ATCC®

Product No.: YF-500

YF MAC-HD 1.0

Kit to detect IgM to Yellow Fever Virus

For Research Use Only (RUO)

Not for use in diagnostic procedures

Store at 4ºC

DO NOT FREEZE

Contents

BACKGROUND

Yellow fever virus (YFV) belongs to the genus *Flavivirus* and is found in tropical and subtropical areas in South America and Africa. It is related to other viruses such as dengue and West Nile viruses. The virus is transmitted to humans by the bite of an infected mosquito. Illness ranges in severity from a self-limited febrile illness to severe liver disease with bleeding. Yellow fever disease is diagnosed based on symptoms, physical findings, laboratory testing, and travel history, including the possibility of exposure to infected mosquitoes. There is no specific treatment for yellow fever; care is based on symptoms but includes rest, fluids, and use of pain relievers and medication to reduce fever. Steps to prevent yellow fever virus infection include using insect repellent, wearing protective clothing, using mosquito nets and getting vaccinated.

- In persons who develop symptoms, the incubation period (time from infection until illness) is typically 3 to 6 days.
- The initial symptoms include sudden onset of fever, chills, severe headache, back pain, general body aches, nausea, and vomiting, fatigue, and weakness. Most persons improve after the initial presentation.
- After a brief remission of hours to a day, roughly 15% of cases progress to develop a more severe form of the disease. The severe form is characterized by high fever, jaundice, bleeding, and eventually shock and failure of multiple organs.
- Among those who develop severe disease, 20% to 50% may die.
- Those who recover from yellow fever generally have lasting immunity against subsequent infection.
- A presumptive diagnosis of yellow fever is often based on the patient's clinical features, places and dates of travel and epidemiologic history of the location where the presumed infection occurred.
- Surveillance for yellow fever is critical to early recognition of outbreaks. Immunoglobulin M (IgM) testing is a means to identify infections in the community.

PRINCIPLE OF THE TEST

The **YF MAC-HD** is a qualitative assay based on the in-house YF MAC-ELISA method developed by CDC which detects IgM to yellow fever virus (see Reference 1, page 21). The YF MAC-HD uses anti-human IgM to capture IgM in human serum. If IgM reactive to yellow fever is present, this reacts with non-infectious yellow fever whole virus antigen, which is detected using a horseradish peroxidase-conjugated flavivirus group-reactive monoclonal antibody, and a measurable colorimetric reaction is produced using a 3,3’,5,5’-tetramethylbenzidine substrate. The assay has been adapted to a kit format with a faster completion time and incorporates stability and ease-of-use making it convenient in a wide variety of laboratory settings. The protocol to resolve equivocal results is included in this instruction for use (Refer to page 7 “YF MAC-ON”).

Intended use

The YF MAC-HD is for research use only (RUO) and is intended for use in the detection of anti-yellow fever virus IgM in human serum. It should be used in a laboratory setting by trained laboratory personnel. The results of this assay are qualitative and should be used in the context of laboratory surveillance for yellow fever. Results should be confirmed according to applicable laboratory guidelines. The assay has not been validated for clinical purposes (i.e., the diagnosis of yellow fever disease).

Specimen collection

Serum specimens should be obtained using serum separator tubes according to tube instructions. Blood may be stored at 4°C to 8°C for up to 24 hours before the serum is separated. Blood should be allowed to clot and retract by leaving it at room temperature for half an hour to an hour. Centrifugation at 1000 x g for 10 minutes will separate the serum from the clot. Serum should immediately be transferred to a new labelled vial and stored at 4°C to 8°C until testing. If no centrifuge is available, serum can be aseptically removed from the retracted clot by means of a sterile pipette and transferred to a sterile labelled vial and stored at 4°C to 8°C until testing. Care should be taken to avoid transferring red blood cells. **Hemolysis may result in incorrect results**. Testing using separated serum should be performed as soon as possible and within 7 days. After testing is completed, remaining sera can be stored at -20°C, or below -60°C if being used for isolation procedure.

Storage and transport

The YF MAC-HD kit should be stored at 4°C until the expiration date after which time the kit should be disposed of (see disposal section). The transport temperature should be maintained at 4°C. A Tempilabel® is affixed to the substrate bottle. **If the dot is black, this indicates that the kit may have been compromised due to high temperature and the kit should be discarded.**

WARNING: POTENTIALLY BIOHAZARDOUS MATERIAL This kit contains reagents made with human serum. The serum is commercially sourced and tested negative for HIV-1 & HIV-2, HIV-Ag, HCV, HBsAg, and RPR by FDA approved methods. Handle all sera and kits as if they contain infectious agents. Observe established precautions against microbiological hazards while performing all procedures and follow the standard procedures for proper disposal of specimens.

Disposal

Remaining materials from the YF MAC-HD kit should be disposed of via autoclaving or in accordance with the laboratory internal policies.

Recommendations

- For the interpretation of data obtained using this kit It is critical to obtain the date of onset of symptoms, collection date of the sample, and place of residence/travel history of the patient and other relevant health information.
- Samples taken in the first few days after onset may not contain IgM antibodies to yellow fever and follow-up samples should be obtained.
- The result must be interpreted according to national surveillance guidelines, testing algorithms and taking into account clinical and vaccination history.

Limitations of the YF MAC-HD

- For use in laboratory research/surveillance of YFV only.
- The presence of false positive and negative results must be considered, including false positives due to acute infection with malaria, high levels of rheumatoid factor, and cross-reactivity with IgM of other flaviviruses.
- As YF IgM can be detected following vaccination, vaccine history should be sought to help determine the source of exposure (to wild virus or following vaccination).
- This kit should be used in accordance with national YF algorithms and confirmation of results performed as indicated.
- This kit cannot distinguish between vaccine-induced antibody and antibodies to wild-type yellow fever virus.
- This YF MAC-HD is considered a high complexity test and should only be performed in laboratories with the appropriate storage and operating conditions, and trained laboratory staff
- The YF MAC-HD kit has only been validated for use with serum; Other potential sources of antibodies (e.g. anticoagulated blood, sputum) have not been validated and the accuracy of these results cannot be assured.

Features of the YF MAC-HD Assay

- **Each assay set is single use (i.e., no storage of remaining assay components even if some are left over after opening)**
- Each assay can be used to test 24 samples singly or 8 samples in triplicate following the overnight protocol when testing equivocals (YF MAC-ON)
- Approx. 3.5 hours start to completion (not including test sample preparation)
- All reagents and diluents are included with exception of water used to dilute wash buffer
- Reagents provided at working dilution or lyophilized and at working dilution after reconstitution. No reagent titrations or further dilutions required
- All reagents stored together (Technical Note #2, page 7)
- Minimum shelf life 1 year from manufacture
- Tolerance of fluctuations in shipping temperature (Technical Note #3, page 7)
- Sensitivity similar to that of the CDC-ELISA (72 h test)
- Specificity similar to that of the CDC-ELISA (Technical Note #4, page 7)
- No shipping restrictions for the stop solution

- Test is optimized for incubations of the serum and conjugate to be performed at 28°C (recommended), but 21°C, 26°C, and 37°C also work with this test, based on limited data
- Equivocal results should be checked by retesting sample using overnight protocol (YF MAC-ON, page 7)
- Tests for IgM to wild-type or vaccine strain virus in serum (Technical Note #5, page 7)
- **Samples should be handled using appropriate infection control measures appropriate for handling clinical specimens. (Technical Note #1, page 7)**

Items included for each assay (10 assays per kit):

1. Wash buffer concentrate (liquid, 10X concentration) (clear bottle) (YF-1)
2. Conjugate concentrate (liquid in stabilizer) (amber vial with white lid) (YF-2)
3. Conjugate diluent (lyophilized) (glass vial with ●–marked silver seal) (YF-3)
4. Sterile water for reconstitution (opaque bottle with green sticker) (YF-4)
5. Negative serum control (lyophilized in buffer) (glass vial with blue seal) (YF-5)
6. Normal antigen – mock-inactivated Vero cell culture supernatant (lyophilized in buffer) (glass vial with green seal) (YF-6) **Normal antigen is required to detect non-specific background reactions which are resulted as NEG. Do not eliminate from test.**
7. One IgM coated and stabilized 96-well microtiter plate (YF-7)
8. YF IgM positive control (humanized monoclonal antibody) (lyophilized in buffer) (glass vial with red seal) (YF-8)
9. Sample diluent (liquid, at working dilution) (opaque bottle with orange sticker) (YF-9)
10. Stop solution (liquid, at working dilution) (opaque bottle) (YF-10)
11. Substrate (liquid, at working dilution) (brown bottle) (Technical Note #6) (YF-11)
12. YF antigen – inactivated YF 17D Vero cell culture supernatant (lyophilized in buffer) (glass vial with silver seal) (YF-12)
13. Three (3) plate sealers
14. "YF MAC-HD Calculations Workbook", available in Excel 2022 version. The workbook may be obtained from: https://www.atcc.org/federal-solutions/global-health-and-biodefense/yellow-fever-surveillance-kits.

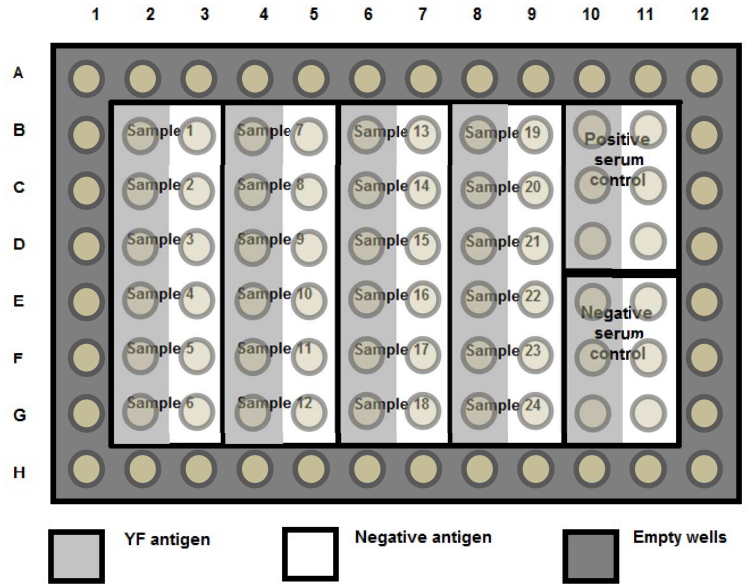
Required items not included in the kit:

1. Calibrated pipettors [(P-10, P-200, P-1000, multichannel (200 µL)]
2. Biosafety cabinet for manipulation of potentially infectious samples (Technical Note #1, page 7)
3. Reagent reservoirs (minimum of 2) – required for use with multichannel pipette
4. Deionized (DI) water for wash buffer
5. 1L PETG bottle or similar container for mixing DI water and wash buffer
6. Plate reader (450 nm filter)
7. Refrigerator (2°C to 8°C)
8. Permanent marker
9. Sample dilution tubes (eg. Polypropylene; 1 mL tubes; 1 per sample tested) and stoppers

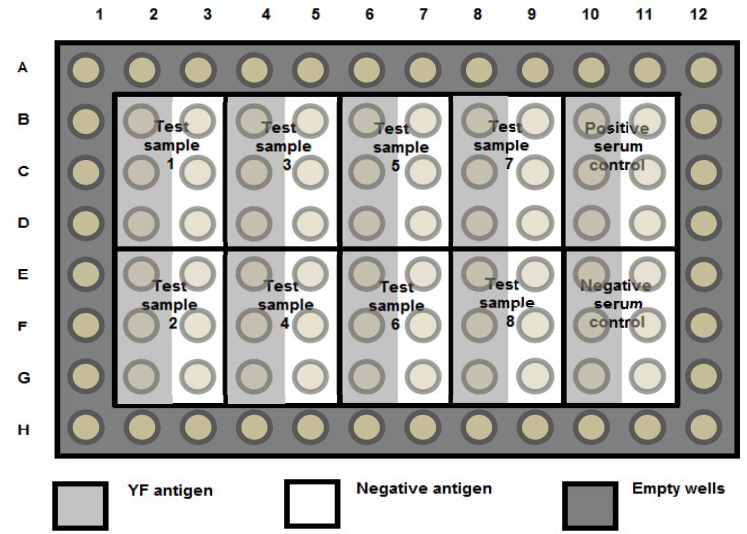
Recommended items not included in the kit:

1. **In-house YFV IgM positive control** – strongly recommended to assure testing consistency
2. Plate washer (preferred over washing plates by hand)
3. Vortexer (useful but not required)
4. Incubator set at 28°C (preferred)
5. Scissors
6. Pipet aid (100 mL into 900 mL) or you may pour the pre-measured 100 mL 10X wash buffer into the premeasured 900 mL DI water.

Routine single plate format (up to 24 test samples)



Equivocal resolution format (8 test-sample plate for overnight protocol YF MAC-ON)



YF MAC-HD PROTOCOL (ROUTINE – UP TO 24 SAMPLES TESTED IN SINGLICATE)

DO NOT ALLOW THE PLATE TO DRY OUT AT ANY TIME

1. Bring all reagents in kit to room temperature.
2. Dilute test serum samples and in-house positive control to 1:100 in sample diluent (provided); mix. (Recommend 4 µL serum in 396 µL sample diluent for a total volume of 400 µL). Use new pipet tip for each sample.
3. Add 400 µL sterile water (provided) to negative and positive control vials; mix and use as soon as possible.
4. Open packet containing plate with scissors; annotate wells using permanent marker (outer wells are not coated). Placement of positive and negative controls is pre-marked.
5. Add 50 µL of test serum to 2 wells (1 viral antigen + 1 normal antigen well per sample). Add 50 µL of negative and positive controls to 6 wells per control (3 viral and 3 normal antigen wells).
6. Cover plate with sealer. Incubate 30 min. at 28°C (Technical Note #7, page 7).
7. During the incubation in step 6, prepare the 1X wash buffer. Add 900 mL deionized water to 100 mL 10X wash buffer (provided); mix.
8. 10 min. prior to the end of the step 6 incubation, reconstitute YF antigen and normal antigen in 1.8 mL sterile water and mix.
9. At the completion of the step 6 incubation, carefully remove sealer and discard.
10. Wash plate using 5 cycles if using plate washer or 3 cycles if using manual washing (Technical Note #8 and #9, page 7).
11. Add 50 µL /well YF antigen to even numbered (left) columns; 50 µL normal antigen to odd numbered (right) columns.
12. Cover the plate with sealer. Incubate 2 hours at 4°C.
13. 5 min. prior to the end of step 12 incubation, add 3.45 mL sterile water to conjugate diluent followed by the addition of 50 µL conjugate to diluent; mix.
14. Following the step 12 incubation, carefully remove sealer and discard.
15. Wash plate using 5 cycles if using plate washer or 3 cycles if using manual washing (Technical Note #8 and #9, page 7).
16. Add 50 µL /well conjugate mixture to all wells. Cover plate with sealer. Incubate 30 min at 28°C.
17. Following the step 16 incubation, carefully remove sealer and discard.

18. Wash plate 15 cycles turning plate every 5 cycles if using a 96-well plate washer, 10 cycles if using a strip washer (Technical Note #8 and #9, page 7) or 6 cycles if using manual washing.
19. Add 75 µL /well substrate to all wells (Technical Note #3 and #6, page 7). Place plate uncovered in the dark. Incubate 10 min. at room temperature.
20. Add 75 µL /well stop solution to all wells including A1-D1 (Technical Note #10, page 8) and seal the plate.
21. Read at 450 nm within 15 minutes (Technical Note #11, page 8). For calculations see pages 9-11 and Addendum pages 15-16.
22. **Discard unused test components**.

YF MAC-ON (UP TO 8 SAMPLES TESTED IN TRIPLICATE OVERNIGHT) – EQUIVOCAL RESOLUTION PROTOCOL

Optional method to help resolve equivocal results. For YF MAC-ON with up to 8 samples tested in triplicate overnight, use the 8-sample format on page 6. Follow the protocol for YF MAC-HD on page 6 with the following exceptions:

5. Add diluted specimen to 3 viral and 3 normal antigen wells.

10. Cover the plate and incubate the antigen overnight (18-24h) at 4°C.

For calculations see pages 9 and 13.

TECHNICAL NOTES

1. Due to the potentially infectious nature of serum samples, minimum containment should include a biosafety cabinet, safety glasses, lab coat and gloves. Wash buffer waste should be treated with bleach or other approved disinfectant. If a biosafety cabinet is not available, samples should be inactivated in a 56°C water bath for 30 minutes prior to use to minimize risk. Note that this does not guarantee inactivation of all infectious agents.

2. Stability has been optimized for storage at 4°C for all kit components. Other temperatures have not been investigated. Freezing will inactivate the conjugate. **DO NOT FREEZE**.

3. If the temperature sensor on the substrate container shows a solid black dot, this indicates the kit has been subjected to unacceptable conditions during shipping. Discard the kit.

4. False positive results occur and may be due to infection with other flaviviruses, malaria, past YF vaccination, and interference due to high levels of rheumatoid factor. Consult your regional WHO guidelines for recommendations on further testing.

5. YF MAC-HD has not been validated for use with sample types other than serum.

6. If substrate has a pale blue color, discard. It is recommended to pour the substrate into a reagent reservoir (not included) and use a multichannel pipettor to dispense, as repeated entry of pipette tips into the substrate will cause color contamination.

7. Place sealed plates in a single layer for incubation. Do not stack. Kit is optimized for serum and conjugate incubations at 28°C.

8. Plates washed with a **strip washer** should be washed using 10 cycles with 250 µL wash buffer for the final wash step.

9. The use of an **automatic plate washer** (either a 96-well washer or strip washer) is advised, with a program as follows:

a. Plate washer (5 cycles): Aspirate 2 sec; wash 1 sec; aspirate 2 sec; wash 1 sec; aspirate 2 sec; wash 1 sec; aspirate 2 sec; wash 1 sec; aspirate 2 sec; wash 1 sec; aspirate 4 sec. Wash volume is 250 µL/well. 100 mL of 10X wash buffer (provided) should be added to 900 mL deionized water.

b. Manual plate washing: Empty contents of plate into disposal container (in biosafety cabinet if samples are potentially infectious). Add 200 µL of wash buffer to each well. Empty plate. Repeat to obtain the required number of cycles. After final cycle, blot plate dry on absorbent surface to remove any remaining wash buffer from the wells. Results from hand-washed plates may vary slightly from those of automatically-washed plates.

10. To avoid creating bubbles, dispense stop solution holding pipette tips against the sides of the wells. Do not dispense past the first stop position of the pipette. Incorrect OD readings will occur if bubbles are present.

11. If you are using the Excel workbook for calculations it is not necessary to set the reader to automatically blank the wells using A1, B1, C1, D1. However, automatic blanking using the plate reader is very helpful if you are performing manual calculations.

AUTOMATED CALCULATIONS

To perform automated calculations, use the Excel workbook **"YF MAC-HD Calculations Workbook"**, available in Excel 2022 version. The workbook may be obtained from: https://www.atcc.org/federal-solutions/global-health-and-biodefense/yellow-fever-surveillance-kits. Follow the directions on the Instructions tab.

For the 24-sample (routine half-day) format, use the YF MAC-HD tab to enter the A_{450} results into cells B2:M9. Validity, control values and test results will be automatically calculated.

For the overnight protocol (triplicate using the 8-sample format), use the YF MAC-ON tab to enter the A_{450} results into cells B2:M9. Validity, control values and test results will be automatically calculated.

Examples for each test format are given on separate tabs in the 2022 Calculations workbook YF MAC-HD.

MANUAL CALCULATIONS

To perform manual calculations, use the template provided below.

YF MAC-HD 24-sample format

Abbreviations

YFVA = Yellow Fever Viral Antigen
NA = Normal Antigen
PCVA = Average Positive Control on YFVA
NCVA = Average Negative Control on YFVA
PCNA = Average Positive Control on NA

Validity

1. Enter sample OD's minus average blank OD [(A1+B1+C1+D1)/4] in template provided on page 12.

2. Calculate PCVA.

Prior to calculating PCVA, address any variability in replicates of positive control on viral antigen (see *Addendum*, page 15-16).

Use the replicates of PCVA that do not exceed the variability limit (VL) of OD 0.3 to calculate PCVA

Example: (B10+C10+D10)/3 = PCVA

3. Calculate NCVA.

Prior to calculating NCVA, address any variability in replicates of negative control on YFVA (see *Addendum*, page 15-16).

IMPORTANT NOTE: if NCVA is <0.05, use NCVA value of 0.05 to calculate P/N values; in this situation, variability calculations are not necessary.

Use the replicates of NCVA that do not exceed the variability limit (VL) of 0.025 to calculate NCVA

Example: (E10+F10+G10)/3 = NCVA

4. Calculate PCNA.

Prior to calculating PCNA, address any variability in replicates of positive control on NA (see *Addendum*, page 15-16).

Example: (B11+C11+D11)/3 = PCNA

Note that OD's of the negative control reacted on NA are not used in the calculation

5. Calculate the P/N of the positive control (P/N): **PCVA/NCVA = P/N**

6. Calculate the normal background ratio (NBR): **PCVA/PCNA = NBR**

7. Determine if the test is valid:

a. If positive control PCVA-VL is not exceeded, test is valid. If not, repeat test.
b. f PCVA is ≥0.6, the test is valid. If not, repeat test.
c. If negative control NCVA-VL is not exceeded, test is valid. If not, repeat test.
d. If the NCVA is <0.2, the test is valid. If not, repeat the test.
e. If P/N of positive control ≥3, the test is valid; if not, repeat the test
f. If NBR of the positive control is ≥2, the test is valid.; if not, repeat the test

Note: If variability of control OD's is present, check pipetting and mixing techniques, and plate washer or manual washing technique.

Analysis of sample results

8. Calculate and enter result (P/N) for each sample: P/N = OD of sample on YFVA/NCVA

IMPORTANT NOTE: if NCVA is <0.05, use NCVA value of 0.05 to calculate P/N values

9. Calculate and enter normal background ratio (NBR) for each sample: NBR = OD of sample on YFVA/OD of sample on NA

Interpretation of results

10. Interpret and circle results using the following:

YF IgM positive (POS)	P/N ≥2	NBR ≥1.5	
YF IgM equivocal (EQ)	P/N ≥1.5 < 2	NBR ≥1.5	Perform optional retest using overnight protocol YF MAC-ON (page 7)
YF IgM negative (NEG)	P/N <1.5	NBR ANY	
YF IgM negative (NEG)	P/N ANY	NBR <1.5	

Results table 24 samples

	YF VA	NA	YF VA	NA	YF VA	NA	YF VA	NA	YF VA	NA
	2	3	4	5	6	7	8	9	10	11
B	Sample name:		Sample name:		Sample name:		Sample name:		Positive Control	Positive Control
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		
	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			
C	Sample name:		Sample name:		Sample name:		Sample name:		PCVA=	PCNA=
									P/N=	NBR=
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		Valid test if all true:
	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			PCVA≥0.6
D	Sample name:		Sample name:		Sample name:		Sample name:			Pos control P/N≥2.0
										NBR≥2.0
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		NCVA<0.2
	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			PCVA/NCVA-VL not exceeded
E	Sample name:		Sample name:		Sample name:		Sample name:		Negative Control	Is test valid?
										Yes/No
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		
	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			
F	Sample name:		Sample name:		Sample name:		Sample name:		NCVA=	
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		
	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			
G	Sample name:		Sample name:		Sample name:		Sample name:			
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		
	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			

Wash method:__Manual / Strip / 96-well (circle one)

Kit Lot No._____

Kit expiration date_____

Plate ID_____

Name_____

Date_____

Lab_____

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Effective Date:01 MAR 2023

YF MAC-ON (UP TO 8 SAMPLES TESTED IN TRIPLICATE OVERNIGHT) – EQUIVOCAL RESOLUTION PROTOCOL

Perform validity calculation steps 1-7 on pages 10-11, followed by analysis and interpretation of sample results as follows:

Analysis of sample results

8. Calculate and enter result (P/N) for each sample: P/N = Average OD of sample on YFVA/NCVA

If any specimen replicate reacted on YFVA or NA is obviously different from the other 2 replicates, discard that replicate and use the other 2 to calculate P/N.

IMPORTANT NOTE: if NCVA is <0.05, use NCVA value of 0.05 to calculate P/N values.

9. Calculate and enter normal background ratio (NBR) for each sample: NBR = Average OD of sample on YFVA/Average OD of sample on NA

Interpretation of results

10. Interpret and circle results using the following:

YF IgM positive (POS)	P/N ≥3	NBR ≥2.0
YF IgM equivocal (EQ)	P/N ≥2.0 < 3.0	NBR ≥2.0
YF IgM negative (NEG)	P/N <2.0	NBR ANY
YF IgM negative (NEG)	P/N ANY	NBR <2.0

Results table 8 samples										
	YF VA	NA	YF VA	NA	YF VA	NA	YF VA	NA	YF VA	NA
	2	3	4	5	6	7	8	9	10	11
	Sample name:		Sample name:		Sample name:		Sample name:		Positive control	Positive control
B										
C										
D										
Mean OD									PCVA=	PCNA=
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=
Interp	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			**Valid test if all true:**
	Sample name:		Sample name:		Sample name:		Sample name:		Negative control	PCVA≥0.6
E										Pos control P/N ≥3.0
F										NBR≥2.0
G										NCVA<0.2
Mean OD									NCVA=	PCVA/NCVA -VL not exceeded
	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=	P/N=	NBR=		**Is test valid?**
Interp	Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ		Pos/Neg/EQ			**Yes/No**

Wash method:__Manual / Strip / 96-well (circle one)

Kit Lot No._____

Kit expiration date_____

Plate ID_____

Name_____

Date_____

Lab_____

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ADDENDUM

Variability among control replicates

OD of Positive control reacted on YFVA

Identify if variability among the replicates exceeds the PCVA variation limit (PCVA-VL) of OD 0.3.

1. Identify the replicate with the middle value and calculate the difference between it and the other 2 values:
   a) If both upper and lower values are <0.3 different from the middle value use all 3 values to calculate the PCVA. PCVA-VL is not exceeded.
   b) If either the upper or lower values are ≥0.3 different from the middle value, discard the value that is ≥0.3 different. Calculate the PCVA using the remaining 2 values. PCVA-VL is not exceeded.
   c) If both the upper and lower values are ≥0.3 different from the middle value, the test exceeds the PCVA-VL; repeat the test. Check on pipetting technique, mixing of reagents, and plate washing method.

Example:

Replicate 1 1.495 Replicate 2 0.975 Replicate 3 1.167

1. *The middle value is Replicate 3 (1.167)*
   Replicate 1 minus Replicate 3 = 0.328
   Replicate 3 minus Replicate 2 = 0.192

   a) *Not applicable*
   b) *Replicate 1 is ≥0.3 different from Replicate 3. Discard Replicate 1. Replicate 3 is <0.3 different from Replicate 2; use Replicates 2 and 3 to calculate the PCVA. PCVA = (0.975+1.167)/2 = 1.071*
   c) *Not applicable*

OD of Negative control reacted on YFVA

Identify if variability among the replicates exceeds the variation NCVA limit (VL) of OD 0.025.

1. Identify the replicate with the middle value and calculate the difference between it and the other 2 values:
   a) If both upper and lower values are <0.025 different from the middle value, use all 3 values to calculate the NCVA. NCVA-VL is not exceeded.
   b) If either the upper or lower values are ≥0.025 different from the middle value, discard the value that is ≥0.025 different. Calculate the NCVA using the remaining 2 values. NCVA-VL is not exceeded.
   c) If both the upper and lower values are ≥0.025 different from the middle value, the test exceeds the NCVA-VL. Repeat the test, and check on pipetting technique, mixing of reagents, and plate washing method.

Example:

Replicate 1 0.053 Replicate 2 0.045 Replicate 3 0.063

1. *The middle value is Replicate 1 (0.053).*
   Replicate 3 minus Replicate 1 = 0.01
   Replicate 1 minus Replicate 2 = 0.008

   a) *Both upper and lower values are <0.025 different from the middle value; use all 3 values to calculate the PCVA. PCVA = (0.053+0.045+0.063)/3 = 0.054*
   b) *Not applicable*
   c) *Not applicable*

OD of Positive control reacted on Normal Antigen

Identify if there is too much variability among the replicates. Note: there is no variability threshold for the PCNA.

1. Identify the replicate with the middle value and calculate the difference between it and the other 2 values:

a) If both upper and lower values are <0.3 different from the middle value, use all 3 values to calculate the PCNA.
b) If either the upper or lower values are ≥0.3 different from the middle value, discard the value that is ≥0.3 different. Calculate the PCNA using the remaining 2 values.
c) If both the upper and lower values are ≥0.3 different from the middle value, calculate the PCNA using all 3 values, but check on pipetting technique, mixing of reagents, and plate washing method.

Example:

Replicate 1 0.102 Replicate 2 1.345 Replicate 3 0.187

1. *The middle value is Replicate 3 (0.187).*
   Replicate 2 minus Replicate 3 = 1.158
   Replicate 3 minus Replicate 1 = 0.085
   a) Not applicable
   b) Replicate 2 is ≥0.3 different from Replicate 3. Discard Replicate 2. Replicate 1 is <0.3 different from Replicate 3. Use Replicates 1 and 3 to calculate the PCNA. PCNA = (0.102+0.187)/2 = 0.145
   c) Not applicable

PERFORMANCE OF THE YF MAC-HD KIT

Shelf life/stability

12 months from the date of manufacture. The YF MAC-HD kit must be stored at 4°C and should not be used after the date of expiration. Each assay (10) in the kit is single-use only and components should not be stored after opening.

Analytical sensitivity (LOD)

In the absence of an international standard, a comparative estimate of analytical sensitivity was determined for YF MAC-HD using 3 replicates of each of 8 concentrations of low, medium, and high YF IgM-positive specimens and compared to similar titrations of the specimens using the CDC YF MAC-ELISA. Each sample was used undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, where dilutions were made in normal human serum. Sample dilutions were subsequently diluted to the assay working dilution for each assay using the appropriate sample diluent and tested. Low, medium, and high YF IgM positive serum samples were used.

Test	Sample No.	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128
YF MAC-HD	1 (Low pos)	POS	POS	**POS**	EQ	EQ	NEG	NEG	NEG
CDC MAC-ELISA		POS	**POS**	EQ	EQ	NEG	NEG	NEG	NEG
YF MAC-HD	2 (Med pos)	POS	POS	POS	POS	**POS**	NEG	NEG	NEG
CDC MAC-ELISA		POS	POS	POS	**POS**	EQ	EQ	EQ	EQ
YF MAC-HD	3 (Med pos)	POS	POS	**POS**	NEG	NEG	NEG	NEG	NEG
CDC MAC-ELISA		POS	**POS**	EQ	NEG	NEG	NEG	NEG	NEG
YF MAC-HD	4 (Hi pos)	POS	POS	POS	POS	POS	**POS**	EQ	NEG
CDC MAC-ELISA		POS	POS	POS	**POS**	EQ	EQ	NEG	NEG
YF MAC-HD	5 (Hi pos)	POS	POS	POS	POS	POS	POS	POS	**POS**
CDC MAC-ELISA		POS	POS	POS	POS	POS	POS	**POS**	EQ

Conclusion: All samples gave positive results in the YF MAC-HD at equal or higher dilutions than the reference CDC MAC-ELISA.

Analytical specificity

Serum specimens containing IgM to flaviviruses other than YFV, to alphaviruses, and to other infections or substances were assayed using the YF MAC-HD kit to determine the assay's potential to solely detect YF specific IgM.

Virus	No. of specimens	YF MAC-HD POS results	YF MAC-HD NEG results	% SPECIFICITY[4]
Flaviviruses				
Dengue[3]	10	4	6	60
Zika[3]	10	1	9	90
West Nile[2]	20	4	16	80
Powassan[2]	19	6	13	68
Japanese encephalitis[3]	9	5	4	44
St. Louis encephalitis[2]	2	0	2	100
Hepatitis C[3]	20	0	20	100
Alphaviruses				
Chikungunya[3]	2	0	2	100
Eastern equine encephalitis[3]	2	0	2	100
Ross River[2]	11	0	11	100
Other Infections or Substances				
Cytomegalovirus[2]	7	2	5	71
Leptospira[2]	7	1	6	85
Epstein-Barr virus[2]	2	1	1	50
Varicella-Zoster virus[1]	1	0	1	100
Malaria[3]	10	9	1	10
Rheumatoid factor[3]	3	2	1	33
Human leukocyte antigen[1]	3	0	3	100
Anti-nuclear antibody[2]	11	1	10	90
Possible obstructive jaundice[1]	9	0	9	100
Sera from rheumatic patients[1]	3	1	2	66
Human anti-mouse antibody[3]	5	0	5	100
Auto-antibody positive[1]	2	0	2	100
Human immunodeficiency virus[5]	9	0	9	100

[1]Data from assays performed by CDC.
[2]Data from CDC and independent laboratory testing.
[3]Data from independent laboratory testing.
[4]Small sample numbers may not reflect actual levels of specificity.
[5]Tested at ATCC using HIV positive serum.

Conclusion: The YF MAC-HD reacts nonspecifically with some arboviral anti-flavivirus IgM positive specimens, especially those that are high titered. This is expected as the antigen used in the YF MAC-HD kit includes the envelope protein which is known to have flavivirus cross-reactive epitopes. It is consistent with the reference CDC YF MAC-ELISA. Specimens with a positive reaction in the YF MAC-HD should be investigated further using additional methods to confirm the YF infection.

Clinical performance panel

Specimens from Africa and the Americas were used to assess clinical performance of the YF MAC-HD

Clinical sensitivity[1]

YF IgM positive sera	No. specimens	YF MAC-HD POS results	YF MAC-HD NEG results	% Clinical sensitivity	95% Confidence Interval
High positive	14	14	0	100%	78.5% - 100%
Medium positive	13	13	0	100%	77.2% - 100%
Low positive	3	1	2	33%	6.2% - 79.2%
Total	30	28	2	93%	78.7% - 98.2%

Clinical specificity[1]

	No. specimens	YF MAC-HD POS results	YF MAC-HD NEG results	% Clinical specificity	95% Confidence Interval
YF IgM negative sera	50	1	49	98%	89.5% - 99.6%

[1]Data from independent laboratory testing.

Additional clinical performance studies

A total of 237 reference positive specimens and 398 reference negative specimens from Africa and S. America gave 95% sensitivity and 97% specificity using YF MAC-HD kits produced by a different manufacturer using the same methodology as ATCC. Proficiency panels consisting of 10-20 YF positive and negative specimens were run in laboratories in 43 countries across the continents of Africa and the Americas using the YF MAC-HD, and 42/43 laboratories obtained 100% correct results.

Use in endemic regions

Forty-three National Laboratories in Africa and the Americas were provided with YF MAC-HD kits produced by an alternative manufacturer and proficiency panels of 10 to 20 samples. Forty-two of the laboratories obtained 100% correct results and 1 obtained 90% correct results. This indicates that the YF MAC-HD is useful under laboratory conditions in YF-endemic regions.

Precision

Intra-assay precision (repeatability) was measured using three replicates each of 10 samples of differing YF IgM positivity assayed in the same test at the same time by the same operator. The coefficient of variation (CV) was 5% (range 0-8%).

Inter-assay precision was measured using three replicates each of 10 samples of differing IgM positivity assayed by three different operators on three different days. The CV was 14.6% (range 10-18%)

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