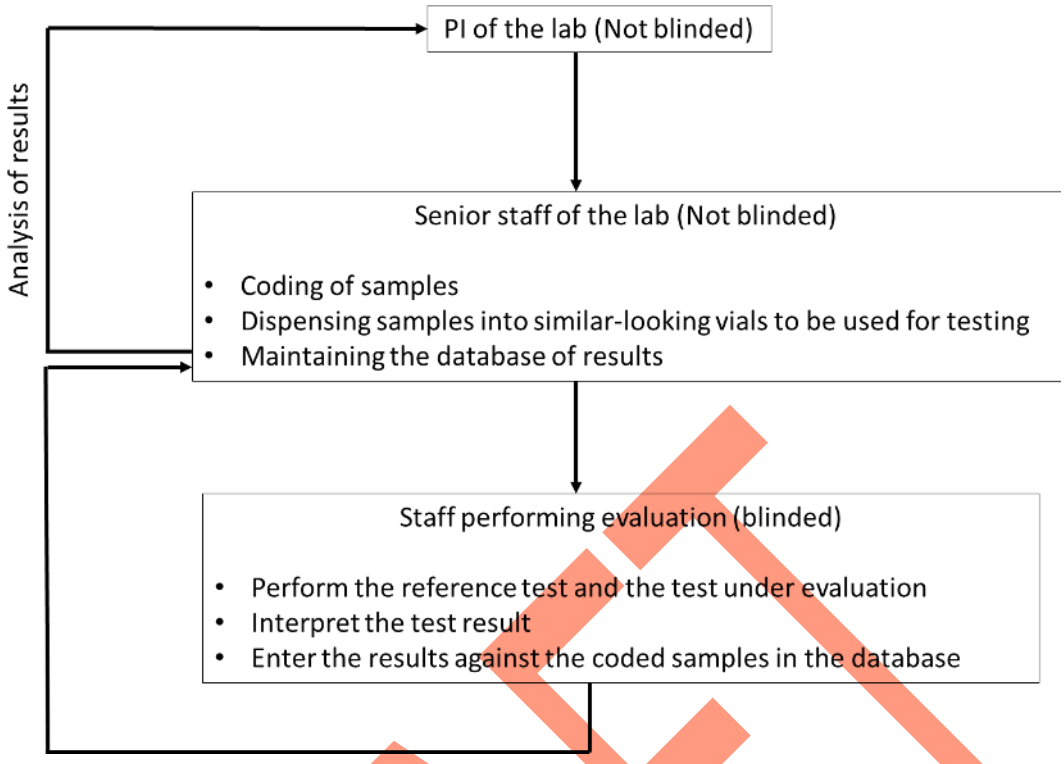
Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥310 positive samples and ≥150 negative samples should be tested for *P vivax*, and ≥80 positive samples and ≥80 negative samples should be tested for *P falciparum*.

11. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



12. Publication Rights

The PI(s) of the evaluating labs shall retain publication rights to the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable. Any request of re-validation from the same manufacturer for the same test type will only be entertained if valid proof of change in the kit composition is submitted.

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Ministry of Health and Family Welfare. Guidelines for Bivalent RDT. Available at: guidelines-for-bivalent-rdt.pdf (mohfw.gov.in)
2. World Health Organization. Malaria Rapid Diagnostic Test Performance - Results of WHO product testing of malaria RDTs: round 8 (2016–2018): Available at: https://iris.who.int/bitstream/handle/10665/276190/9789241514965-eng.pdf?sequence=1
3. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitology. 1993;61:315–20.
4. Krishna S, Bharti PK, Chandel HS, Ahmad A, Kumar R, Singh PP, et al. Detection of Mixed Infections with Plasmodium spp. by PCR, India, 2014. Emerg Infect Dis. 2015;21(10):1853-7.

VII. Performance evaluation report format

REPORT FORMAT

Name of the Laboratory

Name of the Institute, (with station)

Certificate of Analysis

File No.: _____

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number: Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	

	Negative samples (provide details, including cross reactivity panel)	

Results:

		Reference assay …… (name)		
		Positive	Negative	Total
Name of index malaria RDT	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- Details of cross reactivity with other agents:
- **Conclusions:**
  - Sensitivity, specificity
  - Performance: **Satisfactory / Not Satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting on serum samples only, using kits provided by the manufacturer from the batch mentioned above. Results should not be extrapolated for any other sample type.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for …….. Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal ……

**********End of the Report**********

Performance evaluation protocol for Malaria ELISA kits

I. Background:

CDSCO/ICMR, New Delhi, have aimed to facilitate the evaluation and supply of Quality-Assured In Vitro Diagnostics kits suitable for use in India. Hence, the following guidelines shall establish the uniformity during the performance evaluation of IVD kits . The objective of performance evaluation is to independently validate the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

II. Purpose:

To evaluate the performance characteristics of malaria ELISA kits for the diagnosis of malaria parasite infection using irreversibly de-identified leftover archived/ spiked clinical samples. The malaria ELISA kits are designed to detect antigens (hrp2, LDH, aldolases) occurring in subjects infected with species specific (*P. falciparum, P. vivax*) and stage specific antibodies (MSP1, MSP3, CSP, EBA175 etc.- parasite markers for the purpose of sero-survey).

III. Requirements:

a) Instructions for use (IFU)

b) Supply of ELISA kits under evaluation (with batch no./lot no. expiry date & required details). In case the kit to be evaluated is designed to work in a closed system format, the manufacturer needs to supply the required equipment.

c) Evaluation sites/laboratories (With required equipment)

d) Reference test kits

e) Characterised Evaluation panel

f) Laboratory supplies

IV. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. Study design/type: Diagnostic accuracy study using irreversibly de-identified leftover clinical samples.

2. Preparation of Evaluation sites/laboratories:

Identified ELISA kit evaluation laboratories should establish their proficiency through

a) Laboratory accreditation: Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical

Lab (ISO:15189), PT provider ISO: 17043 or CDSCO approved Reference laboratory.

b) It is recommended that malaria Medical Device Testing Labs (MDTLs) participate in Quality Control exercises such as EQAP (External Quality Assurance Programme).

c) **Staff training:** All the staff involved in ELISA kit evaluation should undergo hands on training and competency testing on the following at referral level malaria labs before initiation of MDTL activity:

- Preparation and characterization of evaluation panel for the respective ELISA kit.
- Management of malaria ELISA kits received for performance evaluation (Verification/Storage/Unpacking etc).
- Perform tests , interpretation and documentation of results and reporting.
- Data management and safety and confidentiality

3. Reference sample panel:

To evaluate the performance of ELISA kit a well characterised malaria stage specific antigens/species specific antibody ELISA evaluation sample panel is required. In the absence of WHO Pre-Qualified/US FDA/ ATAGI Australia/ PMDA Japan approved malaria ELISA assay, it is recommended that performance evaluation of ELISA assays be performed on a rigorously well characterized panel of positive and negative samples.

A statistically significant number of sera samples should be collected from malaria confirmed cases from health facilities. *All samples should be further confirmed by PCR assay (Snounou protocol/FDA approved assay).*

A. ***Malaria samples confirmed positive by PCR*** *should be characterized for parasite load on in-house calibrated equipment using ELISA. Samples with analyte values satisfying the range of acceptance criteria (as mentioned in this document) should be included in the positive sample panel for the evaluation of malaria RDT kits.*
For those kits which have other antigen/antibody as target analyte (for which limits of detection have not been established), characterization of samples for that analyte should be performed on calibrated equipment, leading to their classification as low and high parasitemic samples, which will then be used for performance evaluation of the assay.

Range of Parasitemia: Panel members should have low (≤200 parasites per microliter) to high (≥2000 parasites per microliter) range of *Plasmodium falciparum, P. vivax* and/or other *Plasmodium* species, as obtained from ELISA results. Characterized panels **must** contain equal number of samples of both low and high parasitemia.

Consistent ELISA quantification results should be obtained in ≥3 runs of ELISA experiments performed for each of the three antigens (PfHRP2, LDH and aldolase – recombinantly expessed proteins) with the results obtained at the 200 p/µL and the

2,000 p/µL being consistent with each other as well (factor of roughly 10 between results). The limit of detection of Pfhrp2 is 5-10 ng/ µL, and Pvldh is 15-45 ng/ µL.

** *It should be noted that no such limit of detection is defined for aldolase. Where values/standard reference assay not available, standard procedure on calibrated equipment will be followed for obtaining results.*

The above-mentioned activities should not be performed with spiked/contrived samples.

Equal representation of samples positive for Plasmodium (P.*falciparum /P.vivax*) *species preferred.*

B. *Negative panel should constitute malaria negative samples (confirmed by PCR) as described in point 6B.*

The reference sample panel should be stored in appropriate storage conditions, and the quality of the panel should be checked periodically with appropriate tests (including parasite culture) as needed.

Malaria positive samples should be obtained from health facilities, including tertiary care centers and their linked hospitals, private clinics, field practice areas etc.

Wherever any WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved assay is available, it should be used as reference standard.

Sample size and sample panel composition for performance evaluation: Sample sizes of positive and negative samples of each species targeted by the kit against different values of sensitivity and specificity are provided in Table 1 and Table 2, with recommended composition. Sample sizes have been calculated assuming 95% level of significance and an absolute precision of 5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*

- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*

Table 1. Positive sample sizes (per species) and composition for different values of sensitivity claimed by the manufacturer for evaluation of Pf (single/combo) or Pv (single/combo) ELISA

Sensitivity	*Sample size: Minimum number of positive samples#*	*Composition of positive samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07
95%	73 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28
90%	139 (rounded to 140 for better distribution of samples)	Strong positive = 42 Moderate positive = 49 Weak positive = 49
85%	196 (rounded to 200 for better distribution of samples)	Strong positive = 60 Moderate positive = 70 Weak positive = 70
80%	246 (rounded to 255 for better distribution of samples)	Strong positive = 75 Moderate positive = 90 Weak positive = 90
75%	289 (rounded to 295 for better distribution of samples)	Strong positive = 87 Moderate positive = 104 Weak positive = 104

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 2. Negative sample sizes and composition for different values of specificity claimed by the manufacturer for evaluation of Pf (single/combo) or Pv (single/combo) ELISA

Specificity	*Sample size: Minimum number of negative samples #*	*Composition of negative samples*
99%	16 (rounded to 20)	Dengue NS1/IgM positive samples: 03 Chikungunya IgM positive samples:03 Serum reactive for RA factor – low positive and high positive:02 Serum reactive for TPHA/other specific test for syphilis:02 Healthy controls from endemic regions: 10
95%	73 (rounded to 80)	Dengue NS1/IgM positive samples: 10 Chikungunya IgM positive samples:10

		Serum reactive for RA factor – low positive and high positive:10 Serum reactive for TPHA/other specific test for syphilis:10 Healthy controls from endemic regions: 40
90%	139 (rounded to 140)	Dengue NS1/IgM positive samples: 18 Chikungunya IgM positive samples:18 Serum reactive for RA factor – low positive and high positive:18 Serum reactive for TPHA/other specific test for syphilis:18 Healthy controls from endemic regions: 68
85%	196 (rounded to 200)	Dengue NS1/IgM positive samples: 25 Chikungunya IgM positive samples:25 Serum reactive for RA factor – low positive and high positive:25 Serum reactive for TPHA/other specific test for syphilis:25 Healthy controls from endemic regions: 100
80%	246 (rounded to 250)	Dengue NS1/IgM positive samples: 30 Chikungunya IgM positive samples:30 Serum reactive for RA factor – low positive and high positive:30 Serum reactive for TPHA/other specific test for syphilis:30 Healthy controls from endemic regions: 130

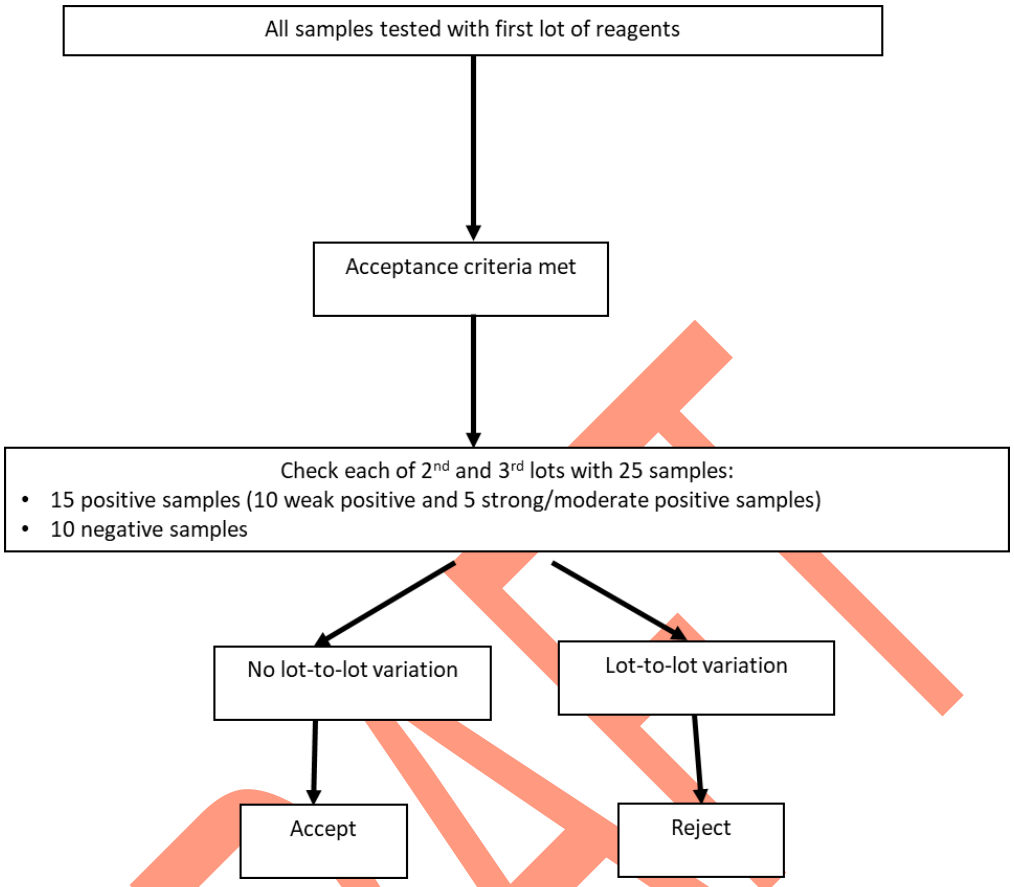
#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

4. Sample size for lot-to-lot reproducibility

Three lots of an assay shall be evaluated. The first lot shall be evaluated on the entire panel of samples (statistically significant sample size). For the subsequent two lots, 25 samples should be used for evaluation (15 positive samples including 10 weak positive samples and 5 moderate/strong positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

Fig.1: Lot-to-lot reproducibility



5. Evaluation Methodology:

The index test should be tested on a rigorously well-characterized panel of samples from confirmed malaria positive and negative cases, which are further tested for the presence of malaria parasite using the Snounou protocol.

6. Interpretation of results:

Results should be interpreted as per the IFU of the reference assay.

7. Resolution of discrepant results:

True positive samples: These are well-characterized samples from confirmed malaria positive cases, which are also positive by the index test.

True negative samples: These are well-characterized samples from confirmed malaria negative cases, which are also negative by the index test.

False positive samples: These are well-characterized samples from confirmed malaria negative cases, which are positive by the index test.

False negative samples: These are well-characterized samples from confirmed malaria positive cases, which are negative by the index test.

8. Acceptance Criteria:

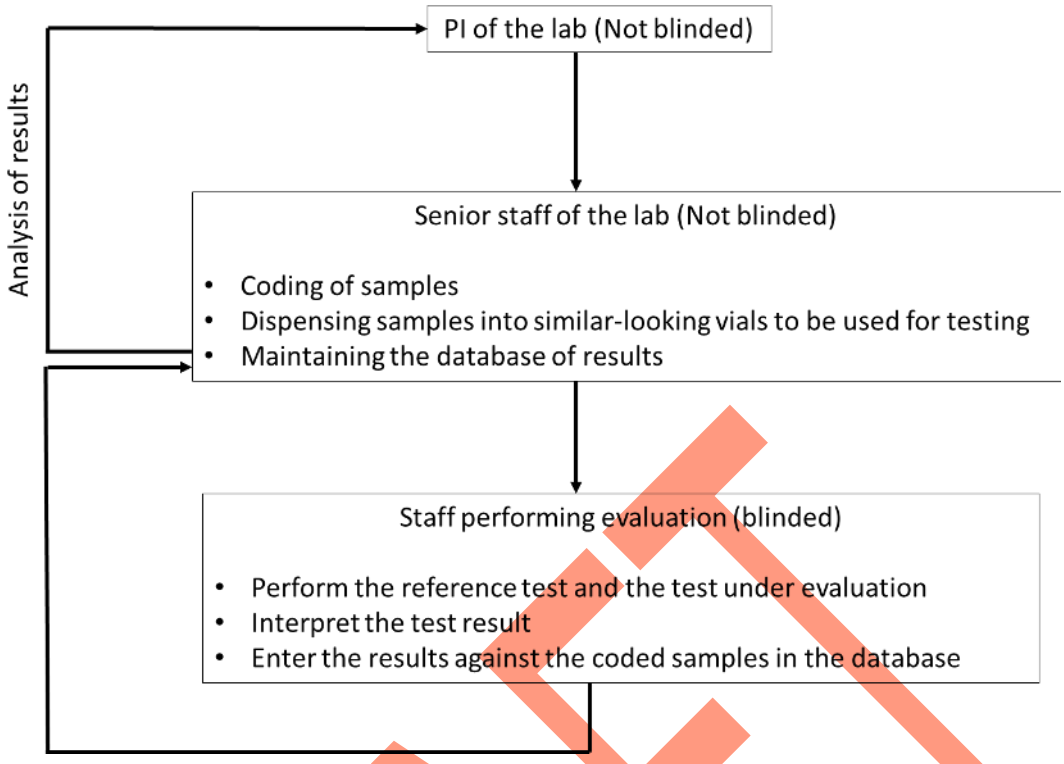
Type of assay	Acceptance criteria	Minimum no. of samples needed to achieve at least the performance characteristics outlined in the acceptance criteria
Malaria antibody ELISA	Sensitivity: ≥90% Specificity: ≥95%	Minimum no. of Positive samples = 140 Minimum no. of Negative samples = 80
Pv ELISA	Sensitivity: ≥75% Specificity: ≥95%	Minimum no. of Positive samples = 295 Minimum no. of Negative samples = 80
Pf ELISA	Sensitivity: ≥90% Specificity: ≥95%	Minimum no. of Positive samples = 140 Minimum no. of Negative samples = 80

Cross-reactivity: Nil

9. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



10. Publication Rights

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitology. 1993;61:315–20.

VII. Performance evaluation report format

REPORT FORMAT

Name of the Laboratory

Name of the Institute, (with station)

Certificate of Analysis

File No.: _____

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number: Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	

	Negative samples (provide details, including cross reactivity panel)	

Results:

		Samples with confirmed disease status (Further confirmed by Snounou protocol/ FDA approved assay)		
		Positive	Negative	Total
Name of malaria ELISA kit	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- Details of cross reactivity with other agents:
- **Conclusions:**
  - Sensitivity, specificity
  - Performance: **Satisfactory / Not Satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting on ……. samples only, using kits provided by the manufacturer from the batch mentioned above. Results should not be extrapolated for any other sample type.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for …….. Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal ……

**********End of the Report**********

Performance evaluation protocol for Malaria real-time PCR kits

I. Background:

CDSCO/ICMR, New Delhi, have aimed to facilitate the evaluation and supply of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding IVD kit performance.

II. Purpose:

To evaluate the performance characteristics of Malaria real-time PCR (RT-PCR) kits using irreversibly de-identified leftover archived/ spiked clinical samples.

III. Requirements:

1. Instructions for use (IFU)
2. Supply of kits under evaluation (with batch no. and lot no. ; Manufacturing and Expiry and other required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.
3. Evaluation sites/laboratories (With required equipment)
4. Reference test kits
5. Characterised Evaluation panel
6. Laboratory supplies

IV. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. **Study design/type**: Diagnostic accuracy study using irreversibly de-identified leftover clinical/spiked samples.
2. **Preparation of Evaluation sites/laboratories:**
   Identified IVD kit evaluation laboratories should establish their proficiency through

a) Laboratory accreditation: Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO: 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

b) It is recommended that malaria Medical Device Testing Labs (MDTLs) participate in Quality Control exercises such as EQAP (External Quality Assurance Programme).

c) **Staff training:** All the staff involved in IVD kit evaluation should undergo hands-on training and competency testing on the following at referral level malaria labs before initiation of MDTL activity:

- Preparation and characterization of evaluation panel for the respective IVD kit.
- Management of RDT kits (specific for *Plasmodium falciparum / Plasmodium vivax*) received for performance evaluation (Verification/Storage/Unpacking etc.).
- Perform tests interpretation and documentation of results, and reporting.
- Data management and safety and confidentiality.

1. Preparation of evaluation sample panel for Malaria

To evaluate the performance of malaria RT-PCR IVD kit, a well characterized species specific malaria whole genome panel is required. Hence, statistically significant number of whole blood samples should be collected from malaria confirmed cases. *The panel should comprise positive and negative samples as described in section 8.*

The reference sample panel should be stored in appropriate storage conditions, and the quality of the panel should be checked periodically with appropriate tests (including parasite culture) as needed.

Malaria positive samples should be obtained from health facilities, including tertiary care centers and their linked hospitals, private clinics, field practice areas etc.

2. DNA extraction

DNA extraction should be performed using a standard protocol/kit as recommended by the manufacturer, or fully automated DNA extractor may be used (as per manufacturer's instruction and compatible reagent kits).

Note: If the manufacturer of the index test recommends a specific DNA extraction kit, it needs to be provided by the manufacturer, if the evaluation lab is unable to procure the same.

3. Real-time PCR system:

PCR should be performed using IVD-approved machines. If any equipment(s) is specified in the IFU of the index test, it should be used for the evaluation, and it should be provided by the manufacturer if not available within the lab's IVD evaluation scope.

Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.

4. Internal Control/Extraction Control:

The index test must have an internal control (housekeeping gene), with or without an extraction control.

5. Reference assay:

Two WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan-approved malaria RT-PCR assays (or one FDA-approved assay and the Snounou protocol) should be used as reference assays for the characterization of samples, with 100% agreement between their results.

All positive samples should be confirmed positive by the reference assay(s).

All negative samples should be confirmed negative by the reference assay(s).

6. Sample size and sample panel composition for performance evaluation:

Sample sizes of positive and negative samples of each species targeted by the kit against different values of sensitivity and specificity are provided in Table 1 and Table 2, with recommended composition. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate of 5%. Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Table 1. Positive sample sizes (per species) and composition for different values of sensitivity claimed by the manufacturer for evaluation of Pf (single/combo RDT) or Pv (single/combo RDT)

Sensitivity	*Sample size: Minimum number of positive samples#*	*Composition of positive samples*
99%	16 (rounded to 20 for better distribution of samples)	Strong positive = 06 Moderate positive = 07 Weak positive = 07
95%	77 (rounded to 80 for better distribution of samples)	Strong positive = 24 Moderate positive = 28 Weak positive = 28
90%	146 (rounded to 155 for better distribution of samples)	Strong positive = 45 Moderate positive = 55 Weak positive =55
85%	207 (rounded to 215 for better distribution of samples)	Strong positive = 63 Moderate positive = 76 Weak positive = 76
80%	259 (rounded to 260 for better distribution of samples)	Strong positive = 78 Moderate positive = 91 Weak positive = 91
75%	304 (rounded to 310 for better distribution of samples)	Strong positive = 92 Moderate positive = 109 Weak positive = 109

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 2. Negative sample sizes and composition for different values of specificity claimed by the manufacturer for evaluation of Pf (single/combo RDT) or Pv (single/combo RDT)

Specificity	*Sample size: Minimum number of negative samples#*	*Composition of negative samples*
99%	16 (rounded to 20)	Dengue NS1/IgM positive samples: 03 Chikungunya IgM positive samples:03 Serum reactive for RA factor – low positive and high positive:02 Serum reactive for TPHA/other specific test for syphilis:02 Healthy controls from endemic regions: 10
95%	77 (rounded to 80)	Dengue NS1/IgM positive samples: 10 Chikungunya IgM positive samples:10 Serum reactive for RA factor – low positive and high positive:10 Serum reactive for TPHA/other specific test for syphilis:10 Healthy controls from endemic regions: 40
90%	146 (rounded to 150)	Dengue NS1/IgM positive samples: 18 Chikungunya IgM positive samples:18

		Serum reactive for RA factor – low positive and high positive:18 Serum reactive for TPHA/other specific test for syphilis:18 Healthy controls from endemic regions: 78
85%	207 (rounded to 210)	Dengue NS1/IgM positive samples: 26 Chikungunya IgM positive samples:26 Serum reactive for RA factor – low positive and high positive:26 Serum reactive for TPHA/other specific test for syphilis:26 Healthy controls from endemic regions: 106
80%	259 (rounded to 260)	Dengue NS1/IgM positive samples: 35 Chikungunya IgM positive samples:35 Serum reactive for RA factor – low positive and high positive:30 Serum reactive for TPHA/other specific test for syphilis:30 Healthy controls from endemic regions: 130

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Sample panel composition:

A. ***Positive samples:*** Malaria positive samples should be obtained from health facilities and confirmed using two FDA approved PCR Kits (including Snounou protocol). Once the positive samples are well-characterized with these two PCR assays (100% agreement between results), they should be classified as per their parasite load using ELISA on *in-house calibrated equipment.* Samples with analyte values satisfying the range of acceptance criteria (as mentioned in this document) should be included in the positive sample panel for the evaluation of malaria RT-PCR kits.

Additional analytes (whose cutoff values have not yet been established) may be used for further sample characterization by ELISA. However, this characterization of samples should also be performed on calibrated equipment, leading to their classification as low and high parasitemia samples, which should then be used for performance evaluation of the assay.

Range of Parasitemia: Panel members should have a low (≤200 parasites per microliter) to high (≥2000 parasites per microliter) range of *Plasmodium falciparum, P. vivax*, as obtained from ELISA results. Characterized panels **must** contain equal number of samples of both low and high parasitemia.

Consistent ELISA quantification results should be obtained in ≥3 runs of ELISA experiments performed for each of the three antigens (PfHRP2, LDH and aldolase), with the results obtained at the 200 p/µL and the 2,000 p/µL being consistent with each other as well (factor of roughly 10 between results). The limit of detection of Pfhrp2 is 5-10 ng/ µL, and Pvldh is 15-45 ng/ µL.

** *It should be noted that no such limit of detection is defined for aldolase. Where values/standard reference assay not available, standard procedure on calibrated equipment will be followed for obtaining results.*

The above mentioned activities should not be performed with spiked/contrived samples.

Equal representation of samples positive for all Plasmodium (P.falciparum /P.vivax) species preferred.

7. Test reproducibility

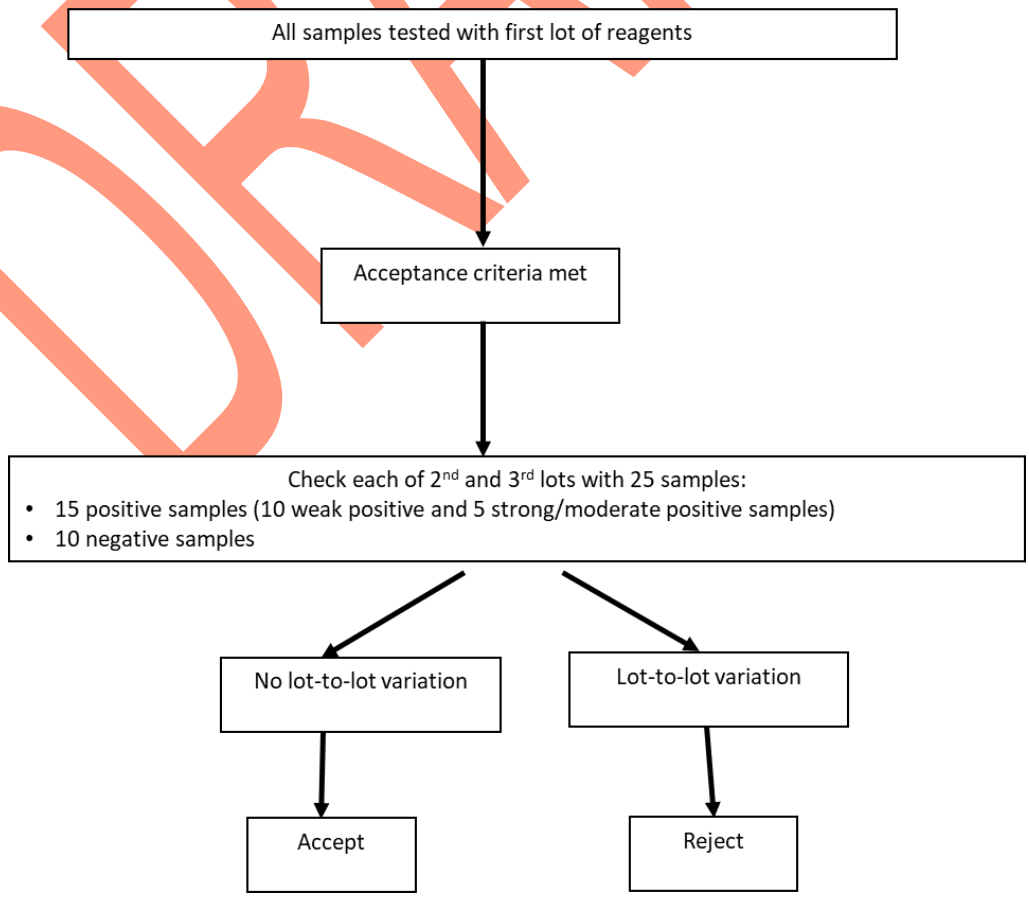
A. Lot-to-lot reproducibility:

- **Sample size for lot-to-lot reproducibility**

Three lots of an assay shall be evaluated. The first lot shall be evaluated on the entire panel of samples (statistically significant sample size). For the subsequent two lots, 25 samples should be used for evaluation (15 positive samples including 10 weak positive samples and 5 moderate/strong positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

Fig.1: Lot-to-lot reproducibility



B. **Reader-to-reader reproducibility:** 25 samples (15 positive samples including 10 weak positive samples and 5 strong/moderate positive samples, and 10 negative samples) need to be tested by at least 2 trained personnel. Agreement should be 100%.

C. **Machine-to-machine reproducibility:** 25 samples (15 positive samples including 10 weak positive samples and 5 strong/moderate positive samples, and 10 negative samples) to be tested on two different platforms (e.g.: ABI 7500 and BioRad CFX96). Agreement should be 100%.

8. Testing Methodology:

The reference assay and the index test should be run on the sample panel in parallel.

9. Interpretation of results:

Results should be interpreted as per the IFU of the reference assay and the index test.

10. Resolution of discrepant results:

True positive samples: These are samples positive by both reference assay and index test.

True negative samples: These are samples negative by both reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

11. Acceptance Criteria:

Target Plasmodium species	**Acceptance criteria**	**Minimum no. of samples needed to achieve at least the performance characteristics outlined in the acceptance criteria**
Pf PCR	Sensitivity ≥98% Specificity ≥98% Limit of detection: 1 parasite/µl Invalid test rate: ≤5%	Minimum no. of Positive samples = 80 Minimum no. of Negative samples = 80
Pv PCR	Sensitivity ≥95% Specificity ≥98% Limit of detection: 1-2 parasites/µl Invalid test rate: ≤5%	Minimum no. of Positive samples = 80 Minimum no. of Negative samples = 80
Multiplex PCR - Pf & Pv	For Pf: • Sensitivity: ≥98% • Specificity: ≥98%	For Pf: Minimum no. of Positive samples = 80

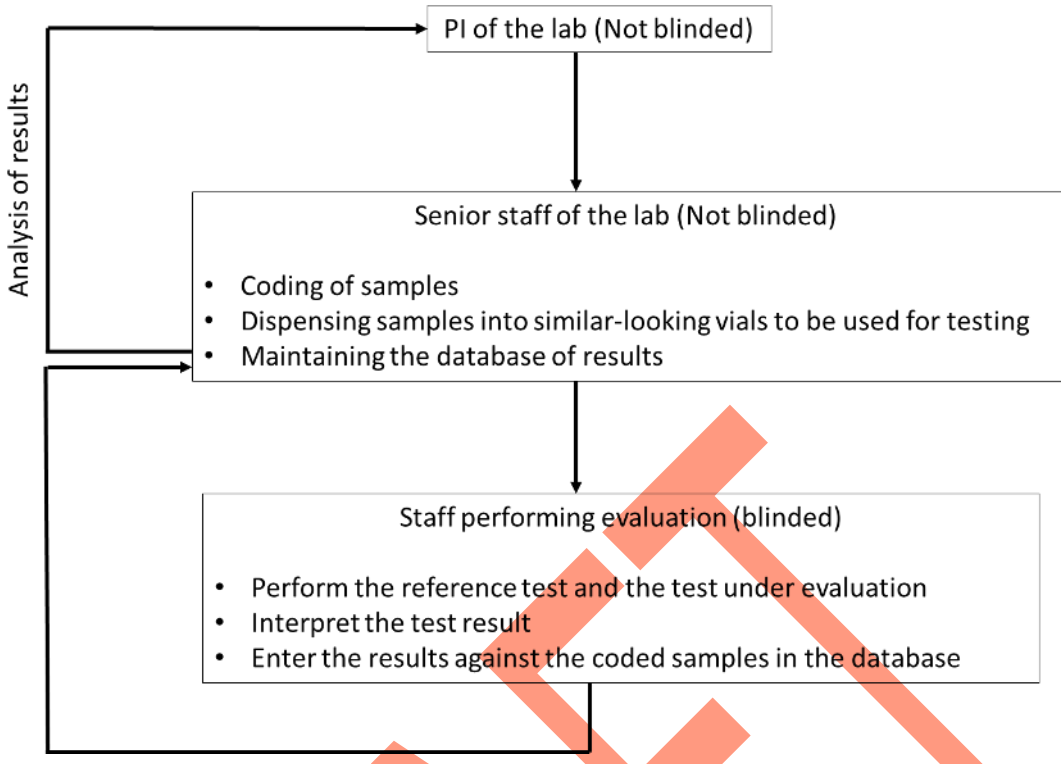
	• Absolute precision 5% • 95% CI • Invalid test rate ≤5% • Limit of detection: 1 parasite/µl For Pv: • Sensitivity: ≥95% • Specificity: ≥98% • Absolute precision 5% • 95% CI • Invalid test rate ≤5% • Limit of detection: 1-2 parasites/µl	Minimum no. of Negative samples = 80 <u>For Pv:</u> Minimum no. of Positive samples = 80 Minimum no. of Negative samples = 80

Cross-reactivity: nil
Invalid test rate: ≤5%

12. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



13. Publication Rights

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested

polymerase chain reaction. Molecular and Biochemical Parasitology. 1993;61:315–20.

2. Ramírez AM, Tang THT, Suárez ML, Fernández AÁ, García CM, Hisam S, Rubio JM. Assessment of Commercial Real-Time PCR Assays for Detection of Malaria Infection in a Non-Endemic Setting. Am J Trop Med Hyg. 2021 Oct 12;105(6):1732-1737. doi: 10.4269/ajtmh.21-0406. PMID: 34662870; PMCID: PMC8641344.

3. Bouzayene, A., Zaffaroullah, R., Bailly, J. *et al.* Evaluation of two commercial kits and two laboratory-developed qPCR assays compared to LAMP for molecular diagnosis of malaria. *Malar J* **21**, 204 (2022). https://doi.org/10.1186/s12936-022-04219-1

4. Aschar M, Sanchez MCA, Costa-Nascimento MJ, Farinas MLRN, Hristov AD, Lima GFMC, Inoue J, Levi JE, Di Santi SM. Ultrasensitive molecular tests for *Plasmodium* detection: applicability in control and elimination programs and reference laboratories. Rev Panam Salud Publica. 2022 Mar 28;46:e11. doi: 10.26633/RPSP.2022.11. PMID: 35355692; PMCID: PMC8959250.

VII. Performance evaluation report format

REPORT FORMAT

Name of the Laboratory

Name of the Institute, (with station)

Certificate of Analysis

File No.: _____

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number: Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	

	Negative samples (provide details, including cross reactivity panel)	

Results:

		Reference assay …… (name)		
		Positive	Negative	Total
Name of malaria real time PCR kit	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- Details of cross reactivity with other agents:
- **Conclusions:**
  - Sensitivity, specificity
  - Performance: **Satisfactory / Not Satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting on ……. samples only, using kits provided by the manufacturer from the batch mentioned above. Results should not be extrapolated for any other sample type.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for …….. Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal ……

**********End of the Report**********

Field evaluation protocol for combo Malaria Rapid Diagnostic Test (RDT) kits (detecting *P vivax* and *P falciparum*)

I. Background:

CDSCO/ICMR, New Delhi, have aimed to facilitate the availability of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding IVD kit performance.

II. Purpose:

To evaluate the performance characteristics of Malaria RDT kits (detecting *P. vivax* and/or *P. falciparum*) in the diagnosis of Malaria parasite infection in individuals with unknown disease status.

III. Requirements:

1. Supply of kits under evaluation (with batch no. and lot no. Manufacturing and Expiry dates other required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.
2. Evaluation sites/laboratories (With required equipment)
3. Reference test kits
4. Laboratory supplies

IV. Ethical approval:

The study will be initiated after approval from the institutional human ethics committee.

V. Procedure:

1. **Study design/type**: Cross-sectional study
2. **Preparation of Evaluation sites/laboratories:**

Identified IVD kit evaluation laboratories should establish their proficiency through

A. Laboratory accreditation: Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO: 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

It is recommended that malaria Medical Device Testing Labs (MDTLs) participate in Quality Control exercises such as EQAP (External Quality Assurance Programme).

B. Staff training: All the staff involved in IVD kit evaluation should undergo hands on training and competency testing on the following at referral level malaria labs before initiation of MDTL activity:

- Preparation and characterization of evaluation panel for the respective IVD kit.
- Management of RDT kits (specific for *Plasmodium falciparum / Plasmodium vivax*) received for performance evaluation (Verification/Storage/Unpacking etc.).

- Perform tests interpretation and documentation of results, and reporting.
- Data management and safety and confidentiality.

3. Sample size for performance evaluation:

Sample sizes of positive and negative samples against different values of sensitivity and specificity are provided in Tables 1 and 2. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate 5%. It is further assumed that at least 5% of the individuals attending the health care facilities for acute febrile illness and suspected for Malaria will be positive for Malaria (*P. vivax* and *P. falciparum*). Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity. Sample sizes are calculated using the following formulae and assumption of 5% for prevalence of the disease:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\ (1 - S_e)}{d^2 \text{ x } (1 - \text{IR}) \text{ x P}}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\ (1 - S_p)}{d^2 \text{ x } (1 - \text{IR}) \text{ x P}}$$

- *n (se) is the minimum number of individuals to be enrolled to obtain the requisite number of positive samples.*
- *n (sp) is the minimum number of individuals to be enrolled to obtain the requisite number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*
- *P is prevalence of the disease*

Sample size has to be calculated based on both the sensitivity and the specificity. The final sample size will be the maximum of the two. For example, at 95% sensitivity and

95% specificity, the sample size required will be 1600 (maximum of 1600 and 84). Please note that since the prevalence is low, the final sample size is generally expected to be governed by the assumed sensitivity.

Table 1. Sample sizes for different values of species-specific sensitivity being claimed

Sensitivity	Minimum no. of positive samples required (rounded figure) #	Minimum number of individuals to be enrolled in the study to obtain requisite number of positive samples
99%	20	400
95%	80	1600
90%	150	3000
85%	210	4200
80%	260	5200
75%	305	6100

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Samples will be collected from individuals attending the health care facilities *(tertiary care centers and their linked hospitals, private clinics, field practice areas etc.)* for acute febrile illness in highly endemic areas.

The disease status of these cases will be unknown.

Table 2. Sample sizes for different values of species-specific specificity being claimed

Specificity	No. of negative samples required (rounded figure)	Minimum number of individuals to be enrolled to obtain requisite number of negative samples
99%	20	21
95%	80	84
90%	150	158
85%	210	221
80%	260	274
75%	305	321

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Samples will be collected from individuals attending the health care facilities *(tertiary care centers and their linked hospitals, private clinics, field practice areas etc.)* for acute febrile illness in highly endemic areas.

Since a large number of febrile cases have to be enrolled to obtain the requisite number of malaria positive samples, enrolling the number of cases mentioned in Table 1 will be sufficient to obtain the requisite number of negative samples.

4. Inclusion criteria:

Individuals with the following clinical features may be enrolled in the study

Fever and any 2 of the following:

- Chills, sweating, headache, tiredness, nausea and vomiting, jaundice, splenomegaly

5. Exclusion criteria

- Individuals not satisfying inclusion criteria
- Individuals with already known positive history for other pathogens

6. Reference assay:

WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved Malaria PCR assay/ Snounou protocol should be used as reference assay.

7. Study implementation:

The patients displaying Malaria like illness will be recruited into the study and five ml of whole blood will be collected in EDTA tubes. The whole blood sample will be subjected to the reference and the index test.

The disease status of the enrolled cases will be unknown.

8. Evaluation method:

The index test and the reference tests should be run simultaneously on the sample panel, and results should be recorded.

9. Interpretation of results:

Reference test and index test results will be interpreted as per kit IFU.

10. Positive samples:

Samples positive by the reference assay will be considered as true positive samples.

11. Negative samples:

Samples negative by the reference assay will be considered as true negative samples.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

A. Cross reactivity:

The RDT kit should have been evaluated against the following cross reactivity panel during the analytical performance evaluation:

- *Dengue NS1 positive samples (n=10 samples)*
- *Chikungunya PCR positive samples (n=10 samples)*
- *Healthy controls from endemic regions (n= 40 samples)*
- *Serum reactive for RA factor – low positive and high positive (n=15 samples)*
- *Serum reactive for TPHA/other specific test for syphilis (n= 10 samples)*

12. Statistical analysis:

Sensitivity and specificity will be calculated.

Interim analysis of data shall be conducted on completing evaluation of 25%, 50% and 75% of samples. If, at any point, the performance of the assay is found to be not satisfactory, the assay shall not be evaluated further. Evaluation fee shall be charged accordingly.

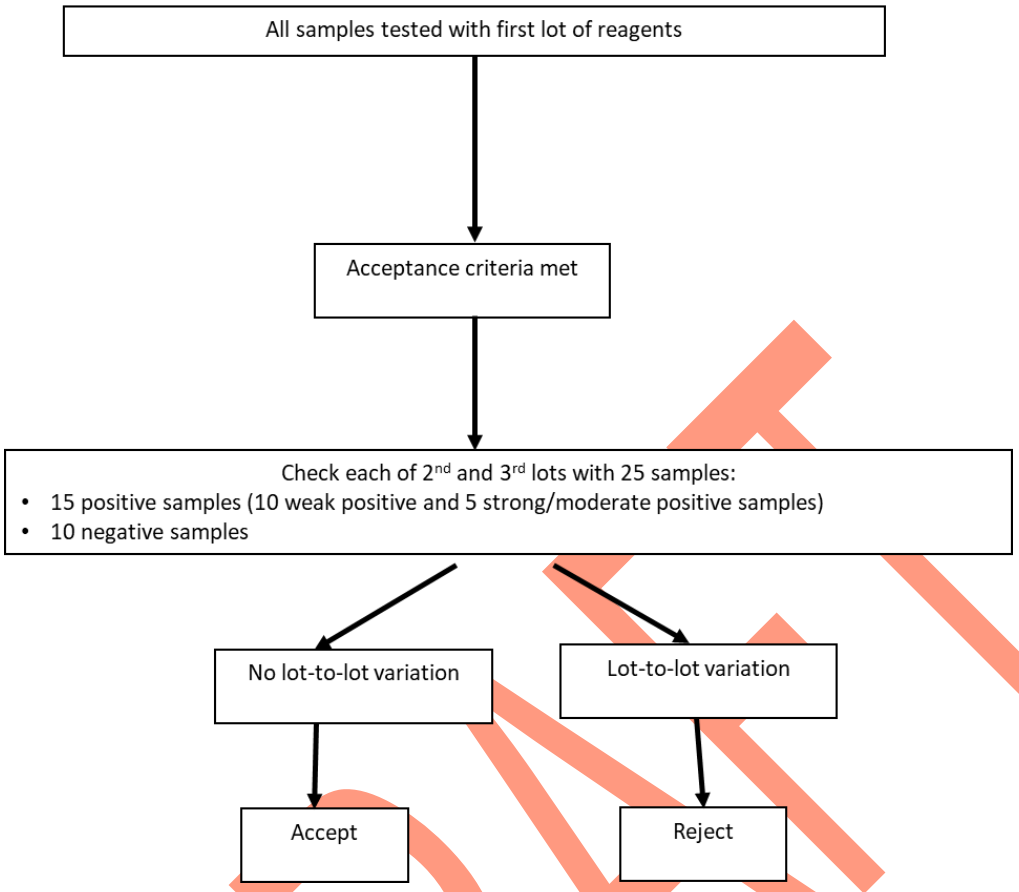
13. Test reproducibility

A. Sample size for lot-to-lot reproducibility

Three lots of an assay shall be evaluated. The first lot shall be evaluated on the entire panel of samples (statistically significant sample size). For the subsequent two lots, 25 samples should be used for evaluation (15 positive samples including 10 weak positive samples and 5 moderate/strong positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

Fig.1: Lot-to-lot reproducibility



B. Reader-to-reader reproducibility: 25 samples (15 positive samples including 10 weak positive samples and 5 strong/moderate positive samples, and 10 negative samples) need to be tested by at least 2 trained personnel. Agreement should be 100%.

14. Resolution of discrepant results:

True positive samples: These are samples positive by both reference assay and index test.

True negative samples: These are samples negative by both reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

15. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the results of the reference assay. The PI of the evaluation exercise should remain unblinded, i.e., privy to the results of the reference test. Another senior laboratory staff selected by the PI may remain unblinded for overseeing the activity and

maintaining the database of results.. The data should be analyzed only by the PI of the evaluating lab.

16. Acceptance criteria:

Expected sensitivity: ≥75% for *P. vivax* and ≥95% for *P. falciparum*
Expected specificity: ≥90% for *P. vivax* and ≥95% for *P. falciparum*
Cross-reactivity: Nil
Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria for *P vivax*, ≥6100 individuals satisfying the case definition need to be enrolled to obtain the requisite number of positive samples. This sample size is sufficient for requisite number of negative samples.
To achieve at least the performance characteristics outlined in the acceptance criteria for *P falciparum*, ≥1600 individuals satisfying the case definition need to be enrolled to obtain the requisite number of positive samples. This sample size is sufficient for requisite number of negative samples.

Recruitment should be terminated once the desired number of positive cases is enrolled and tested.

17. Publication Rights

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Ministry of Health and Family Welfare. Guidelines for Bivalent RDT. Available at: guidelines-for-bivalent-rdt.pdf (mohfw.gov.in)
2. World Health Organization. Malaria Rapid Diagnostic Test Performance - Results of WHO product testing of malaria RDTs: round 8 (2016–2018): Available at: https://iris.who.int/bitstream/handle/10665/276190/9789241514965-eng.pdf?sequence=1
3. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested

polymerase chain reaction. Molecular and Biochemical Parasitology. 1993;61:315–20.

4. Integrated Disease Surveillance Project Training Manual For State & District Surveillance Officers - Case Definitions Of Diseases & Syndromes Under Surveillance (Module-5). Available at: https://idsp.mohfw.gov.in/WriteReadData/OldSite/2WkDSOSept08/Resources_files/DistrictSurvMan/Module5.pdf [Accessed on 25th June 2024]
5. CDC. National Notifiable Diseases Surveillance System (NNDSS). Malaria (Plasmodium spp.) 2014 Case Definition. Available at: https://ndc.services.cdc.gov/case-definitions/malaria-2014/ [Accessed on 28th June, 2024]
6. Kannambath R, Rajkumari N, Sivaradjy M. Prevalence of malaria: A 7-year trend analysis from a tertiary care center, Puducherry. Trop Parasitol. 2023 Jan-Jun;13(1):28-33. doi: 10.4103/tp.tp_41_22. Epub 2023 May 19. PMID: 37415756; PMCID: PMC10321582.

VII. Performance evaluation report format

REPORT FORMAT

Name of the Laboratory

Name of the Institute, (with station)

Certificate of Analysis

File No.: _____

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number: Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	

	Negative samples (provide details, including cross reactivity panel)	

Results:

		Reference assay ……. (name)		
		Positive	Negative	Total
Name of index malaria RDT	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- Details of cross reactivity with other agents:
- Conclusions:
    - Sensitivity, specificity
    - Performance: Satisfactory / Not Satisfactory

(Sensitivity and specificity have been assessed in field/controlled lab setting on……. samples only, using kits provided by the manufacturer from the batch mentioned above. Results should not be extrapolated for any other sample type.)

Disclaimers

1. This validation process does not approve / disapprove the kit design

2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for …….. Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal

……

**********End of the Report**********

Information on Operational and Test Performance Characteristics Required from Manufacturers for Malaria IVD

The manufacturer should provide the following details about the IVD:

1. Instructions for Use
2. Scope of the IVD: to diagnose Malaria (Pf and/or Pv)
3. Intended Use Statement
4. Principle of the assay
5. Intended testing population (cases of acute febrile illness/suspected cases of Malaria)
6. Intended user(laboratory professional and/or health care worker at point-of-care)
7. Detailed test protocol
8. Lot/batch No.
9. Date of manufacture
10. Date of Expiry
11. Information on operational Characteristics
    - i. Configuration of the kit/device
    - ii. Requirement of any additional equipment, device
    - iii. Requirement of any additional reagents
    - iv. Operation conditions
    - v. Storage and stability before and after opening
    - vi. Internal control provided or not
    - vii. Quality control and batch testing data
    - viii. Biosafety aspects- waste disposal requirements
10. Information on Test Performance Characteristics
    - i. Type of sample-serum/plasma/whole blood/other specimen (specify)
    - ii. Volume of sample
    - iii. Sample rejection criteria (if any)
    - iv. Any additional sample processing required
    - v. Any additional device/consumable like sample transfer device, pipette, tube, etc required
    - vi. Name of analyte to be detected
    - vii. Pathogens targeted by the kit

viii. Time taken for testing

ix. Time for result reading and interpretation

x. Manual or automated(equipment)reading

xi. Limit of detection

xii. Diagnostic sensitivity

xiii. Diagnostic specificity

xiv. Stability and reproducibility (including data)

xv. Training required for testing (if any)

xvi. If yes, duration

xvii. Details of Cut-off and /or Equivocal Zone for interpretation of test

xviii. Details of cross reactivity, if any

xix. Interpretation of invalid and indeterminate results to be provided

xx. It is recommended to provide data demonstrating the precision

xxi. Limit of detection

*Please mention “Not applicable” against sections not pertaining to the kit.

**********End of the Document**********

STANDARD PERFORMANCE EVALUATION PROTOCOL

DRAFT FOR STAKEHOLDER COMMENTS

NIPAH VIRUS REAL TIME PCR KIT

ICMR-CDSCO/IVD/GD/PROTOCOLS/08/2025

AUGUST, 2025

New Delhi, India

Table of Contents

Performance evaluation protocol for Nipah virus real-time PCR kit

I. Background:

CDSCO and ICMR, New Delhi, have aimed at facilitating the availability of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

This recommendation focuses on the laboratory performance evaluation of Nipah virus real time PCR kit. All clinical samples tested in the study should be evaluated in accordance with the candidate test's instructions for use.

II. Purpose:

To evaluate the performance characteristics of Nipah virus real-time PCR kits in the diagnosis of Nipah virus infection/ disease using irreversibly de-identified leftover archived/ spiked clinical samples.

III. Requirements:

1. Supply of kits under evaluation (Along with batch/lot No. Expiry & required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.
2. Evaluation sites/laboratories (With required equipment)
3. Reference test kits
4. Characterised Evaluation panel
5. Laboratory supplies

IV. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. **Study design/type**: Diagnostic accuracy study using spiked/clinical samples (human specimens).
2. **Preparation of Evaluation sites/laboratories:**

Identified IVD kit evaluation laboratories should be well-equipped and establish their proficiency through ALL of the following:

A. Availability of BSL-4 facility for handling of Nipah virus positive specimens

B. Accreditation for at least one Quality management system for at least one respiratory viral pathogen molecular testing (accreditation for Testing Lab / Calibration Lab as per ISO/IES 17025, Medical Lab as per ISO 15189, PT provider as per ISO/IEC 17043), or CDSCO approved Reference laboratory.

C. Staff training: All the staff involved in Nipah virus IVD evaluation should undergo hands on training and competency testing on following

- BSL-4 practices
- Nipah virus culture and handling
- Preparation & characterization of reference sample panel
- Handling of Nipah virus RT-PCR kits received for performance evaluation (Verification/Storage/Unpacking etc).
- Testing, interpreting, recording of results & reporting
- Data handling, data safety & confidentiality

3. Preparation of Nipah virus RNA evaluation panel

This is a zoonotic disease, and well characterised Nipah virus positive human samples is a critical requirement for evaluation of RT-PCR IVD kits. A statistically significant number of clinical samples should be used for the evaluation.

4. RNA extraction

RNA extraction should be performed as per manufacturer's instruction for reference assay as well as the assay under evaluation. If any extraction system is specified in the IFU, that shall be used for the test and shall be provided by the manufacturer.

5. Real-Time PCR System

PCR shall be performed using IVD-approved machines. If any equipment(s) is specified in the IFU, that shall be used for the test and shall be provided by the manufacturer.

Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.

6. Internal control/Extraction control

Assays must have an internal control (housekeeping gene), with or without an extraction control (RNA added before extraction to a sample).

7. Reference assay:

The Nipah virus Real Time PCR Assay developed by ICMR-NIV Pune, or a WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved real time PCR assay should be used as the Reference Standard.

All positive samples should be confirmed positive by the reference assay.

All negative samples should be confirmed negative by the reference assay.

8. Sample size for performance evaluation: Sample size is calculated assuming 95% sensitivity and specificity of the index test, 95% confidence level, absolute precision of 5% and ≤5% invalid test rate. A minimum of 77 (rounded to 80) positive clinical samples and a minimum of 77 (rounded to 80) negative clinical samples are required. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\ (1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\ (1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Nipah virus is detectable from throat swab, urine, CSF. The assay should be validated with positive clinical/spiked samples, and negative samples for all the formats claimed by the manufacturer. However, if a particular sample matrix is used to evaluate the assay (as opposed to all the sample types claimed by the manufacturer), the performance evaluation report should clearly mention the performance characteristics of the assay against the sample type used for validation. There should be no ambiguity about the sample type used for assay validation.

9. Sample panel composition:

A. Human samples

A.1 Positive samples (Minimum n=80 for each sample type): Clinical/ Spiked samples positive by the reference real-time PCR assay

A.1.1 Strong positive (Ct value <25) = 24 samples

A.1.2. Moderate positive (Ct value between 25-30) = 28 samples

A.1.3 Weak positive (Ct value >30 to 34) = 28 samples

The sample type should be as per the index test IFU. If an assay claims to detect Nipah virus RNA in several sample types, attempt should be made to use 80 positive samples across each sample type, or at least the sample types available with the evaluating lab. This relaxation is provided since clinical samples are scarce and obtained only during outbreaks occurring every few years in India, which necessitates using spiked clinical samples. The latter is difficult since Nipah virus is a BSL-4 level pathogen and its handling requires sophisticated laboratory setup and trained manpower.

In case the requisite number of specimens for a particular sample type are not available and a smaller number of samples are used for performance evaluation (i.e., sample size calculated assuming higher performance characteristics), it is necessary to ensure that the study has adequate power for acceptance of the evaluation results in case the assay falls short of the assumed performance characteristics.

Note:

If clinical samples positive for Nipah virus are not available, tissue culture fluid (Heat-inactivated) from reference laboratories can be used, spiked in serum/urine/Throat swab samples to obtain the panel with Ct value <25, 25-30 and >35 and tested by the reference assay, and the positive samples can be used for evaluation.

Confirmed negative samples would be used for spiking with Nipah virus.isolate.

A.2 Negative samples (number of samples will depend on sample type): All negative samples should be negative by reference real-time PCR assay. Distribution of the negative samples should be as follows

Categories of samples as per the sample type	Sample type		
	NP/TS (Minimum n= 80)	Serum (Minimum n= 80)	Urine (Minimum n=80)

A.2.1 Samples from cases having similar illness/ spiked samples which are RT-PCR positive for common pathogens but negative for Nipah virus	Samples from individuals presenting with **ARI/ILI/SARI** (n=45): 5 positive clinical/ spiked samples from each of the following diseases: 1. Influenza A virus @ 2. Influenza B virus @ 3. SARS-CoV-2 @ 4. RSV A/B @ 5. HPIV @ 6. HMPV @ 7. Adenovirus @ 8. Seasonal Coronaviruses * 9. Rhinovirus/Enterovirus* *Cross reactivity panel is arranged in descending order of priority.* *The pathogens marked @ are essentially to be tested.* *It is recommended to test for all pathogens listed in the cross reactivity panel. However, if there is an acute shortfall or non-availability of clinical samples, one may consider reducing only the pathogens of lower priority marked by * , while ensuring that the actual numbers of cross reactive sample panel remain the same by compensating with the available "essentially to be tested" samples.*	Samples from cases of **AES** (n=35): 5 positive clinical/ spiked samples from each of the following diseases: 1. Japanese Encephalitis @ 2. Dengue @ 3. HSV @ 4. VZV @ 5. West Nile Virus * 6. Chandipura virus * 7. Rabies virus * *Cross reactivity panel is arranged in descending order of priority.* *The pathogens marked @ are essentially to be tested.* *It is recommended to test for all pathogens listed in the cross reactivity panel. However, if there is an acute shortfall or non-availability of clinical samples, one may consider reducing only the pathogens of lower priority marked by * , while ensuring that the actual numbers of cross reactive sample panel remain the same by compensating with the available "essentially to be tested" samples.*	5 positive clinical/ spiked samples from each of the following diseases, presenting with **respiratory and/or encephalitis symptoms** (n=20): 1. Measles 2. Rubella 3. Mumps 4. SARS-CoV-2
A.2.2 Samples from cases with acute respiratory disease/ acute encephalitis/ acute febrile illness and RT-PCR negative for the above-mentioned pathogens and Nipah virus	25	35	40

A.2.3 Healthy/ asymptomatic cases from endemic regions negative for Nipah virus	10	10	20
Serum/ throat swab/ urine samples collected from the same case may be used for evaluation.			

10. Evaluation method:

The index test and the reference tests should be run simultaneously on the sample panel, and results should be recorded.

11. Interpretation of results:

Reference test and index test results will be interpreted as per kit IFU.

12. Resolution of discrepant results:

True positive samples: These are samples positive by reference assay and index test.
True negative samples: These are samples negative by reference assay and index test.
False positive samples: These are samples negative by reference assay and positive by index test.
False negative samples: These are samples positive by reference assay and negative by index test.

13. Test reproducibility

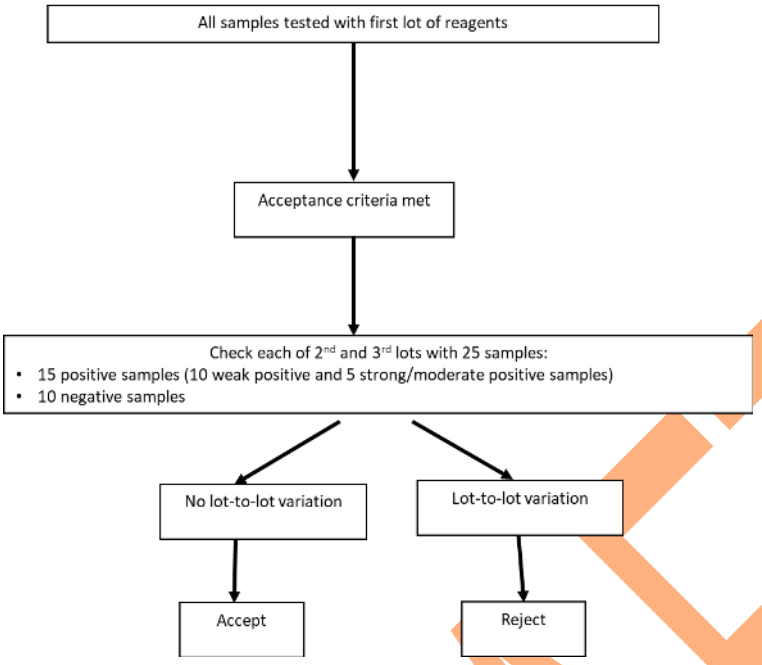
A. Sample size for lot-to-lot reproducibility

Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

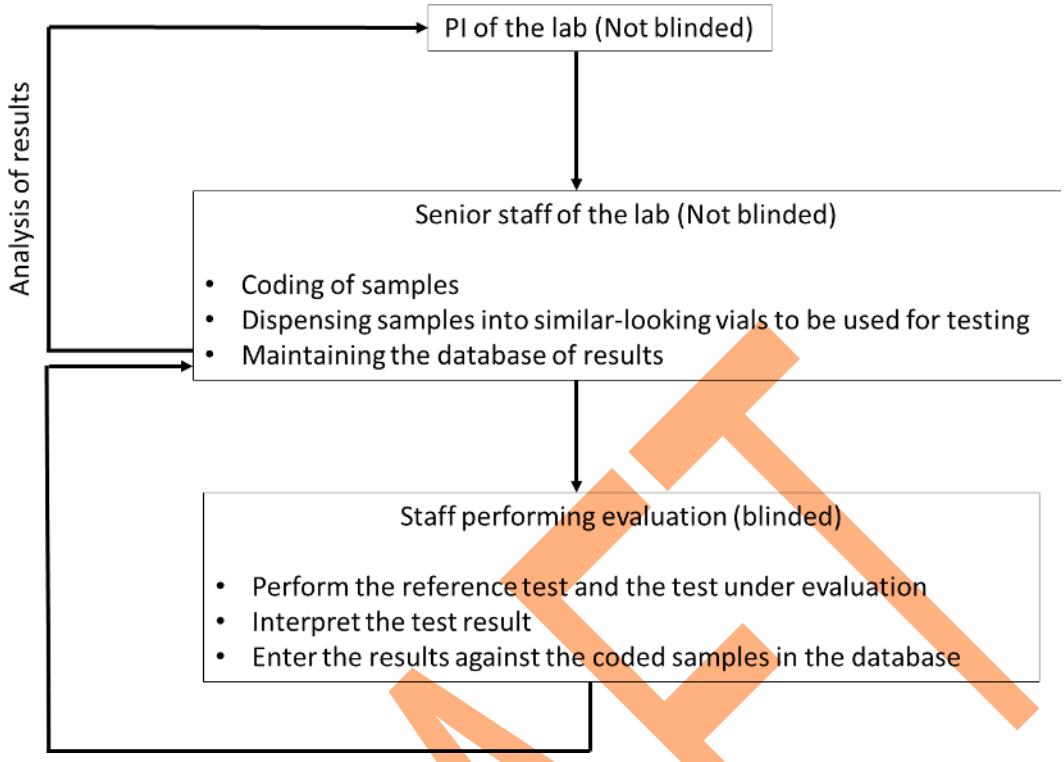
Fig.1: Sample size for Lot-to-lot reproducibility



14. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



15. Acceptance Criteria

Expected sensitivity: ≥95%

Expected specificity: ≥98%

Cross reactivity with other viruses as outlined in the negative sample panel: Nil

Invalid test rate: ≤5%

16. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Yadav PD, Majumdar T, Gupta N, Kumar MA, Shete A, Pardeshi P, Sultana S, Sahay RR, Manoj MN, Patil S, Floura S, Gangakhedkar R, Mourya DT. Standardization & validation of Truenat™ point-of-care test for rapid diagnosis of Nipah. Indian J Med Res. 2021 Apr;154(4):645-649. doi: 10.4103/ijmr.IJMR_4717_20. PMID: 34854433; PMCID: PMC9205002.
2. World Health Organization. Technical Guidance Series (TGS) for WHO Prequalification – Diagnostic Assessment TGS-3. 2017. Available at: https://iris.who.int/bitstream/handle/10665/258985/WHO-EMP-RHT-PQT-TGS3-2017.03-eng.pdf;sequence=1

VII. Performance evaluation report format

PERFORMANCE EVALUATION REPORT FOR NIPAH VIRUS REAL-TIME PCR KITS

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: clinical/spiked, strong, moderate, weak)	
	Negative samples (provide details (clinical/spiked,), including cross reactivity panel)	

Results

		Reference assay …….…… (name)		
		Positive	Negative	Total
Name of Nipah virus real-time PCR	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- Details of cross reactivity with other Paramyxoviruses:
- **Conclusions:**

- Sensitivity, specificity
- Cross reactivity
- Invalid test rate
- Performance: **Satisfactory / Not satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting using kits provided by the manufacturer from the batch mentioned above using ..... sample. Results should not be extrapolated to other sample types.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for Nipah virus…….. Kit (Lot No……) manufactured by …… (supplied by …….)

Evaluation Done on ……

Evaluation Done by ……

Signature of Director/ Director-In-charge …… Seal …….

**********End of the Report**********

Annexure-1: Information on Operational and Test Performance Characteristics Required from Manufacturers

The manufacturer should provide the following details about the IVD:

1. Instructions for Use
2. Scope of the IVD: to diagnose Nipah virus
3. Intended Use Statement
4. Principle of the assay
5. Intended testing population (cases of AES/ARI/SARI)
6. Intended user (laboratory professional and/or health care worker at point-of-care)
7. Lot/batch No.
8. Date of manufacture
9. Date of Expiry
10. Information on operational Characteristics

i. Configuration of the kit/device

ii. Requirement of any additional equipment, device

iii. Requirement of any additional reagents

iv. Operation conditions

v. Storage and stability before and after opening

vi. Internal control provided or not

vii. Quality control and batch testing data

viii. Biosafety aspects- waste disposal requirements

11. Information on Test Performance Characteristics

i. Type of sample- Nasopharyngeal swab/Throat swab/ CSF/Serum / Other specimen

ii. Volume of sample

iii. Any specific sample NOT to be tested

iv. Any additional sample processing required

v. Any additional device/consumable like sample transfer device, pipette, tube, etc required

vi. Name of analyte to be detected

vii. Pathogen(s) targeted by the kit

viii. Time taken for testing

ix. Time for result reading and interpretation

x. Manual or automated(equipment)reading

xi. Limit of detection

xii. Diagnostic sensitivity

xiii. Diagnostic specificity

xiv. Stability and reproducibility

xv. Training required for testing

xvi. If yes, duration

xvii. Details of Cut-off and /or Equivocal Zone for interpretation of test

xviii. Interpretation of invalid and indeterminate results to be provided

xix. It is recommended to provide data demonstrating the precision

*Please mention "Not applicable" against sections not pertaining to the kit.

**********End of the Document**********

STANDARD PERFORMANCE EVALUATION PROTOCOL

DRAFT FOR STAKEHOLDER COMMENTS

CHANDIPURA VIRUS REAL TIME PCR KIT

ICMR-CDSCO/IVD/GD/PROTOCOLS/07/2025

AUGUST, 2025

New Delhi, India

Table of Contents

Performance evaluation protocol for Chandipura virus real-time PCR kits

I. Background

CDSCO and ICMR, New Delhi, have aimed at facilitating the availability of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

This recommendation focuses on the laboratory performance evaluation of Chandipura virus (CHPV) virus real time PCR kit. All clinical samples tested in the study should be evaluated in accordance with the candidate test's instructions for use.

II. Purpose:

To evaluate the performance characteristics of CHPV real-time PCR kits in the diagnosis of CHPV infection/ disease using irreversibly de-identified leftover archived/ spiked clinical samples.

III. Requirements:

1. **Kits Under Evaluation**: Include detailed information such as batch number, lot number, expiry date, and other relevant specifications. For kits designed to operate within a closed system, manufacturers must provide the necessary equipment and consumables for testing.

2. **Evaluation Sites/Laboratories**: Identify laboratories equipped with the required instruments and infrastructure to conduct the evaluation.

3. **Reference Test Kits**: Use reference kits or in-house kits developed by the reference laboratory, which have been validated to demonstrate satisfactory performance.

4. **Evaluation Panel**: Prepare a panel of well-characterised clinical samples from confirmed cases or spiked samples for a comprehensive evaluation.

5. **Laboratory Supplies**: Ensure all necessary laboratory materials and supplies are available for the evaluation process.

IV. Ethical Approvals:

Performance evaluation activities using irreversibly de-identified clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. Study design/type: Diagnostic accuracy study using irreversibly de-identified archived clinical/spiked samples

2. Preparation of Evaluation sites/laboratories:

Identified IVD kit evaluation laboratories should establish their proficiency through the following:

A) Accreditation for at least one of the Quality management systems, such as

- Testing Laboratory or Calibration Laboratory (ISO/IEC 17025)
- Medical Laboratory (ISO 15189)
- Proficiency Testing Provider (ISO/IEC 17043)

OR

- CDSCO-approved reference laboratory

B) Staff training: All staff involved in IVD kit evaluation process should undergo hands on training and competency assessment in the following areas:

- Preparation and characterization of kit evaluation panel
- Handling of Chandipura real-time PCR kits received for performance evaluation (verification/storage/unpacking etc.).
- Testing procedures, interpretation and recording of results, and reporting
- Data handling, data safety & confidentiality

3. Preparation of Chandipura RNA evaluation panel:

A well characterised panel of CHPV positive clinical samples is a critical requirement for evaluation of these RT-PCR IVD kits. A statistically significant number of clinical samples should be used for the evaluation.

The sample type for CHPV detection is Cerebrospinal fluid (CSF) and serum. If a kit claims to detect CHPV in both sample types, attempt should be made to evaluate the assay across both serum and CSF using statistically significant sample size for each sample type. In case all the sample types mentioned in the IFU are not available with the lab, the performance evaluation report should clearly mention the sample type against which the kit is evaluated, ensuring statistical rigor. There should be no ambiguity about the type of sample used for evaluation.

4. RNA extraction:

RNA extraction should be performed as per manufacturer's instruction for reference assay as well as the assay under evaluation. If the manufacturer of the index test recommends a specific RNA

extraction kit, it needs to be provided by the manufacturer if the evaluation lab is unable to procure the same.

5. Real-time PCR system:

PCR should be performed using IVD-approved machines. If any equipment(s) is specified in the IFU of the index test, it should be used for the evaluation, and it should be provided by the manufacturer if not available within the lab's IVD evaluation scope.

Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.

6. Internal Control/Extraction Control:

The index test must have an internal control (housekeeping gene), with or without an extraction control (RNA added before extraction to a sample).

7. Reference assay:

A WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved real time CHPV PCR assay/ ICMR-National Institute of Virology, Pune developed protocol for detection of Chandipura virus RNA will serve as the reference assay.

All positive samples should be confirmed positive by the reference assay.

All negative samples should be confirmed negative by the reference assay and CHPV IgM.

8. Sample size for performance evaluation:

1. Sample size is calculated assuming 95% sensitivity and specificity of the index test, 95% confidence level, absolute precision of 5% and ≤5% invalid test rate. A minimum of 77 (rounded to 80) positive clinical samples and a minimum of 77 (rounded to 80) negative clinical samples for each sample type are required for performance evaluation. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*

- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

9. Sample panel composition:

A) Positive samples (Minimum n=80 for each sample type): These samples should be clinical/spiked samples positive by reference real-time PCR assay and preferably represent all genetic variants. The distribution of samples should be as follows:

Characteristic of positive sample	Minimum no. of serum samples needed (for kits detecting CHPV in serum)	Minimum no. of CSF samples needed (for kits detecting CHPV in CSF)
A.1 Strong positive [Ct value ≤ 25]	24	24
A.2 Moderate positive [Ct value between >25 and ≤31]	28	28
A.3 Weak positive [Ct value >31 and ≤ 37]	28	28

For kits detecting CHPV in both serum and CSF, 80 positive serum samples and 80 positive CSF samples should be used for performance evaluation. One sample type should not be substituted by the other to reach the desired sample size in case there is paucity of samples.

Note: *Since such large number of positive clinical samples may **NOT** be available for Chandipura virus, pre-titrated and inactivated virus obtained from tissue culture fluid prepared in the laboratory will be used to spike serum and CSF samples [dilution factor: 1:10 to 1:1000 to generate samples with different intensities of positivity]. These spiked samples will be stored at -80°C, after being tested by the reference assay.*

B) Negative samples (n=80 for each sample type): All negative samples should be negative by reference assay and CHPV IgM. Distribution of the negative samples should be as follows:

Categories of samples as per the sample type	Sample type	
	Serum/plasma (Minimum n=80, (B.1 + B.2))	CSF (Minimum n=80, (B.1+B.2))

B.1 Samples from cases of AES/ spiked samples which are RT-PCR positive for known pathogens but negative for CHPV (CHPV RNA and serology)	**30** 5 positive clinical/ spiked samples from each of the following diseases (confirmed by PCR): 1. Dengue virus @ 2. Japanese Encephalitis @ 3. HSV 1/2 * 4. West Nile Virus* 5. VSV *	**35** 1. Seven (07) positive clinical/ spiked samples from each of the following diseases: a) Japanese Encephalitis @ b) Dengue virus @ c) HSV 1/2 * d) West Nile Virus * **2.** Rabies virus (n=4)* **3.** VSV (n=3)*
B.2 Samples from cases with acute encephalitis and RT-PCR negative for the above-mentioned pathogens and CHPV (CHPV RNA and serology)	**50**	**45**
B.3 Healthy/ asymptomatic cases from endemic regions negative for CHPV (CHPV RNA and serology)	5 (desirable, not mandatory)	20 (desirable, not mandatory)
Serum/plasma and CSF samples collected from the same case may be used for evaluation. *Cross reactivity panel is arranged in descending order of priority.* *The pathogens marked @ are essentially to be tested.* *It is recommended to test for all pathogens listed in the cross-reactivity panel. However, if there is an acute shortfall or non-availability of clinical samples, one may consider reducing only the pathogens of lower priority marked by * , while ensuring that the actual numbers of cross-reactive sample panel remain the same by compensating with the available "essentially to be tested" samples.* *Testing for Rabies and VSV is recommended since both the viruses belong to the same family as Chandipura virus (Rhabdoviridae). Spiked specimens/ synthetic transcripts may be used for these viruses.*		

10. Evaluation method:

The index test and reference tests should be conducted simultaneously on the sample panel to minimize the risk of false-negative results from the index test due to freeze-thaw cycles or sample degradation from prolonged storage.

11. Interpretation of results:

Reference test and index test results will be interpreted as per kit IFU.

12. Resolution of discrepant results:

True positive samples: These are samples positive by both the reference assay and index test.

True negative samples: These are samples negative by both the reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

13. Test reproducibility:

A) Sample size for lot-to-lot reproducibility:

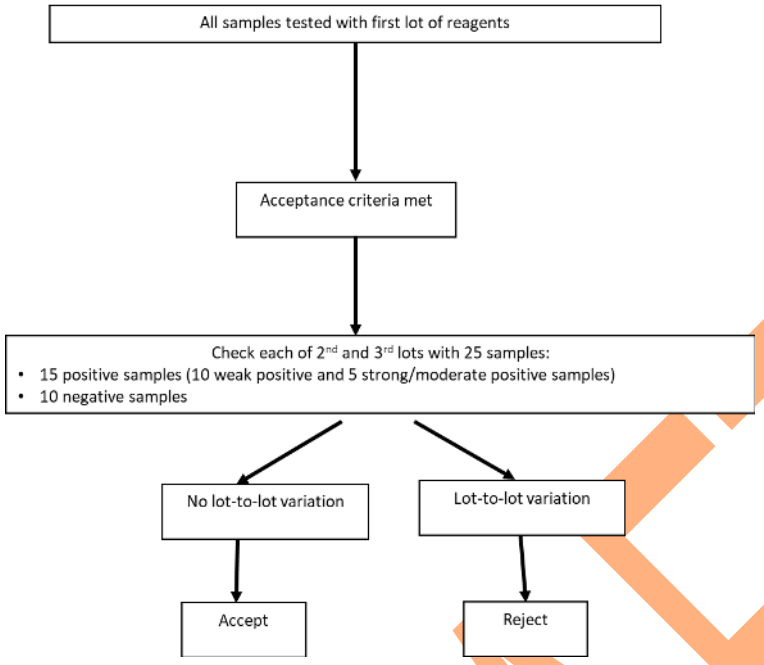
Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol above
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positives and 5 moderate/high positives, and 10 negative samples)
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positives and 5 moderate/high positives, and 10 negative samples)

If there is no lot-to-lot variation, accept the assay.

Refer the flowchart below (Fig. 1):

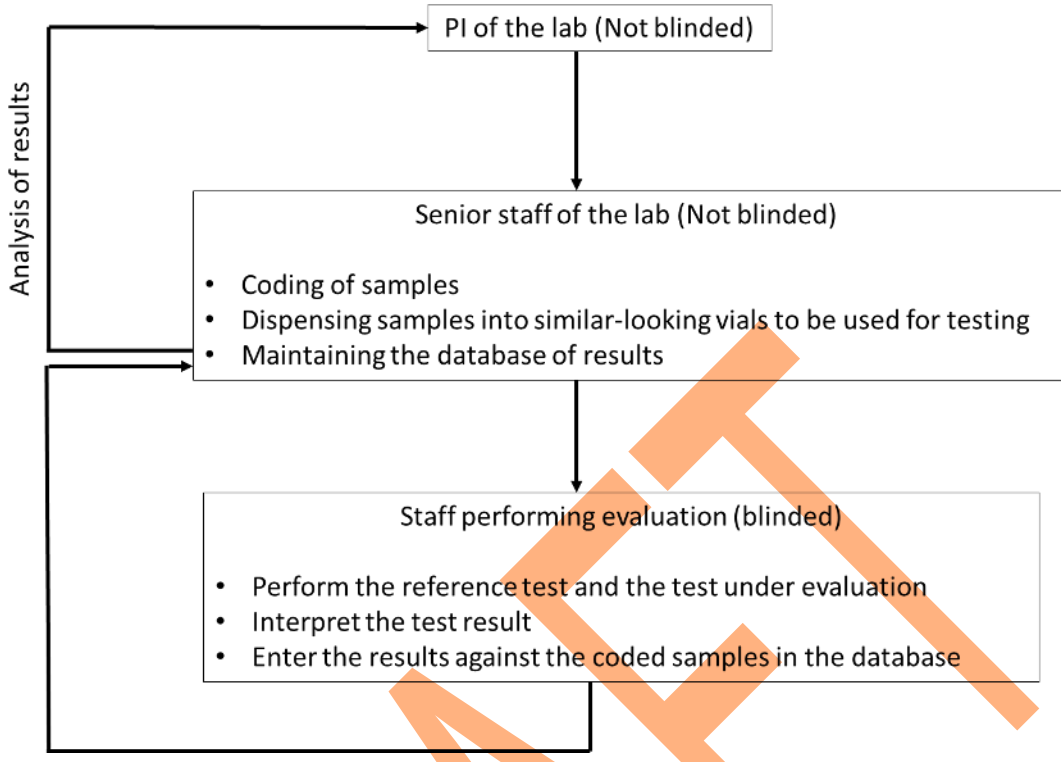
Fig.1: Sample size for Lot-to-lot reproducibility



14. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



15. Acceptance criteria:

Expected sensitivity: ≥ 95%

Expected specificity: ≥ 98%

Cross-reactivity with other rhabdoviruses: Nil

Invalid test rate ≤5%

16. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the field evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References

1. Sudeep AB, Gurav YK, Bondre VP. Changing clinical scenario in Chandipura virus infection. *Indian J Med Res*. 2016;143(6):712-721. doi:10.4103/0971-5916.191929.

2. Sapkal GN, Sawant PM, Mourya DT. Chandipura Viral Encephalitis: A Brief Review. Open Virol J. 2018 Aug 31;12:44-51. doi: 10.2174/1874357901812010044. PMID: 30288194; PMCID: PMC6142667.

3. World Health Organization. Technical Guidance Series (TGS) for WHO Prequalification-Diagnostic assessment TGS-3. 2017. Available at: https://iris.who.int/bitstream/handle/10665/258985/WHO-EMP-RHT-PQT-TGS3-2017.03-eng.pdf;sequence=1

VII. Performance Evaluation Report Format

PERFORMANCE EVALUATION REPORT FOR CHANDIPURA VIRUS REAL-TIME PCR KITS

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Positive samples (provide details: type,strong, moderate, weak)	
	Negative samples (provide details, type,including cross reactivity panel)	

Results

		Reference assay …….…… (name)		
		Positive	Negative	Total
Name of Chandipura real-time PCR kits	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- **Conclusions:**
  - Cross reactivity with related viruses:
  - Invalid test rate:
  - **Performance: Satisfactory / Not satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting using kits provided by the manufacturer from the batch mentioned above using ..... sample. Results should not be extrapolated to other sample types.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for Chandipura…….. Kit (Lot No……) manufactured by …… (supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal ……

**********End of the Report**********

Annexure-1: Information on Operational and Test Performance Characteristics Required from Manufacturers

The manufacturer should provide the following details about the IVD:

1. Instructions for Use
2. Scope of the IVD: to diagnose Chandipura virus
3. Intended Use Statement
4. Principle of the assay
5. Intended testing population (cases of Acute Febrile Illness/ AES)
6. Intended user (laboratory professional and/or health care worker at point-of-care)
7. Lot/batch No.
8. Date of manufacture
9. Date of Expiry
10. Information on operational Characteristics
    - i. Configuration of the kit/device
    - ii. Requirement of any additional equipment, device
    - iii. Requirement of any additional reagents
    - iv. Operation conditions
    - v. Storage and stability before and after opening
    - vi. Internal control provided or not
    - vii. Quality control and batch testing data
    - viii. Biosafety aspects- waste disposal requirements
11. Information on Test Performance Characteristics
    - i. Type of sample-CSF/Serum/Other specimen
    - ii. Volume of sample
    - iii. Any specific sample NOT to be tested
    - iv. Any additional sample processing required
    - v. Any additional device/consumable like sample transfer device, pipette, tube, etc required

vi. Name of analyte to be detected

vii. Pathogen(s) targeted by the kit

viii. Time taken for testing

ix. Time for result reading and interpretation

x. Manual or automated (equipment) reading

xi. Limit of detection

xii. Diagnostic sensitivity

xiii. Diagnostic specificity

xiv. Stability and reproducibility

xv. Training required for testing

xvi. If yes, duration

xvii. Details of Cut-off and /or Equivocal Zone for interpretation of test

xviii. Interpretation of invalid and indeterminate results to be provided

xix. It is recommended to provide data demonstrating the precision

xx. Limit of detection

*Please mention "Not applicable" against sections not pertaining to the kit.

**********End of the Document**********

STANDARD PERFORMANCE EVALUATION PROTOCOL

DRAFT FOR STAKEHOLDER COMMENTS

MULTIPLEX RESPIRATORY VIRUS REAL TIME PCR

ICMR-CDSCO/IVD/GD/PROTOCOLS/09/2025

AUGUST, 2025

New Delhi, India

Table of Contents

Performance evaluation protocol for multiplex respiratory virus real-time PCR kit

I. Background:

CDSCO and ICMR, New Delhi, have aimed at facilitating the availability of Quality-Assured diagnostic kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding IVD performance.

This recommendation focuses on the laboratory performance evaluation of multiplex respiratory virus real time PCR kit. All clinical samples tested in the study should be evaluated in accordance with the candidate test's instructions for use.

II. Purpose:

To evaluate the performance characteristics of multiplex respiratory virus real-time PCR kits using irreversibly de-identified leftover archived clinical/spiked samples.

III. Scope of the document:

This document outlines performance evaluation protocol for multiplex real time PCR assays detecting the following respiratory viruses of utmost importance in human clinical specimens (Table 1), as determined by ICMR appointed working group and expert group of physicians and clinical microbiologists following extensive literature review and real-life experience. This pathogen list has been developed as part of the National One Health Mission.

Table 1: List of respiratory viruses within the scope of this performance evaluation protocol

1. Influenza virus A
2. Influenza virus B
3. SARS Coronavirus-2
4. Respiratory syncytial virus
5. Adenovirus
6. Human Respiroviruses 1 and 3 and Human Rubulaviruses 2 and 4 (erstwhile Human Parainfluenzaviruses 1-4)
7. Human metapneumovirus
8. Measles virus
9. Rhinovirus
10. Human Bocavirus
11. Enterovirus
12. Cytomegalovirus

IV. Requirements:

1. Supply of kits under evaluation (Along with batch/lot No. Expiry & required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.
2. Evaluation sites/laboratories (With required equipment)
3. Reference test kits
4. Characterised Evaluation panel
5. Laboratory supplies

V. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

VI. Procedure:

1. **Study design/type**: Diagnostic accuracy study using irreversibly de-identified archived/ spiked clinical samples
2. **Preparation of Evaluation sites/laboratories:**
   Identified IVD kit evaluation laboratories should be well-equipped and establish their proficiency through ALL of the following:

A. Accreditation at least one of the Quality management systems for at least one respiratory viral pathogen molecular testing (accreditation for Testing Lab / Calibration Lab as per ISO 17025, Medical Lab as per ISO 15189, PT provider as per ISO/IEC 17043), or CDSCO approved Reference laboratory.

B. Staff training: All the staff involved in IVD evaluation should undergo hands-on training and competency testing on the following:

- Preparation & characterization of reference sample panel
- Handling of multiplex respiratory virus RT-PCR kits received for performance evaluation (Verification/Storage/Unpacking etc).
- Testing
- Data handling, data safety & confidentiality

3. **Preparation of multiplex respiratory virus evaluation panel**

A well characterised panel of positive and negative clinical samples is a critical requirement for evaluation of these RT-PCR IVD kits. Also, a statistically significant number of clinical samples should be used for the evaluation.

The sample type for respiratory virus detection is usually nasopharyngeal/oropharyngeal swab. If a kit claims to detect these viruses across several sample types, attempt should be made to evaluate the assay across all the sample types. In case all the sample types mentioned in the IFU are not available with the lab, the performance evaluation report should clearly mention the sample type against which the kit is evaluated. There should be no ambiguity about the type of sample used for evaluation.

4. Nucleic acid extraction

Nucleic acid extraction should be performed using standard techniques. If the manufacturer of the index test recommends a specific nucleic acid extraction kit, it needs to be provided by the manufacturer if the evaluation lab is unable to procure the same.

**Caution is advised in the selection of a nucleic acid extraction kit since the target pathogens comprise both RNA and DNA viruses.*

5. Real-Time PCR System

PCR should be performed using IVD-approved machines. If any equipment(s) is specified in the IFU of the index test, it should be used for the evaluation, and it should be provided by the manufacturer if not available within the lab's IVD evaluation scope.

Real-time closed systems/devices awaiting evaluation should be provided by the manufacturer along with all necessary components, supplies and reagents.

6. Internal control/Extraction control

The test under evaluation (index test) must have an internal control (housekeeping gene), with or without an extraction control (nucleic acid added before extraction to a sample).

7. Reference assay:

The following points are to be noted:

i. A WHO Pre-Qualified/ US FDA/ ATAGI Australia/ PMDA Japan approved single plex (for a particular target pathogen) or multiplex real-time PCR assay/ ICMR-NIV Pune in-house single plex (for a particular target pathogen) or multiplex Real Time PCR Assay should be used as the reference assay.
ii. Since the list of target pathogens is extensive, a combination of single plex and/or multiplex assays may be used as the reference assay(s), as long as these reference assays satisfy the criteria outlined in point 7(i).

All samples positive for a particular pathogen should be confirmed positive by the reference assay.

All samples negative for a particular pathogen should be confirmed negative by the reference assay.

8. **Sample size for performance evaluation:** The 2009 FDA guidance document "*Respiratory Viral Panel Multiplex Nucleic Acid Assay - Class II Special Controls Guidance for Industry and FDA Staff*", recommends including a sufficient number of prospectively collected samples for each specimen type to generate a result with at least 90% sensitivity with a lower bound of the two-sided 95% confidence interval (CI) greater than 80, and demonstrate specificity with a lower bound of the two-sided 95% CI greater than 90%. In accordance with these guidelines and for feasibility of evaluation of these extensive multiplex panels, sample size for each pathogen is calculated assuming ≥90% sensitivity and specificity of the index test, 95% confidence level, absolute precision of 7.5%, and ≤5% invalid test rate. A minimum of 65 positive clinical samples (rounded to 70) and a minimum of 65 negative clinical samples for each target pathogen are required for performance evaluation of the assay. However, 120 negative samples are recommended per pathogen to account for an extensive cross reactivity panel. Sample sizes are calculated using the formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

The details of sample requirement are outlined in Table 2.

Table 2: No. of samples required for performance evaluation:

Pathogen	Minimum no. of positive samples needed per pathogen	Minimum no. of negative samples recommended per pathogen
1. Influenza virus A*	70	120
2. Influenza virus B*	70	120
3. SARS Coronavirus-2	70	120

4. Respiratory syncytial virus*	70	120
5. Adenovirus*	70	120
6. Human Respirovirus 1 and Human Respirovirus 3 and Human Rubulavirus 2 and Human Rubulavirus 4*	70	120
7. Human metapneumovirus *	70	120
8. Measles virus	70	120
9. Rhinovirus**	70	120
10. Human Bocavirus	70	120
11. Enterovirus**	70	120
12. Cytomegalovirus	70	120

***If a kit claims to differentiate between virus types/subtypes, please use minimum 70 positive samples and minimum 120 negative samples for each virus type/subtype. If such type/subtype specific samples are not available (only for predicate device) or if the kit does not claim to differentiate between pathogen types/subtypes, and the kit is evaluated against the pathogen as a whole, the reports should be issued with a disclaimer that performance characteristics against pathogen types/subtypes have not been evaluated separately. However, in such a scenario, the evaluating centre should try to include all types/subtypes of the pathogen in the evaluation panel (even if the numbers are not statistically significant for each pathogen type).**

****If clinical samples positive separately for Rhinovirus/Enterovirus are not available (only for predicate device), or if the kit does not differentiate between Enteroviruses and Rhinoviruses, please use minimum 70 samples positive for Rhinovirus/Enterovirus in the positive sample panel and issue the reports with a disclaimer that performance characteristics against Rhinovirus/Enterovirus have not been evaluated separately.**

Influenza virus, SARS Coronavirus 2, Respiratory Syncytial Virus and Human Metapneumovirus positive samples used for evaluation should have been collected within the past 1 year.

Notes for Table 2:

1. Samples positive for currently circulating virus strains should be used in the positive sample panel, with representation from all virus types/subtypes.
2. Sample positive for a particular virus type and negative for the target pathogen being considered may be used in the negative sample panel for the target pathogen, e.g.: a sample positive for SARS-CoV-2 may be used as a negative sample for RSV.

9. Sample panel composition:

A. Human samples

A.1 Positive samples for each pathogen/ type or subtype of pathogen (Minimum n=70): Clinical samples positive by the reference real-time PCR assay should be included, as per the following criteria

A.1.1 Strong positive (Ct value <25) = 20 samples

A.1.2. Moderate positive (Ct value between 25-30) = 25 samples

A.1.3 Weak positive (Ct value >30-36) = 25 samples

A.2 Negative samples for each pathogen/ type or subtype of pathogen (Minimum n=120): All negative samples should be negative for the target pathogen/ its type or subtype by the reference real-time PCR assay. Distribution of the negative samples should be as follows:

A.2.1 NP/OP swab from individuals with respiratory infection that are negative for the target pathogen/its type or subtype = 35 samples **

A.2.2 NP/OP swab from apparently healthy individuals with no respiratory symptoms = 23 samples **

A.2.3 Cross reactivity panel (Table 3): Samples negative for the target pathogen but positive for other common respiratory viruses = 62 samples ***

Archived frozen sample aliquots if used for the evaluation, should not be thawed more than once.

*** If samples are available with the evaluating lab that satisfy these criteria and are negative for all the pathogens targeted by the kit, the same samples may be included in the negative sample panel for all target pathogens to prevent wastage of resources.*

**** Same positive samples may be included in the cross-reactivity panel of several target pathogens to prevent wastage of resources e.g.: the same Influenza A virus positive sample may be included in the cross-reactivity panel for RSV, Human Metapneumovirus, SARS-CoV-2 etc.*

Table 3: Cross reactivity panel for performance evaluation of multiplex respiratory virus real time PCR kit

Target Pathogen	Virus-wise no. of samples needed for cross reactivity analysis														Total no. of cross reactive samples per pathogen
	Influenza virus A *	Influenza virus B*	SARS Coronavirus-2 *	Respiratory syncytial virus *	Adenovirus @	Human Respiroviruses 1 and 3 , Human Rubulaviruses 2 and 4 #	Human metapneumovirus @	Measles virus *	Rhinovirus @ $	Human Bocavirus	Enterovirus $	Cytomegalovirus	Seasonal coronaviruses*	Rubella	
1. Influenza virus A	0	5	5	5	5	5	5	5	5	5	5	5	5	2	62
2. Influenza virus B	5	0	5	5	5	5	5	5	5	5	5	5	5	2	62
3. SARS Coronavirus-2	5	5	0	5	5	5	5	5	5	5	5	5	5	2	62
4. Respiratory syncytial virus	5	5	5	0	5	5	5	5	5	5	5	5	5	2	62
5. Adenovirus	5	5	5	5	0	5	5	5	5	5	5	5	5	2	62
6. Human Respiroviruses 1 and 3, Human Rubulaviruses 2 and 4	5	5	5	5	5	0	5	5	5	5	5	5	5	2	62
7. Human metapneumovirus	5	5	5	5	5	5	0	5	5	5	5	5	5	2	62
8. Measles virus	5	5	5	5	5	5	5	0	5	5	5	5	5	2	62
9. Rhinovirus	5	5	5	5	5	5	5	5	0	5	5	5	5	2	62
10. Human Bocavirus	5	5	5	5	5	5	5	5	5	0	5	5	5	2	62
11. Enterovirus	5	5	5	5	5	5	5	5	5	5	0	5	5	2	62
12. Cytomegalovirus	5	5	5	5	5	5	5	5	5	5	5	0	5	2	62

*Include all currently circulating strains/types/subtypes
@It is desirable to have representation from all types of the pathogen, since even approved assays may not always differentiate between pathogen types.
Include at least 1 of each

$ If clinical samples positive separately for Rhinovirus/Enterovirus are not available, please use total 10 samples positive for Rhinovirus/Enterovirus in the cross-reactivity panel for remaining pathogens. • Can use lower respiratory tract specimen **If a kit claims to differentiate between virus types/subtypes, please use 5 positive samples for each virus type in the cross reactivity panel for other target pathogens. If such type specific samples are not available and the kit is evaluated against the pathogen as a whole, it should be clearly mentioned in the report.**
If available, samples positive for relevant bacterial pathogens and other relevant viruses (with which majority of the population is likely to be infected), should also be included in the cross-reactivity panel. ***Influenza virus, SARS Coronavirus 2, Respiratory Syncytial Virus and Human Metapneumovirus positive samples used for evaluation should have been collected within the past 1 year.***

B. Contrived samples:

Contrived positive and negative samples may be used for evaluation in case of paucity/unavailability of human clinical samples. Positive contrived samples should be positive and negative contrived samples should be negative for the target pathogen/type/subtype using the reference assay. The number and distribution of positive and negative samples, including the cross reactivity panel, should remain the same.

Contrived positive samples (as part of positive sample panel/ cross-reactivity panel) should be prepared by spiking a sample matrix negative for the pathogen with a pathogen-infected cell line, genomic DNA plasmids or RNA transcripts.

It is recommended to demonstrate equivalence between contrived and clinical specimens. Serial dilutions of clinical sample and serial dilutions of contrived sample with targeted levels of analyte should be compared for demonstrating equivalence.

10. Evaluation method:

The index test and the reference assay should be run simultaneously on the sample panel, and results should be recorded.

11. Interpretation of results:

Reference test and index test results will be interpreted as per kit IFU.

12. Resolution of discrepant results:

True positive samples: These are samples positive by reference assay and index test.

True negative samples: These are samples negative by reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

13. Test reproducibility

A. Sample size for lot-to-lot reproducibility

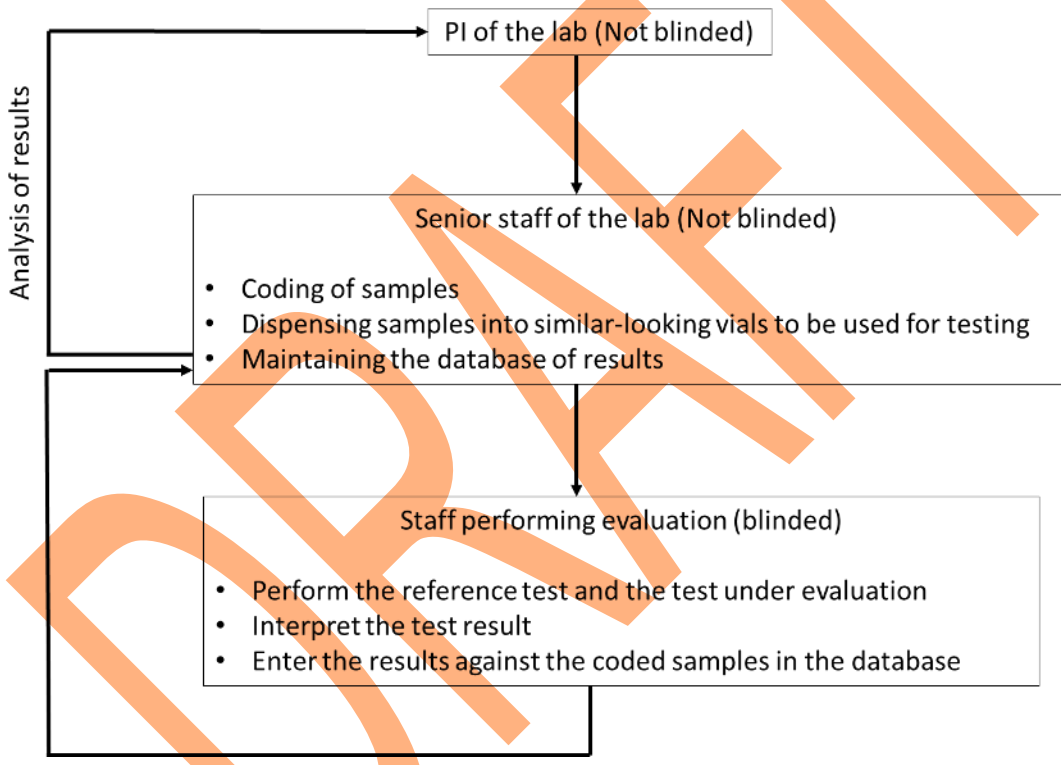
Three lots of an assay should be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples for each pathogen/type of pathogen as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples for each pathogen/type of pathogen (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples for each pathogen/type of pathogen (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- There should be no lot-to-lot variability.

14. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



15. Acceptance Criteria

Expected sensitivity for each pathogen/type/subtype: ≥90%

Expected specificity for each pathogen/type/subtype: ≥95%

Cross reactivity with other viruses as outlined in the negative sample panel: Nil

Invalid test rate: ≤5%

16. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VII. References:

1. Food and Drug Administration. Respiratory Viral Panel Multiplex Nucleic Acid Assay - Class II Special Controls Guidance for Industry and FDA Staff. Available at: https://www.fda.gov/medical-devices/guidance-documents-medical-devices-and-radiation-emitting-products/respiratory-viral-panel-multiplex-nucleic-acid-assay-class-ii-special-controls-guidance-industry-and [Accessed on 22nd January, 2025].
2. Food and Drug Administration. 510(k) Substantial Equivalence Determination Decision Summary, Biofire Diagnostics LLC, FilmArray Pneumonia Panel. Available at: https://www.accessdata.fda.gov/cdrh_docs/reviews/K180966.pdf [Accessed on 19th January 2025]
3. Food and Drug Administration: Testing for Human Metapneumovirus (hMPV) Using Nucleic Acid Assays - Class II Special Controls Guidance for Industry and FDA Staff. 2009. Available at: https://www.fda.gov/medical-devices/guidance-documents-medical-devices-and-radiation-emitting-products/testing-human-metapneumovirus-hmpv-using-nucleic-acid-assays-class-ii-special-controls-guidance#3 [Accessed on January 11, 2025]

VIII. Performance evaluation report format

PERFORMANCE EVALUATION REPORT FOR MULTIPLEX RESPIRATORY VIRUS REAL-TIME PCR KITS

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Pathogens detected by the assay		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: clinical/spiked, strong, moderate, weak)	
	Negative samples (provide details: clinical/spiked, including cross reactivity panel)	

Results: Tables 1 and 2 should be made for each pathogen/type of pathogen targeted by the kit under evaluation

Table 1: 2x2 table for sensitivity and specificity calculation (prepare 1 table for each target pathogen /type/ subtype)

		Reference assay …….…… (name)		
		Positive	Negative	Total
Name of …….. virus real-time PCR	Positive			
	Negative			
	Total			

Table 2: Sensitivity and specificity

	Estimate (%)	95% CI
Sensitivity		
Specificity		

- Details of cross reactivity with other viruses in the cross-reactivity panel:
- **Conclusions:**
  - Sensitivity, specificity
  - Cross reactivity
  - Invalid test rate
  - Performance: **Satisfactory / Not satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting using kits provided by the manufacturer from the batch mentioned above using ..... sample. Results should not be extrapolated to other sample types.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note:

This report is exclusively for Human Metapneumovirus…….. Kit (Lot No……) manufactured by …… (supplied by ……..)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal …….

**********End of the Report**********

Annexure-1: Information on Operational and Test Performance Characteristics Required from Manufacturers

1. The manufacturer should provide the following details about the IVD:
2. Instructions for Use
3. Scope of the IVD:
4. Pathogens/type/subtype of pathogens targeted by the kit
5. Intended Use Statement
6. Principle of the assay
7. Intended testing population (cases of ARI/ILI/SARI)
8. Intended user (laboratory professional and/or health care worker at point-of-care)
9. Lot/batch No.
10. Date of manufacture
11. Date of Expiry
12. Information on operational Characteristics
    - i. Configuration of the kit/device
    - ii. Requirement of any additional equipment, device
    - iii. Requirement of any additional reagents
    - iv. Operation conditions
    - v. Storage and stability before and after opening
    - vi. Internal control provided or not
    - vii. Quality control and batch testing data
    - viii. Biosafety aspects- waste disposal requirements
11. Information on Test Performance Characteristics
    - i. Type of sample-NP/OP swab, other respiratory specimen
    - ii. Volume of sample
    - iii. Any specific sample NOT to be tested
    - iv. Any additional sample processing required
    - v. Any additional device/consumable like sample transfer device, pipette, tube, etc required
    - vi. Name of analyte to be detected

vii. Pathogens targeted by the kit

viii. Time taken for testing

ix. Time for result reading and interpretation

x. Manual or automated(equipment)reading

xi. Limit of detection

xii. Diagnostic sensitivity

xiii. Diagnostic specificity

xiv. Stability and reproducibility

xv. Training required for testing

xvi. If yes, duration

xvii. Details of Cut-off and /or Equivocal Zone for interpretation of test

xviii. Interpretation of invalid and indeterminate results to be provided

xix. It is recommended to provide data demonstrating the precision

xx. Limit of detection

*Please mention "Not applicable" against sections not pertaining to the kit.

**********End of the Document**********

STANDARD PERFORMANCE EVALUATION PROTOCOLS

DRAFT FOR STAKEHOLDER COMMENTS

DENGUE IgG BASED ASSAYS

ICMR-CDSCO/IVD/GD/PROTOCOLS/10/2025

AUGUST, 2025

New Delhi, India

Table of Contents

Performance evaluation protocol for Dengue IgG RDT kits

I. Background:

CDSCO/ICMR, New Delhi, have aimed at facilitating the availability of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

II. Purpose:

To evaluate the performance characteristics of Dengue IgG RDT kits in the diagnosis of primary and secondary dengue infections using irreversibly de-identified leftover archived clinical samples.

III. Requirements:

a) Supply of kits under evaluation (Along with batch/lot No. Expiry & required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.

b) Evaluation sites/laboratories (With required equipment)

c) Reference test kits

d) Characterised Evaluation panel

e) Laboratory supplies

IV. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. **Study design/type**: Diagnostic accuracy study using irreversibly de-identified archived/ spiked leftover clinical samples
2. **Preparation of Evaluation sites/laboratories:**
   Identified IVD kit evaluation laboratories should establish their proficiency through ALL of the following:

A. Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO: 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

B. Staff training: All the staff involved in IVD kit evaluation should undergo hands on training and competency testing on following

- Preparation & characterization of kit evaluation panel
- Handling of Dengue IgG Rapid IVD kits received for performance evaluation (Verification/Storage/Unpacking etc).
- Testing, interpreting, recording of results & reporting
- Data handling, data safety & confidentiality

3. Preparation of Dengue IgG Rapid IVD kit evaluation panel

Well characterised Dengue IVD kit evaluation panel is a critical requirement for performance evaluation of IVD kits. Hence statistically significant number of sera samples should be collected from Dengue NS1/PCR/IgM confirmed cases. Further characterised for Dengue IgG positivity by using approved reference kits having high sensitivity and specificity.

Dengue IgG performance evaluation panel need to be tested again by the reference assays at the time of evaluating a particular index test to confirm the positive and negative status of the samples.

4. Reference assay:

Positive and negative samples should be characterized using composite reference standard of Dengue IgG AND one additional marker of Dengue (NS1 or IgM or PCR). The following kits should be used for characterization of the sample panel:

- *Panbio Dengue IgG capture ELISA kit*
- *WHO Pre-Qualified/ US-FDA/ ATAGI Australia/ PMDA Japan approved Dengue IgM ELISA kit*
- *NS1 antigen status to be assessed using WHO Pre-Qualified/ US-FDA/ ATAGI Australia/ PMDA Japan approved NS1 ELISA kit*
- *Serotype status to be assessed using a combination of CDC/NIV real-time PCR serotyping protocols.*

5. Sample size for performance evaluation:

Sample sizes of positive and negative samples and sample panel composition against different values of sensitivity and specificity are provided in Tables 1 and 2. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5% using the following formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity.

Positive samples: The panel of positive samples should include samples positive for IgG by the reference assay. The samples should also be positive for either dengue NS1 antigen or dengue IgM antibodies.

Negative samples: Samples which are negative by reference dengue IgG test should form the negative sample panel.

Table 1. Sample sizes and panel composition of positive dengue IgG samples for different values of sensitivity claimed by the manufacturer.

Sensitivity	*Calculated sample size*	*Minimum no. of Positive Samples required [Sample size rounded off] #*	*Sample Panel Composition*
99%	16	20	Strong Positive: 6 Moderate Positive: 7 Weak Positive: 7

95%	77	80	Strong Positive: 24 Moderate Positive: 28 Weak Positive: 28
90%	145	150	Strong Positive: 44 Moderate Positive: 53 Weak Positive: 53
85%	206	210	Strong Positive: 62 Moderate Positive: 74 Weak Positive: 74
80%	258	260	Strong Positive: 78 Moderate Positive: 91 Weak Positive: 91
The samples need to be classified as strong, moderate and weak positives based on ELISA units of the reference assay. *#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.*			

Table 2. Sample sizes and panel composition of negative dengue IgG samples for different values of specificity claimed by the manufacturer.

Specificity	*Calculated sample size*	*Minimum no. of Negative Samples required [Sample size rounded off] #*	*Sample Panel Composition*
99%#	16	20	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **7** 2.Acute febrile illness cases: **8** • Chikungunya positive samples:2 • Dengue (NS1 & IgM & IgG & PCR) negative samples:6 3.Samples from other flavivirus disease cases (cross-reactive panel): **3** • Japanese Encephalitis IgM/IgG positive: 1 @ • West Nile Virus IgM/IgG positive: 1 * • Zika Virus IgM/IgG positive: 1 * 4. [a]Healthy subjects from endemic regions: **2**
95%	77	80	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **27** 2.Acute febrile illness cases: **32**

			• Chikungunya positive samples:8 • Dengue (NS1 & IgM & IgG & PCR) negative samples:24 3.Samples from other flavivirus disease cases(cross-reactive panel): **9** • Japanese Encephalitis IgM/IgG positive: 3 @ • West Nile Virus IgM/IgG positive: 3 * • Zika Virus IgM/IgG positive: 3 * 4. [a]Healthy subjects from endemic regions: **12**
90%	145	150	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **50** 2.Acute febrile illness cases: **60** • Chikungunya positive samples:15 • Dengue (NS1 & IgM & IgG & PCR) negative samples:45 3.Samples from other flavivirus disease cases(cross-reactive panel): **15** • Japanese Encephalitis IgM/IgG positive: 5 @ • West Nile Virus IgM/IgG positive: 5 * • Zika Virus IgM/IgG positive: 5 * 4. [a]Healthy subjects from endemic regions: **25**
85%	206	210	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **70** 2.Acute febrile illness cases: **84** • Chikungunya positive samples:21 • Dengue (NS1 & IgM & IgG & PCR) negative samples:63 3.Samples from other flavivirus disease cases(cross-reactive panel): **21** • Japanese Encephalitis IgM/IgG positive: 7 @ • West Nile Virus IgM/IgG positive: 7 * • Zika Virus IgM/IgG positive: 7 * 4. [a]Healthy subjects from endemic regions: **35**
80%	258	260	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **85** 2.Acute febrile illness cases: **104** • Chikungunya positive samples:26 • Dengue (NS1 & IgM & IgG & PCR) negative samples:78 3.Samples from other flavivirus disease cases(cross-reactive panel): **27** • Japanese Encephalitis IgM/IgG positive: 9 @ • West Nile Virus IgM/IgG positive: 9 * • Zika Virus IgM/IgG positive: 9 * 4. [a]Healthy subjects from endemic regions: **44**

[a] Samples from healthy subjects from endemic regions negative for all dengue markers (NS1, IgM, IgG, RNA)

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Cross reactivity panel is arranged in descending order of priority.
The pathogens marked @ are essentially to be tested.
*It is recommended to test for all pathogens listed in the cross-reactivity panel. However, if there is an acute shortfall or non-availability of clinical samples, one may consider reducing only the pathogens of lower priority marked by * , while ensuring that the actual numbers of cross-reactive sample panel remain the same by compensating with the available "essentially to be tested" samples.*

Note: If IgM/IgG positive samples for cross reactive flaviviruses are not available, commercially available IgM/IgG sera panel for different viruses can be procured and used to test cross reactivity.

6. Evaluation method:

The index test and the reference assay should be run simultaneously on the sample panel, and results should be recorded.

7. Interpretation of results:

Reference test and index test results will be interpreted as per kit IFU.

8. Resolution of discrepant results:

True positive samples: These are samples positive by reference assay and index test.
True negative samples: These are samples negative by reference assay and index test.
False positive samples: These are samples negative by reference assay and positive by index test.
False negative samples: These are samples positive by reference assay and negative by index test.

9. Test reproducibility

A. Sample size for lot-to-lot reproducibility

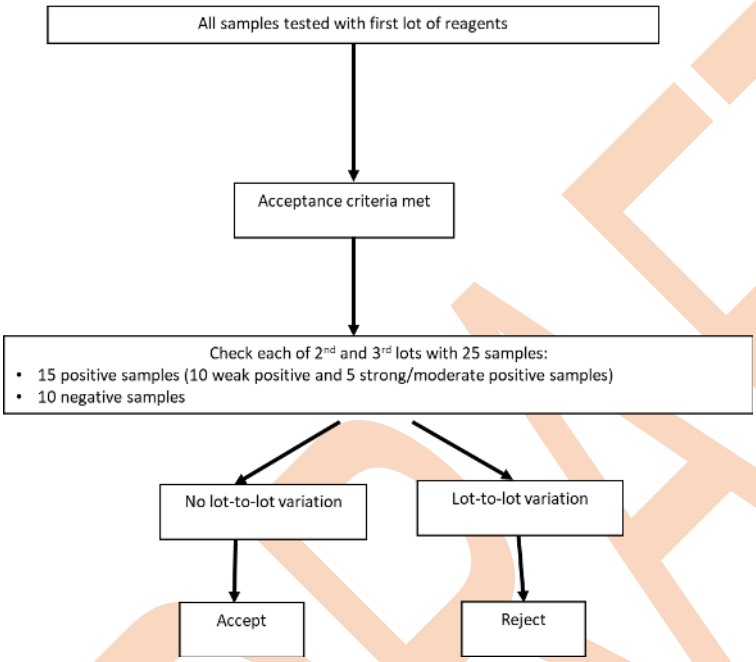
Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.

- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

Fig.1: Sample size for Lot-to-lot reproducibility



B. Sample size for reader-to-reader reproducibility

For reader-to-reader reproducibility, sample size should be 25 (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

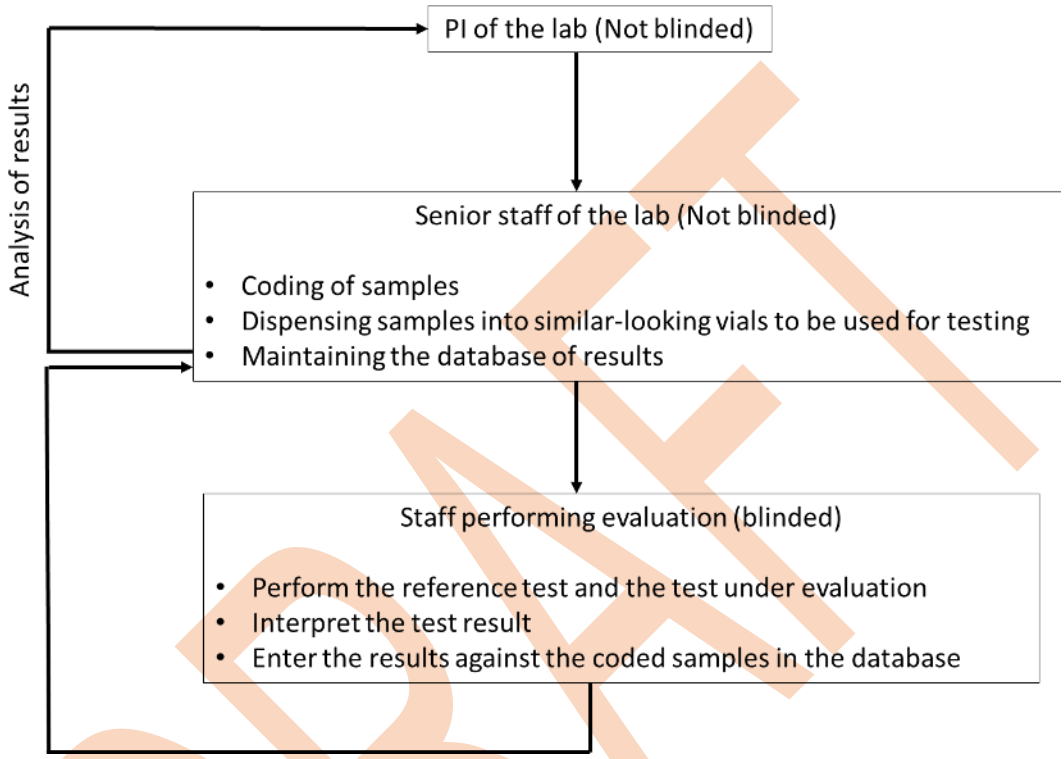
Two operators will be reading the test results independently as per manufacturer's instruction. Agreement should be 100% between the operators.

10. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of

results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



11. Acceptance Criteria

Expected sensitivity: ≥80%

Expected specificity: ≥90%

Cross reactivity: Nil

Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥260 positive samples and ≥150 negative samples should be used for evaluation.

12. Publication Rights:

The PI (s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Vazquez S, Hafner G, Ruiz D, Calzada N, Guzman MG. Evaluation of immunoglobulin M and G capture enzyme-linked immunosorbent assay Panbio kits for diagnostic dengue infections. J Clin Virol. 2007 Jul;39(3):194-8. doi: 10.1016/j.jcv.2007.04.003..
2. WHO, Evaluation of commercially available anti-Dengue virus immunoglobulin M tests. (Diagnostics evaluation series, 3). ISBN 978 92 4 159775 3.
3. Central Drugs Standard Control Organization. Guidance on Performance Evaluation of In-vitro Diagnostic Medical Devices. 2018. Available at: https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/medical device/guidanceperformanceivd.pdf
4. Central Drugs Standard Control Organization. In-Vitro Diagnostic (IVD) Medical Devices Frequently Asked Questions. 2022. Available at: https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/IVD/FAQs/CDSCO-IVD-FAQ-03-2022-.pdf
5. U.S. Food and Drug Administration. Dengue Virus Serological Reagents - Class II Special Controls Guideline for Industry and Food and Drug Administration Staff. 2014. Available at: https://www.fda.gov/medical-devices/guidance-documents-medical-devices-and-radiation-emitting-products/Dengue-virus-serological-reagents-class-ii-special-controls-guideline-industry-and-food-and-drug
6. World Health Organization. Technical Guidance Series (TGS) for WHO Prequalification – Diagnostic Assessment TGS-3. 2017. Available at: https://iris.who.int/bitstream/handle/10665/258985/WHO-EMP-RHT-PQT-TGS3-2017.03-eng.pdf;sequence=1

***The validation protocols need to be revisited after introduction of Dengue vaccines and the acceptance criteria needs revisiting every year so as to enable the availability of best diagnostic kits.**

VII. Performance evaluation report format

PERFORMANCE EVALUATION REPORT FOR DENGUE IgG RDT KIT

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	
	Negative samples (provide details: clinical/spiked, including cross reactivity panel)	

Results:

		Reference assay …….…… (name)		
		Positive	Negative	Total
Name of Dengue IgG antibody - based RDT kit	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

Conclusions:

- Sensitivity, specificity
- Cross-reactivity:
- Invalid test rate:

- Performance: **Satisfactory / Not satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting using kits provided by the manufacturer from the batch mentioned above using ..... sample. Results should not be extrapolated to other sample types.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for ……Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal …….

**********End of the Report**********

Performance evaluation protocol for Dengue IgM and IgG RDT combo kits

I. Background:

CDSCO/ICMR, New Delhi, have aimed at facilitating the availability of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

II. Purpose:

To evaluate the performance characteristics of Dengue IgM and IgG RDT combo kits in the diagnosis of dengue and discriminating primary and secondary dengue infections using irreversibly de-identified leftover archived clinical samples.

III. Requirements:

f) Supply of kits under evaluation (Along with batch/lot No. Expiry & required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.

g) Evaluation sites/laboratories (With required equipment)

h) Reference test kits

i) Characterised Evaluation panel

j) Laboratory supplies

IV. Ethical approvals:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. **Study design/type**: Diagnostic accuracy study using irreversibly de-identified archived/ spiked leftover clinical samples
2. **Preparation of Evaluation sites/laboratories:**
   Identified IVD kit evaluation laboratories should establish their proficiency through ALL of the following:

A.Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO: 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

B.Staff training: All the staff involved in IVD kit evaluation should undergo hands on training and competency testing on following

- Preparation & characterization of kit evaluation panel
- Handling of Dengue IgM and IgG Rapid IVD kits received for performance evaluation (Verification/Storage/Unpacking etc).
- Testing, interpreting, recording of results & reporting
- Data handling, data safety & confidentiality

3. Preparation of Dengue IgM and IgG Rapid IVD kit evaluation panel

Well characterised Dengue IVD kit evaluation panel is a critical requirement for performance evaluation of IVD kits. Hence statistically significant number of sera samples should be collected from Dengue NS1/PCR/IgM confirmed cases. Further characterised for Dengue IgG positivity by using approved reference kits having high sensitivity and specificity.

Dengue IgG performance evaluation panel need to be tested again by the reference assays at the time of evaluating a particular index test to confirm the positive and negative status of the samples.

4. Reference assay:

Positive and negative samples should be characterized using reference standard for Dengue IgG (and one additional marker of Dengue - NS1 or IgM or PCR) AND IgM. The following kits should be used for characterization of the sample panel:

- *Panbio Dengue IgG capture ELISA kit*
- *WHO Pre-Qualified/ US-FDA/ ATAGI Australia/ PMDA Japan approved Dengue IgM ELISA kit*
- *NS1 antigen status to be assessed using WHO Pre-Qualified/ US-FDA/ ATAGI Australia/ PMDA Japan approved NS1 ELISA kit*
- *Serotype status to be assessed using a combination of CDC/NIV real-time PCR serotyping protocols.*

Sample size and sample panel composition: Sample sizes of positive and negative samples of Dengue against different values of sensitivity and specificity are provided in Tables 1 and 2. Sample sizes have been calculated assuming 95% level of significance, an absolute precision of 5%, and invalid test rate ≤5% using the following formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\ (1 - S_e)}{d^2 \text{ x } (1 - \text{IR})}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\ (1 - S_p)}{d^2 \text{ x } (1 - \text{IR})}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
- *IR is the invalid test rate*

Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity.

Positive samples: The samples should be positive for dengue IgM antibodies. The panel of positive samples should include 50% of samples positive for IgG by the reference assay. Samples should be representative of varying degrees of positivity:

Negative samples: These should include samples negative by all the reference assays (True negatives).

Table 1. Sample sizes and panel composition of positive Dengue samples for different values of sensitivity claimed by the manufacturer.

Sensitivity	*Calculated sample size*	*Minimum no. of Positive Samples required [Sample size rounded off for balanced allocation] #*	*Sample Panel Composition*
99%	16	20	1. 10 samples positive for Dengue IgM • Strong positive:3 • Moderate positive: 3

			• Weak positive: 4 2. 10 samples positive for both Dengue IgM and IgG • Strong positive IgG:3 • Moderate positive IgG: 3 • Weak positive IgG: 4
95%	77	80	40 samples positive for Dengue IgM • Strong positive:12 • Moderate positive: 14 • Weak positive: 14 40 samples positive for both Dengue IgM and IgG • Strong positive IgG:12 • Moderate positive IgG: 14 • Weak positive IgG: 14
90%	145	150	75 samples positive for Dengue IgM • Strong positive:23 • Moderate positive: 26 • Weak positive: 26 75 samples positive for both Dengue IgM and IgG • Strong positive IgG: 23 • Moderate positive IgG: 26 • Weak positive IgG: 26
85%	206	210	105 samples positive for Dengue IgM • Strong positive:31 • Moderate positive: 37 • Weak positive: 37 105 samples positive for both Dengue IgM and IgG • Strong positive IgG: 31 • Moderate positive IgG: 37 • Weak positive IgG: 37
80%	258	260	130 samples positive for Dengue IgM • Strong positive:38 • Moderate positive: 46 • Weak positive: 46

			130 samples positive for both Dengue IgM and IgG • Strong positive IgG: 38 • Moderate positive IgG: 46 • Weak positive IgG: 46

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 2. Sample sizes and panel composition of negative Dengue samples for different values of specificity claimed by the manufacturer.

Specificity	*Calculated sample size*	*Minimum no. of Negative Samples required [Sample size rounded off for balanced allocation] #*	*Sample Panel Composition*
99%	16	20	1.[a]Samples from acute febrile illness cases negative for dengue: **9** • Samples positive for chikungunya: 2 • Other Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR):7 2.Samples from other flavivirus disease cases (cross-reactive panel): **3** • Japanese Encephalitis IgM/IgG positive: 1@ • West Nile Virus IgM/IgG positive:1* • Zika Virus IgM/IgG positive: 1 * 3. [b]Healthy subjects from endemic regions: **8**
95%	77	80	1.[a]Samples from acute febrile illness cases negative for dengue: **44** • Samples positive for chikungunya: 8 • Other Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR):36 2.Samples from other flavivirus disease cases (cross-reactive panel): **6**

			• Japanese Encephalitis IgM/IgG positive: 2@ • West Nile Virus IgM/IgG positive:2* • Zika Virus IgM/IgG positive: 2 * 3. [b]Healthy subjects from endemic regions: **30**
90%	145	150	1.[a]Samples from acute febrile illness cases negative for dengue: **80** • Samples positive for chikungunya: 15 • Other Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR):65 2.Samples from other flavivirus disease cases (cross-reactive panel): **15** • Japanese Encephalitis IgM/IgG positive: 5 @ • West Nile Virus IgM/IgG positive:5* • Zika Virus IgM/IgG positive: 5* 3. [b]Healthy subjects from endemic regions: **55**
85%	206	210	1.[a]Samples from acute febrile illness cases negative for dengue: **110** • Samples positive for chikungunya: 21 • Other Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR):89 2.Samples from other flavivirus disease cases (cross-reactive panel): **24** • Japanese Encephalitis IgM/IgG positive: 8 @ • West Nile Virus IgM/IgG positive:8* • Zika Virus IgM/IgG positive: 8* 3. [b]Healthy subjects from endemic regions: **76**
80%	258	260	1.[a]Samples from acute febrile illness cases negative for dengue: **138**

			• Samples positive for chikungunya: 26 • Other Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR):112 2.Samples from other flavivirus disease cases (cross-reactive panel): **27** • Japanese Encephalitis IgM/IgG positive: 9 @ • West Nile Virus IgM/IgG positive:9* • Zika Virus IgM/IgG positive: 9* 3. [b]Healthy subjects from endemic regions: **95**

[a] Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR)
[b] Samples from healthy subjects from endemic regions negative for all Dengue markers (NS1, IgM, IgG, RNA)

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Cross reactivity panel is arranged in descending order of priority.
The pathogens marked @ are essentially to be tested.
*It is recommended to test for all pathogens listed in the cross reactivity panel. However, if there is an acute shortfall or non-availability of clinical samples, one may consider reducing only the pathogens of lower priority marked by * , while ensuring that the actual numbers of cross reactive sample panel remain the same by compensating with the available "essentially to be tested" samples.*

Note: If IgM/IgG positive samples for cross reactive flaviviruses are not available, commercially available IgM/IgG sera panel for different viruses can be procured and used to test cross reactivity.

5. Evaluation method:

The index test and the reference assay should be run simultaneously on the sample panel, and results should be recorded.

6. Resolution of discrepant results:

True positive samples: These are samples positive by reference assay and index test.
True negative samples: These are samples negative by reference assay and index test.

False positive samples: These are samples negative by reference assay and positive by index test.

False negative samples: These are samples positive by reference assay and negative by index test.

7. Test reproducibility

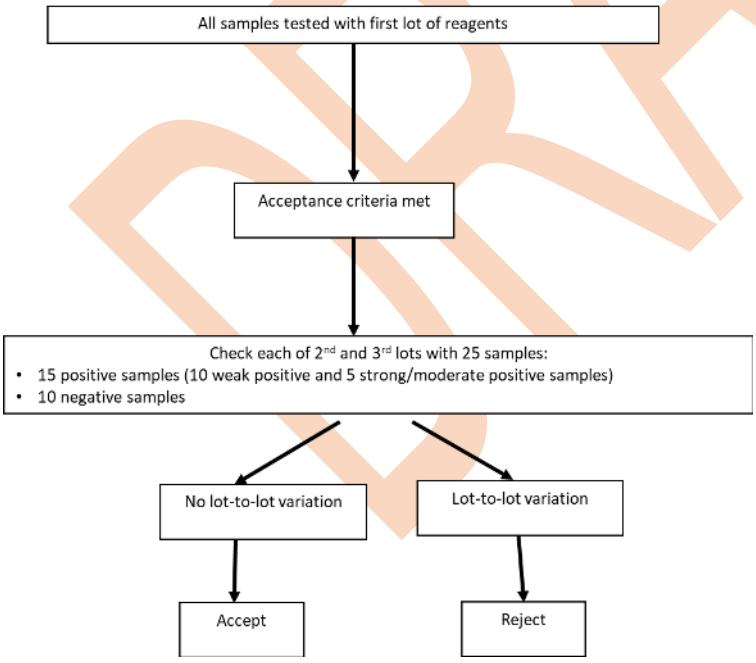
C. Sample size for lot-to-lot reproducibility

Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

Fig.1: Sample size for Lot-to-lot reproducibility



D. Sample size for reader-to-reader reproducibility

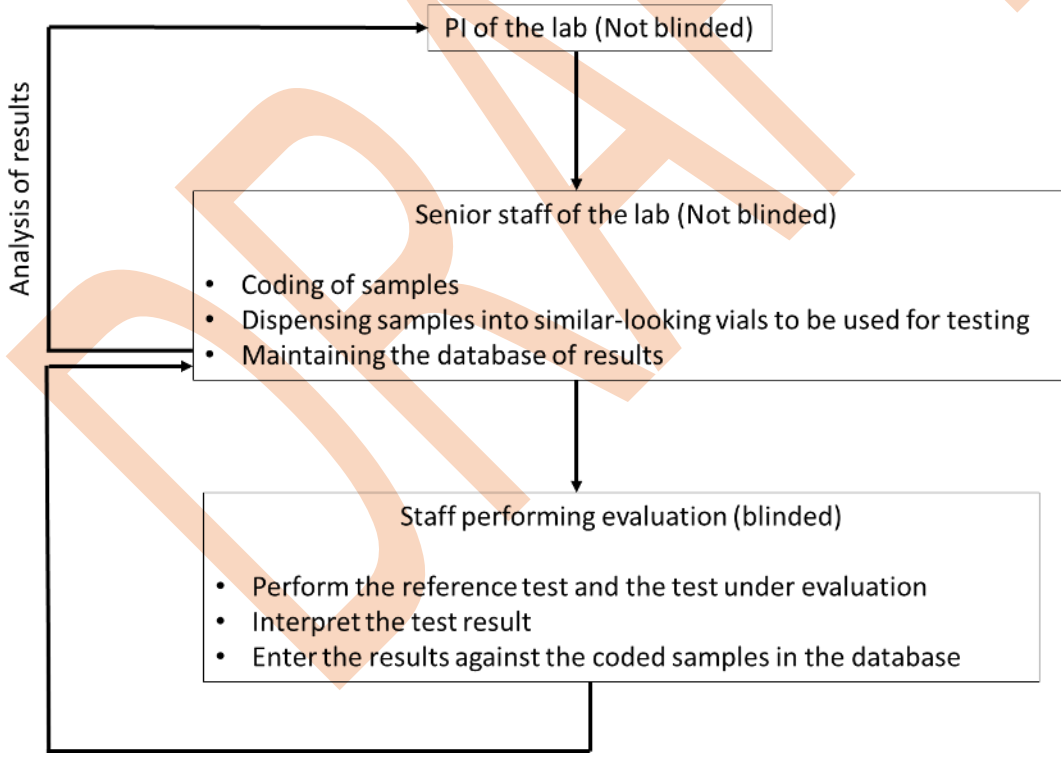
For reader-to-reader reproducibility, sample size should be 25 (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

Two operators will be reading the test results independently as per manufacturer's instruction. Agreement should be 100% between the operators.

8. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



9. Acceptance Criteria

Expected sensitivity for each analyte: ≥80%

Expected specificity for each analyte: ≥90%

Cross-reactivity: Nil

Invalid test rate: ≤5%

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥260 positive samples and ≥150 negative samples should be used for evaluation.

10. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Vazquez S, Hafner G, Ruiz D, Calzada N, Guzman MG. Evaluation of immunoglobulin M and G capture enzyme-linked immunosorbent assay Panbio kits for diagnostic dengue infections. J Clin Virol. 2007 Jul;39(3):194-8. doi: 10.1016/j.jcv.2007.04.003..
2. WHO, Evaluation of commercially available anti-Dengue virus immunoglobulin M tests. (Diagnostics evaluation series, 3). ISBN 978 92 4 159775 3.
3. Central Drugs Standard Control Organization. Guidance on Performance Evaluation of In-vitro Diagnostic Medical Devices. 2018. Available at: https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/medical device/guidanceperformanceivd.pdf
4. Central Drugs Standard Control Organization. In-Vitro Diagnostic (IVD) Medical Devices Frequently Asked Questions. 2022. Available at: https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/IVD/FAQs/CDSCO-IVD-FAQ-03-2022-.pdf
5. U.S. Food and Drug Administration. Dengue Virus Serological Reagents - Class II Special Controls Guideline for Industry and Food and Drug Administration Staff. 2014. Available at: https://www.fda.gov/medical-devices/guidance-documents-medical-devices-and-radiation-emitting-products/Dengue-virus-serological-reagents-class-ii-special-controls-guideline-industry-and-food-and-drug

6. World Health Organization. Technical Guidance Series (TGS) for WHO Prequalification – Diagnostic Assessment TGS-3. 2017. Available at: https://iris.who.int/bitstream/handle/10665/258985/WHO-EMP-RHT-PQT-TGS3-2017.03-eng.pdf;sequence=1

***The validation protocols need to be revisited after introduction of Dengue vaccines and the acceptance criteria needs revisiting every year so as to enable the availability of best diagnostic kits.**

VII. Performance evaluation report format

PERFORMANCE EVALUATION REPORT FOR DENGUE IgM and IgG COMBO RDT KIT

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	
	Negative samples (provide details:,clinical/spiked, including cross reactivity panel)	

Results for IgM:

		Reference assay …….…… (name)		
		Positive	Negative	Total
Name of Dengue antibody combo RDT kit	Positive			
	Negative			
	Total			

Results for IgG:

		Reference assay …….…… (name)		
		Positive	Negative	Total

Name of Dengue antibody combo RDT kit	Positive			
	Negative			
	Total			

- Details of cross reactivity with other flavivirus antibodies:
- Invalid test rate:

- **Conclusions:**
  - Sensitivity, specificity for dengue IgM:
  - Sensitivity, specificity for dengue IgG:
  - Performance:
    - **Satisfactory / Not satisfactory for Dengue IgM**
    - **Satisfactory / Not satisfactory for Dengue IgG**

(Sensitivity and specificity have been assessed in controlled lab setting using kits provided by the manufacturer from the batch mentioned above using ….. sample. Results should not be extrapolated to other sample types.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for …….. Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by ……

Signature of Director/ Director-In-charge …… Seal ……

**********End of the Report**********

Performance evaluation protocol for Dengue IgG ELISA kits

I. Background:

CDSCO/ICMR, New Delhi, have aimed at facilitating the availability of Quality-Assured Diagnostics kits appropriate for use in India. Hence the following guidelines shall establish the uniformity in performance evaluation of in-vitro diagnostic kits (IVD). The performance evaluation is to independently verify the manufacturer's claim regarding in-vitro diagnostic kit (IVD) performance.

II. Purpose:

To evaluate the performance characteristics of Dengue IgG ELISA kits in the diagnosis of Dengue infection using irreversibly de-identified leftover archived clinical samples.

III. Requirements:

1. Supply of kits under evaluation (Along with batch/lot No. Expiry & required details). If the kit to be evaluated works in a closed system format, the manufacturer needs to supply the required equipment.
2. Evaluation sites/laboratories (With required equipment)
3. Reference test kits
4. Characterised Evaluation panel
5. Laboratory supplies

IV. Ethical approval:

Performance evaluation activities using irreversibly de-identified leftover clinical samples are exempt from ethics approval as per ICMR's Guidance on Ethical Requirements for Laboratory Validation Testing, 2024.

Investigators are required to submit a self-declaration form, as outlined in the ICMR guidelines, to the institutional authorities and ethics committee for information.

V. Procedure:

1. **Study design/type**: Diagnostic accuracy study using irreversibly de-identified archived/ spiked leftover clinical samples
2. **Preparation of Evaluation sites/laboratories:**
   Identified IVD kit evaluation laboratories should establish their proficiency through ALL of the following:

A. Accreditation for at least one of the Quality management systems (accreditation for Testing Lab / Calibration Lab (ISO: 17025), Medical Lab (ISO: 15189), PT provider (ISO: 17043) or CDSCO approved Reference laboratory.

B. Staff training: All the staff involved in IVD kit evaluation should undergo hands on training and competency testing on following

- Preparation & characterization of kit evaluation panel
- Handling of Dengue IgG ELISA IVD kits received for performance evaluation (Verification/Storage/Unpacking etc).
- Testing, interpreting, recording of results & reporting
- Data handling, data safety & confidentiality

3. Preparation of Dengue IgG ELISA IVD kit evaluation panel

Well characterised Dengue IVD kit evaluation panel is a critical requirement for performance evaluation of IVD kits. Hence statistically significant number of sera samples should be collected from Dengue NS1/PCR/IgG confirmed cases. Further characterised for Dengue IgM positivity by using approved reference kits having high sensitivity and specificity.

Dengue IgG performance evaluation panel need to be tested again by the reference assays at the time of evaluating a particular index test to confirm the positive and negative status of the samples.

4. Reference assay:

Positive and negative samples should be characterized using composite reference standard of Dengue IgG AND one additional marker of Dengue (NS1 or IgM or PCR). The following kits should be used for characterization of the sample panel:

- *Panbio Dengue IgG capture ELISA kit*
- *WHO Pre-Qualified/ US-FDA/ ATAGI Australia/ PMDA Japan approved Dengue IgM ELISA kit*
- *NS1 antigen status to be assessed using WHO Pre-Qualified/ US-FDA/ ATAGI Australia/ PMDA Japan approved NS1 ELISA kit*
- *Serotype status to be assessed using a combination of CDC/NIV real-time PCR serotyping protocols.*

5. Sample size for performance evaluation:

Sample sizes of positive and negative samples and sample panel composition against different values of sensitivity and specificity are provided in Tables 1 and 2. Sample sizes have been calculated assuming 95% level of significance, and an absolute precision of 5% using the following formulae:

$$n_{se} \geq \frac{Z^2 \text{ x } S_e\,(1 - S_e)}{d^2}$$

$$n_{sp} \geq \frac{Z^2 \text{ x } S_p\,(1 - S_p)}{d^2}$$

- *n (se) is the minimum number of positive samples.*
- *n (sp) is the minimum number of negative samples.*
- *Z^2 is the critical value from the standard normal distribution corresponding to the desired confidence level (95% CI corresponds to Z^2 =1.96).*
- *Se is the predetermined sensitivity.*
- *Sp is the predetermined specificity.*
- *d is the predetermined marginal error (5%)*
-

Appropriate sample size has to be chosen from the tables according to the values of sensitivity and specificity being claimed by the manufacturer. If a claimed sensitivity/specificity is not present in the table, the manufacturer needs to consider the sample size associated with the largest sensitivity/specificity provided in the table that is smaller to the claimed value (that is, as per the next smaller value of the sensitivity/ specificity available in the table). For example, if a manufacturer claims a sensitivity of 93%, they are required to use a sample size mentioned against 90% sensitivity. Similarly, a claim of 87% specificity would require usage of the sample size outlined for 85% specificity.

Positive samples: The panel of positive samples should include samples positive for IgG by the reference assay. The samples should also be positive for either dengue NS1 antigen or dengue IgM antibodies. Samples should be representative of varying degrees of positivity:

Negative samples: These should include samples negative by the reference assays for dengue IgG.

Table 1. Sample sizes and panel composition of positive Dengue samples for different values of sensitivity claimed by the manufacturer.

Sensitivity	*Calculated sample size*	*Minimum no. of Positive Samples required [Sample size rounded off] #*	*Sample Panel Composition*
99%	15	20	Strong Positive: 6 Moderate Positive: 7 Weak Positive: 7
95%	73	80	Strong Positive: 24 Moderate Positive: 28

			Weak Positive: 28
90%	138	140	Strong Positive: 42 Moderate Positive: 49 Weak Positive: 49
85%	196	200	Strong Positive: 60 Moderate Positive: 70 Weak Positive: 70
80%	246	250	Strong Positive: 75 Moderate Positive: 87 Weak Positive: 88

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Table 2. Sample sizes and panel composition of negative Dengue samples for different values of specificity claimed by the manufacturer.

Specificity	*Calculated sample size*	*Minimum no. of Negative Samples required [Sample size rounded off]#*	*Sample Panel Composition*
99%	15	20	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **7** 2.Acute febrile illness cases: **8** • Chikungunya positive samples:2 • Dengue (NS1 & IgM & IgG & PCR) negative samples:6 3.Samples from other flavivirus disease cases (cross-reactive panel): **3** • Japanese Encephalitis IgM/IgG positive: 1 @ • West Nile Virus IgM/IgG positive: 1 * • Zika Virus IgM/IgG positive: 1 * 4. [a]Healthy subjects from endemic regions: **2**
95%	73	80	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **27** 2.Acute febrile illness cases: **32** • Chikungunya positive samples:8 • Dengue (NS1 & IgM & IgG & PCR) negative samples:24

			3.Samples from other flavivirus disease cases(cross-reactive panel): **9** • Japanese Encephalitis IgM/IgG positive: 3 @ • West Nile Virus IgM/IgG positive: 3 * • Zika Virus IgM/IgG positive: 3 * 4. [a]Healthy subjects from endemic regions: **12**
90%	138	140	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **45** 2.Acute febrile illness cases: **60** • Chikungunya positive samples:15 • Dengue (NS1 & IgM & IgG & PCR) negative samples:45 3.Samples from other flavivirus disease cases(cross-reactive panel): **15** • Japanese Encephalitis IgM/IgG positive: 5 @ • West Nile Virus IgM/IgG positive: 5 * • Zika Virus IgM/IgG positive: 5 * 4. [a]Healthy subjects from endemic regions: **20**
85%	196	200	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **65** 2.Acute febrile illness cases: **84** • Chikungunya positive samples:21 • Dengue (NS1 & IgM & IgG & PCR) negative samples:63 3.Samples from other flavivirus disease cases(cross-reactive panel): **21** • Japanese Encephalitis IgM/IgG positive: 7 @ • West Nile Virus IgM/IgG positive: 7 * • Zika Virus IgM/IgG positive: 7 * 4. [a]Healthy subjects from endemic regions: **30**
80%	246	250	1.Samples positive for dengue IgM/NS1/RNA but negative for IgG: **80** 2.Acute febrile illness cases: **104** • Chikungunya positive samples:26 • Dengue (NS1 & IgM & IgG & PCR) negative samples:78 3.Samples from other flavivirus disease cases(cross-reactive panel): **27** • Japanese Encephalitis IgM/IgG positive: 9 @ • West Nile Virus IgM/IgG positive: 9 * • Zika Virus IgM/IgG positive: 9 *

			4. [a]Healthy subjects from endemic regions: **39**

[a] Acute febrile cases negative for Dengue (NS1 & IgM & IgG & PCR)
[b] Samples from healthy subjects from endemic regions negative for all Dengue markers (NS1, IgM, IgG, RNA)

#It is recommended to calculate the sample size as per manufacturer's claims of sensitivity and specificity; however, a higher sample size is suggested to ensure adequate power of the study in case the kit falls short of claimed performance characteristics.

Cross reactivity panel is arranged in descending order of priority.
The pathogens marked @ are essentially to be tested.
*It is recommended to test for all pathogens listed in the cross reactivity panel. However, if there is an acute shortfall or non-availability of clinical samples, one may consider reducing only the pathogens of lower priority marked by * , while ensuring that the actual numbers of cross reactive sample panel remain the same by compensating with the available "essentially to be tested" samples.*

Note: If IgM/IgG positive samples for cross reactive flaviviruses are not available, commercially available IgM/IgG sera panel for different viruses can be procured and used to test cross reactivity.

6. Evaluation method:

The index test and the reference assay should be run simultaneously on the sample panel, and results should be recorded.

7. Interpretation of results:

Reference test and index test results will be interpreted as per kit IFU.

8. Resolution of discrepant results:

True positive samples: These are samples positive by reference assay and index test.
True negative samples: These are samples negative by reference assay and index test.
False positive samples: These are samples negative by reference assay and positive by index test.
False negative samples: These are samples positive by reference assay and negative by index test.

9. Test reproducibility

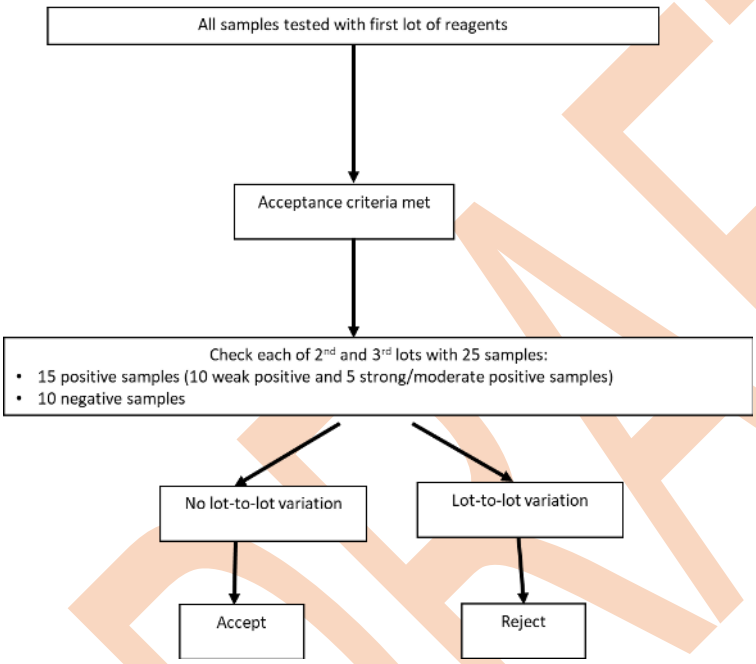
A. Sample size for lot-to-lot reproducibility

Three lots of an assay shall be evaluated. Sample size for lot-to-lot reproducibility should be as follows:

- First lot of the assay: should be tested on statistically significant number of positive and negative samples as calculated in the protocol.
- Second lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).
- Third lot of the assay: should be tested on 25 samples (15 positive samples comprising 10 low positive **AND** 5 moderate/high positive samples, and 10 negative samples).

Refer the flowchart below (Fig. 1):

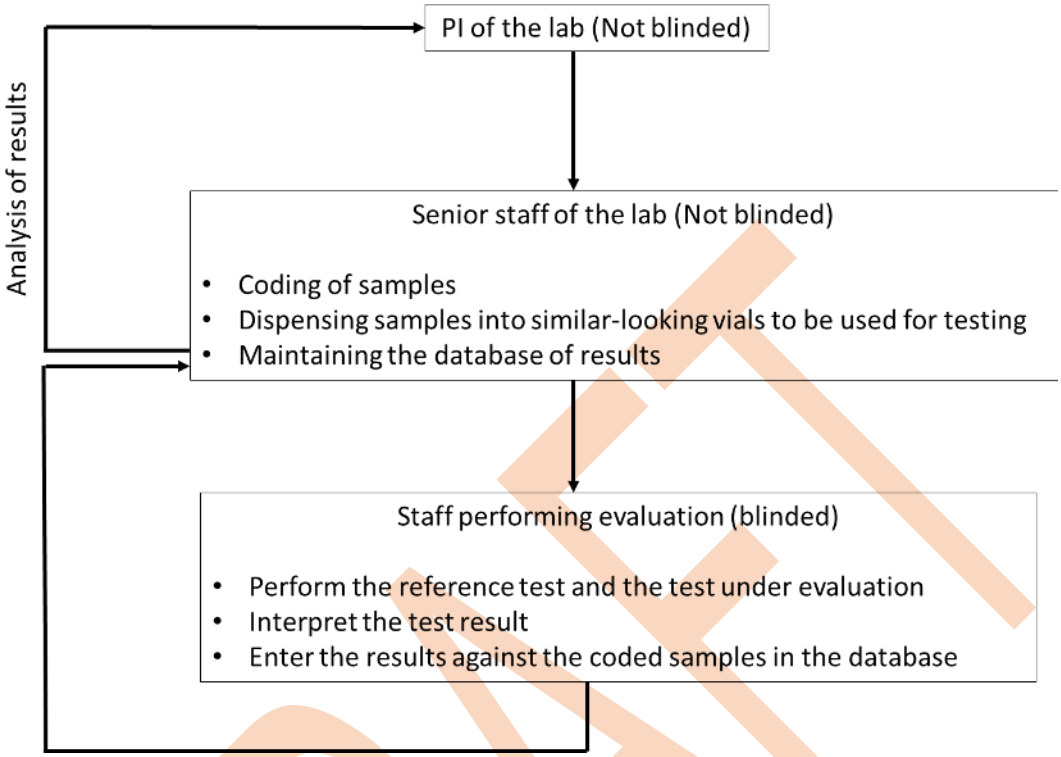
Fig.1: Sample size for Lot-to-lot reproducibility



10. Blinding of laboratory staff

To ensure rigor of the evaluation process, laboratory staff performing the evaluation should be blinded to the status of the clinical samples. The PI of the evaluation exercise should remain unblinded, i.e., privy to the status of the samples. Another senior laboratory staff selected by the PI may remain unblinded and carry out coding of samples and dispensing them into similar-looking vials to be used for testing, and maintaining the database of results. Staff performing the reference test and the test under evaluation, interpretation of the test result, and entering the results against the coded samples in the database, should remain blinded to the status of samples till the completion of evaluation. The data should be analyzed only by the PI of the evaluating lab. Refer to Fig. 2.

Fig.2: Blinding in evaluation exercise



11. Acceptance criteria

Expected sensitivity: ≥90%

Expected specificity: ≥95%

Cross-reactivity: Nil

To achieve at least the performance characteristics outlined in the acceptance criteria, ≥140 positive samples and ≥80 negative samples should be used for evaluation.

12. Publication Rights:

The PI(s) of the evaluating labs shall retain publication rights of the evaluation as lead author(s).

After following due procedure as defined in this document, once any kit is found to be Not of Standard Quality, thereafter, no request for repeat testing of the same kit will be acceptable.

Any request of re-validation from the same manufacturer for the same test type will only be entertained after a minimum of 3 months and only if a high-level technical summary of modifications or functional improvements to the kit design is submitted, without explicit disclosure of proprietary information.

Clinical samples are precious, therefore, repeat evaluation of a kit using the same/ different well-characterized sample panel at a different laboratory may be considered only for kits which claim high performance characteristics (sensitivity and specificity 95% and above), but which fail the performance evaluation by a margin of 5%.

VI. References:

1. Vazquez S, Hafner G, Ruiz D, Calzada N, Guzman MG. Evaluation of immunoglobulin M and G capture enzyme-linked immunosorbent assay Panbio kits for diagnostic dengue infections. J Clin Virol. 2007 Jul;39(3):194-8. doi: 10.1016/j.jcv.2007.04.003..
2. **WHO, Evaluation of commercially available anti-Dengue virus immunoglobulin M tests. (Diagnostics evaluation series, 3). ISBN 978 92 4 159775 3.**
3. Central Drugs Standard Control Organization. Guidance on Performance Evaluation of In-vitro Diagnostic Medical Devices. 2018. Available at: https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/medicaldevice/guidanceperformanceivd.pdf
4. Central Drugs Standard Control Organization. In-Vitro Diagnostic (IVD) Medical Devices Frequently Asked Questions. 2022. Available at: https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/IVD/FAQs/CDSCO-IVD-FAQ-03-2022-.pdf
5. U.S. Food and Drug Administration. Dengue Virus Serological Reagents - Class II Special Controls Guideline for Industry and Food and Drug Administration Staff. 2014. Available at: https://www.fda.gov/medical-devices/guidance-documents-medical-devices-and-radiation-emitting-products/Dengue-virus-serological-reagents-class-ii-special-controls-guideline-industry-and-food-and-drug
6. World Health Organization. Technical Guidance Series (TGS) for WHO Prequalification – Diagnostic Assessment TGS-3. 2017. Available at: https://iris.who.int/bitstream/handle/10665/258985/WHO-EMP-RHT-PQT-TGS3-2017.03-eng.pdf;sequence=1

***The validation protocols need to be revisited after introduction of Dengue vaccines and the acceptance criteria needs revisiting every year so as to enable the availability of best diagnostic kits.**

VII. Performance evaluation report format

PERFORMANCE EVALUATION REPORT FOR DENGUE IgG ELISA KIT

Name of the product (Brand /generic)		
Name and address of the legal manufacturer		
Name and address of the actual manufacturing site		
Name and address of the Importer		
Name of supplier: Manufacturer/Importer/Port office of CDSCO/State licensing Authority		
Lot No / Batch No.:		
Product Reference No/ Catalogue No		
Type of Assay		
Kit components		
Manufacturing Date		
Expiry Date		
Pack size (Number of tests per kit)		
Intended Use		
Number of Tests Received		
Regulatory Approval: Import license / Manufacturing license/ Test license License Number:Issue date: Valid Up to:		
Application No.		
Sample Panel	Sample type	
	Positive samples (provide details: strong, moderate, weak)	
	Negative samples (provide details: clinical/spiked, including cross reactivity panel)	

Results:

		Reference assay …….…… (name)		
		Positive	Negative	Total
Name of Dengue IgG antibody -based ELISA kit	Positive			
	Negative			
	Total			

	Estimate (%)	95% CI
Sensitivity		
Specificity		

Conclusions:

- Sensitivity, specificity
- Cross-reactivity:
- Invalid test rate:
- Performance: **Satisfactory / Not satisfactory**

(Sensitivity and specificity have been assessed in controlled lab setting using kits provided by the manufacturer from the batch mentioned above using ….. sample. Results should not be extrapolated to other sample types.)

Disclaimers

1. This validation process does not approve / disapprove the kit design
2. This validation process does not certify user friendliness of the kit / assay

Note: This report is exclusively for ……Kit (Lot No……) manufactured by …… (Supplied by …….)

Evaluation Done on ……

Evaluation Done by …….

Signature of Director/ Director-In-charge …… Seal……

**********End of the Report**********

Information on Operational and Test Performance Characteristics Required from Manufacturers for Dengue IgG Based Assays

The manufacturer should provide the following details about the IVD:

1. Instructions for Use
2. Scope of the IVD: to diagnose Dengue
3. Intended Use Statement
4. Principle of the assay
5. Intended testing population(cases of acute febrile illness/suspected cases of Dengue)
6. Intended user(laboratory professional and/or health care worker at point-of-care)
7. Detailed test protocol
8. Lot/batch No.
9. Date of manufacture
10. Date of Expiry
11. Information on operational Characteristics
    - i. Configuration of the kit/device
    - ii. Requirement of any additional equipment, device
    - iii. Requirement of any additional reagents
    - iv. Operation conditions
    - v. Storage and stability before and after opening
    - vi. Internal control provided or not
    - vii. Quality control and batch testing data
    - viii. Biosafety aspects- waste disposal requirements
10. Information on Test Performance Characteristics
    - i. Type of sample-serum/plasma/whole blood/other specimen (specify)
    - ii. Volume of sample
    - iii. Sample rejection criteria (if any)
    - iv. Any additional sample processing required
    - v. Any additional device/consumable like sample transfer device, pipette, tube, etc required
    - vi. Name of analyte to be detected
    - vii. Pathogens targeted by the kit

viii. Time taken for testing

ix. Time for result reading and interpretation

x. Manual or automated(equipment)reading

xi. Limit of detection

xii. Diagnostic sensitivity

xiii. Diagnostic specificity

xiv. Stability and reproducibility (including data)

xv. Training required for testing (if any)

xvi. If yes, duration

xvii. Details of Cut-off and /or Equivocal Zone for interpretation of test

xviii. Details of cross reactivity, if any

xix. Interpretation of invalid and indeterminate results to be provided

xx. It is recommended to provide data demonstrating the precision

*Please mention "Not applicable" against sections not pertaining to the kit.

**********End of the Document**********