InBios International, Inc.

Scrub Typhus *Detect*™IgM ELISA System

For In Vitro Diagnostic Use Only
Not for Sale or Distribution in the United States of America

INTENDED USE

The Scrub Typhus *Detect*™ IgM ELISA test for exposure to *Orientia tsutsugamushi* (OT; formerly *Rickettsia*) is an ELISA assay system for the detection of IgM antibodies in human serum to OT-derived recombinant antigen (1-10). This test is to aid in the detection of human exposure to OT species It is not intended to screen blood or blood components. For in vitro diagnostic use only. Not for sale or distribution in the United States of America.

SUMMARY AND EXPLANATION OF THE TEST

Scrub Typhus is an infectious disease that is caused by *Orientia tsutsugamushi* (formerly *Rickettsia*), a tiny parasite about the size of bacteria that belongs to the family Rickettsiaceae. A bite from the larval trombiculid mite, a parasite of rodents, will transmit the disease. An ulcer of the skin is characteristic of a bite from a trombiculid mite, followed by symptoms including fever, a spotted rash on the torso, and swelling of the lymph glands. Scrub typhus generally occurs after exposure to areas with secondary (scrub) vegetation, from which its name is derived. However, the disease can also be prevalent in sandy, mountainous, and tropical areas. Scrub Typhus is a worldwide illness, but particular to South East Asia and the Western Pacific. It accounts for approximately 20% of fever in some regions in South East Asia, where it is endemic. Illness lasts for a period of 10 to 12 days after the initial bite. With therapy, the fever will break within 36 hours, but if left untreated, complications or death may occur. Recently scrub typhus was reported in 24% of patients with unknown febrile illness in India (11, 12, 13). In one of the reports (11), the prevalence of AKI (infection related acute kidney injury) and scrub typhus infection was discussed.

PRINCIPLE OF THE TEST

The Scrub Typhus *Detect*™ IgM ELISA system is a qualitative ELISA for the detection of IgM antibodies to *O. tsutsugamushi* (OT) in serum. Wells of each plate have been coated with unique recombinant antigen mix. During testing, the serum samples are diluted in InBios sample diluent and applied to each well. See "Example for Sera Application" below. After incubation and washing, the wells are treated with polyclonal Goat anti-human IgM antibodies labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the tetramethylbenzidine (TMB) substrate. An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by absorbance measurement at 450nm. The absorbance measured is directly proportional to the concentration of IgM antibodies to OT present. A set of positive and negative controls are provided as internal controls. These are provided to monitor the integrity of the kit components. Because of the endemic nature of this disease in many countries, no fixed cut-off control is supplied. End users are required to determine a robust locally relevant cut-off using appropriate controls from each endemic location including but not limited to normal and unrelated disease specimens. For optimal use of this kit, paired samples from the same patient, collected at different timepoints, should be tested simultaneously to determine if a rise in titer or seroconversion has occurred during the time interval (see results section below).

MATERIALS SUPPLIED

Warning:

- ***Do not use any reagents where damage to the packaging has occurred.***
- ***Controls (*) must be centrifuged for 10 seconds at high speed prior to opening the vial to avoid loss of contents.***

The Scrub Typhus *Detect*™ IgM ELISA system contains sufficient reagents for 96 wells. Each kit contains the following reagents:

1. **Scrub Typhus ELISA Plate:**
   One strip holder in ziplock foil, containing 96 polystyrene microtiter wells coated with OT-derived recombinant antigens in each well. Stable at 2-8°C until the expiration date.

2. **Sample Dilution Buffer for Scrub Typhus:**
   Two bottles, 25 mL each, to be used for preparing sample dilutions. A slight precipitate may form. Mix gently before use. Stable at 2-8°C until the expiration date.

3. **Scrub Typhus IgM Positive Control*:**
   One vial, 50 µL. The positive control will aid in monitoring the integrity of the kit. Stable at 2-8°C until the expiration date. Before use, quickly centrifuge the vial so that contents can be collected at the bottom.

4. Scrub Typhus Negative Control*:
One vial, 50 µL. The negative control will aid in monitoring the integrity of the kit as well. Stable at 2-8°C until the expiration date. Before use, quickly centrifuge the vial so that contents can be collected at the bottom.

5. Ready to Use Enzyme Conjugate-HRP for Scrub Typhus IgM:
One bottle, 12 mL of a pre-diluted conjugate to be used as is in the procedure below. Stable at 2-8°C until the expiration date.
Note: The conjugate should be kept in a light-protected bottle at all times as provided.

6. 10X Wash Buffer:
One bottle, 120 mL of 10X concentrate Wash Buffer to be diluted and used in all the washing steps of this procedure. Stable at 2-8°C until the expiration date.
Note: See Preparation of Reagents in Test Procedure section to prepare 1X Wash Buffer.

7. EnWash: One bottle, 20 mL of *En*Wash to be used in between the washing steps after enzyme conjugate-HRP and before liquid TMB addition. Stable at 2-8°C until the expiration date.

8. Liquid TMB Substrate:
One bottle, 12 mL of liquid substrate to be used in this procedure. Stable at 2-8°C until the expiration date.
Note: The substrate should be kept in a light-protected bottle at all times.

9. Stop Solution:
One bottle, 6 mL to be used to stop the reaction. Stable at 2-8°C until the expiration date.
Caution: strong acid, wear protective gloves, lab coat and safety goggles. Dispose of all materials according to safety rules and regulations.

NOTE: *All reagents and controls must be allowed to reach room temperature (20℃~25℃) and mixed thoroughly by gentle inversion prior to use.*

MATERIALS REQUIRED BUT NOT SUPPLIED

- Microtiter plate reader capable of absorbance measurement at 450 nm
- Biological or High-Grade Water
- 37°C incubator without CO_2 supply or humidification
- Plate washer
- Multi-channel pipettors
- Timer

PRECAUTIONS

- **FOR IN VITRO DIAGNOSTIC USE.**
- Not for sale or distribution in the Unites States of America.

❖ **General Precautions**
- A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert.
- Wear protective clothing, eye protection and disposable gloves while performing the assay. Wash hands thoroughly afterwards.
- Do not eat, drink, smoke or apply cosmetics where immunodiagnostic materials are being handled.
- Do not pipette by mouth.
- Use a clean disposable pipette tip for each reagent, Standard, Control or specimen.
- Cover working area with disposable absorbent paper.

❖ **Sample Precautions**
- All human source material used in the preparation of controls has been tested using FDA-approved methods for antibody to Human Immunodeficiency Virus 1 & 2 (HIV 1&2), Hepatitis C (HCV) as well as Hepatitis B surface antigen and found to be negative. However, no test method can offer complete assurance and all human controls and antigen should be handled as potentially infectious material. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.
- This test must be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.
- It is advised that icteric or lipaemic sera, or sera exhibiting hemolysis or microbial growth not be used.
- Do not heat inactivate sera.
- Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.

- To avoid cross contamination, a new pipet tip must be used for dispensing each control and test sera.

❖ **Kit Reagents Precautions**

- All reagents must be equilibrated to room temperature (20-25°C) before commencing the assay. The assay will be affected by temperature changes.
- Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.
- Unused microwells must be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.
- Substrate System:
  (a) As the Liquid TMB Substrate is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces.
  (b) Avoid prolonged exposure to direct light.
  (c) Some detergents may interfere with the performance of the Liquid TMB Substrate.
  (d) The Liquid TMB Substrate may have a faint blue color. This will not affect the activity of the substrate or the results of the assay.
- Do not mix lots of any kit component within an individual assay microtiter plate.
- Do not use any component beyond the expiration date shown on its label.
- Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.
- Some reagents may form a slight precipitate; mix gently before use.
- Incomplete washing will adversely affect the outcome and assay precision.
- To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the Liquid TMB Substrate solution.
- Avoid microbial contamination of reagents, especially of the Ready to use Enzyme Conjugate-HRP. Avoid contamination of the Liquid TMB Substrate Solution with the Ready-to-Use Enzyme Conjugate-HRP.
- Do not use a humidified chamber for 37°C incubations, as this may affect assay performance.

WARNING: POTENTIAL BIOHAZARDOUS MATERIAL
This kit contains reagents made with human serum or plasma. The serum or plasma used has been heat inactivated unless otherwise stated. Handle all sera and kits used 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.

CHEMICAL HAZARD:
Material Safety Data Sheets (MSDS) are available for all components of this kit. Review all appropriate MSDS before performing this assay. Avoid all contact between hands and eyes or mucous membranes during testing. If contact does occur, consult the applicable MSDS for appropriate treatment.

SPECIMEN COLLECTION AND PREPARATION

- Human serum must be used with this assay. Whole blood or plasma cannot be tested directly.
- Remove serum from the clot of red cells as soon as possible to avoid hemolysis.
- Testing should be performed as soon as possible after collection. Do not leave sera at room temperature for prolonged periods.
- Serum should be used and the usual precautions for venipuncture should be observed. The samples may be stored at 2-8°C for up to 48 hours or frozen at -20°C or lower for up to 30 days. To maintain long-term longevity of the serum, store at -70°C. Avoid repeated freezing and thawing of samples.
- Do not use hemolyzed or lipemic samples.
- Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use.
- If sera are shipped, pack in compliance with Federal Regulations covering transportation of infectious agents.

TEST PROCEDURE

Caution: This kit has not been optimized by InBios for use with any particular automated ELISA processing system. Use with an automated ELISA processing system will require proper validation to ensure results are equivalent to the expectations described in this package insert. Modifications to the protocol of these systems and/or different volumes of reagents may be required.

Bring all kit reagents and specimens to room temperature (~25°C) before use. Thoroughly mix the reagents and samples before use by gentle inversion.

Preparation of Reagents:

- 1X Wash Buffer

Dilute the 10X Wash Buffer to 1X using Biological or High-Grade Water (Mix the provided 120mL of 10X Wash Buffer with 1080mL of Biological or High-Grade Water). After it is diluted to 1X, store at room temperature for a maximum of six months.
Note: Discard the 1X Wash Buffer if any microbial growth is observed.

- Microtiter Wells
  Select the number of coated wells required for the assay. The remaining unused wells should be placed back into the pouch, sealed with desiccant, and stored at 2-8°C until ready to use or expiration.

Note: For long-term storage, sera cannot be repeatedly thawed and frozen. Sera should be further aliquoted in a smaller volume and stored at –20°C to -70°C.

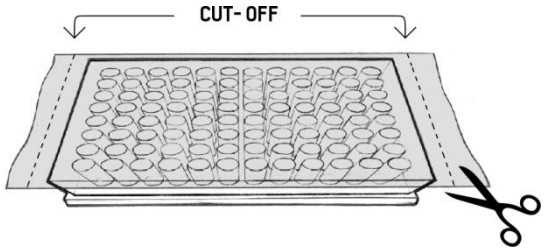
Assay Procedure:
Allow all reagents to reach room temperature (~25°C) and mix thoroughly by gentle inversion before use. Positive and negative controls should be assayed in duplicate. Test samples may be assayed in singlet.

1. Determine number of sera to be tested.
2. Organize sera according to the "Example for Sera Application" provided below or any preferred arrangement. Dilutions can be made either in tubes or in ELISA-type plastic wells (untreated plastics; not provided).

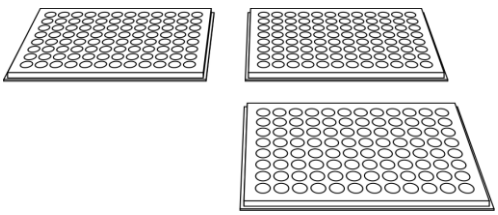
Example for Sera Application, 1/100 Diluted Samples, 100µL/Well

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative Control	Negative Control	S# 13	S# 21	S# 29	S# 37	S# 45	S# 53	S# 61	S# 69	S# 77	S# 85
B	Positive Control	Positive Control	S# 14	S# 22	S# 30	S# 38	S# 46	S# 54	S# 62	S# 70	S# 78	S# 86
C	S# 1	S# 7	S# 15	S# 23	S# 31	S# 39	S# 47	S# 55	S# 63	S# 71	S# 79	S# 87
D	S# 2	S# 8	S# 16	S# 24	S# 32	S# 40	S# 48	S# 56	S# 64	S# 72	S# 80	S# 88
E	S# 3	S# 9	S# 17	S# 25	S# 33	S# 41	S# 49	S# 57	S# 65	S# 73	S# 81	S# 89
F	S# 4	S# 10	S# 18	S# 26	S# 34	S# 42	S# 50	S# 58	S# 66	S# 74	S# 82	S# 90
G	S# 5	S# 11	S# 19	S# 27	S# 35	S# 43	S# 51	S# 59	S# 67	S# 75	S# 83	S# 91
H	S# 6	S# 12	S# 20	S# 28	S# 36	S# 44	S# 52	S# 60	S# 68	S# 76	S# 84	S# 92

3. Dilute test sera to 1/100 by using the provided Sample Dilution Buffer for Scrub Typhus (e.g. 4µL serum plus 396µL Sample Dilution Buffer for Scrub Typhus). Mix well.
   Note: Do not use less than 4µL of serum and controls.
4. Apply 100µL per well of the 1/100 diluted test sera and controls to marked Scrub Typhus ELISA plate.
5. Cover the plate with parafilm or plate covers just on the well opening surface, so the bottom of the plate is not covered. (Please read the important note below.) Incubate the plate at 37°C for 30 minutes in an incubator.



Note: *This is to make sure the temperature distribution is evenly spread out in all wells from bottom and sides; any extra parafilm should be cut off once the top is sealed to block evaporation.*
Note: *Do not stack plates on top of each other. They should be spread out as a single layer. This is very important for even temperature distribution. Do not use CO_2, or any other gases used for tissue culture.*



CORRECT METHOD

6. After the incubation is complete, wash the strips six (6) times with the 1X Wash Buffer using an automatic plate washer. Use 300µL per well of 1X Wash Buffer in each wash cycle for all plate washing.
7. Add 100µL per well of Ready to Use Enzyme-HRP Conjugate for Scrub Typhus IgM into all wells by multi-channel pipettor.
8. Cover the plate with parafilm just on the well opening surface, so the bottom of the plate is not covered (*as described in step 5).*
9. Incubate the plate at 37°C for 30 minutes in an incubator.
10. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer, 300µL per well.
11. Add 150µL per well of EnWash into all wells by a multi-channel pipettor.
12. Incubate the plate at room temperature (20-25°C) for 5 minutes without any cover on the plate.
13. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer, 300µL per well.
14. Add 100µL per well of Liquid TMB substrate into all wells by multi-channel pipettor.
15. Incubate the plate at room temperature (20-25°C) in a dark place (or container) for 10 minutes without any cover on the plate.
16. After the incubation, add 50µL per well of Stop Solution into all wells by multi-channel pipettor and incubate at room temperature (20-25°C) for 1 minute without any cover on the plate.
    Note: Care should be taken to apply Stop Solution at the same speed and order as Liquid TMB Substrate for accurate results.
17. After the incubation, read the optical density (OD) at 450nm with a Microtiter plate reader.

For accurate results, do not subtract blank background

RESULTS

Determination of the cut-off using specimens from endemic locations MUST be performed as described below. To optimize the data obtained from the use of this kit, it is highly recommended that paired samples collected from the same patient, at different time points, should be tested simultaneously. Use of these paired sera, will help determine if there has been a rise in antibody titer or whether seroconversion has occurred in the time interval between sera collections.

Calculation of Cut-off value:
No fixed cut-off value is provided as the cut-off will vary depending on scrub typhus disease prevalence in the geographical location where the kit is being used. Therefore, it is required that the end users MUST calculate cut-off values first using geographically relevant specimens. A minimum of 100 specimens from each of 3 categories - diseased (confirmed with scrub typhus), confirmed unrelated febrile diseases (e.g. brucellosis, enteric infections, etc.), and normal healthy adults from endemic areas – are recommended for determination of cut-off. Receiver Operating Characteristic (ROC) curves can be used to determine a cut-off value.

Note: *Once a cut-off value is determined from a given location, the value can be used for future reference and calculation. The fixed cut-off must be verified/validated for consistency using a set of in-house panel samples available to the end users. They are to be used in combination with controls provided with the kits.*

Interpretation of the results:

1. Samples with spectrophotometric readings > Cut-off are considered to be "Reactive" and samples below this criterion are considered to be "Non-Reactive".
2. Any "Reactive" sample must be repeated to verify the result. Values near the Cut-off are considered to be doubtful and the assay must be repeated in triplicate or more.

Ensuring Assay Performance:
The results on the table below must be obtained using provided positive and negative controls to calculate discrimination capacity of the assay. Non-fulfillment of these criteria is an indication of deterioration of reagents or an error in the test procedure and the assay must be repeated.

Factor	Tolerance
Negative Control (NC) OD	< 0.200
Positive Control (PC) OD	> 0.500
Discrimination Capacity ($R_{PC/NC}$)	≥ 5.0

LIMITATIONS

- **InBios Scrub Typhus *Detect*™ IgM ELISA kit has not been validated with sera from HIV/OT co-infected population and is not recommended for this population.**
- All positive ELISA test results are presumptive and require confirmation by the clinician.

- Testing should only be performed on patients with clinical symptoms. This test is not intended for screening the general population. The positive predictive value depends on the likelihood of the disease being present.
- Serological cross-reactivity across the mycobacterium group may be present.
- Positive results should be interpreted in the context of clinical and other laboratory findings and may not indicate active Scrub typhus.
- Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.
- The reagents supplied in this kit are optimized to measure OT-derived antigen reactive antibody levels in serum.
- Repeated freezing and thawing of reagents supplied in the kit and of specimens must be avoided. Do not freeze the liquid TMB substrate.
- Hemolyzed and lipemic specimens may give false values and should not be used.
- The assay performance characteristics have not been established for visual result determination.
- Results from immunosuppressed patients must be interpreted with caution.
- Generally primary responders exhibit mainly monotypic antibody responses; however, during successive infections the antibody response broadens to include heterotypic reactivity to other related bacteria in the same or different antigenic groups [5].
- Serum and Plasma Comparisons: The assay described here has been optimized with serum. Care should be taken on the quality of sample. Particulate, lipemic, hemolysed and aged samples should not be used. Use of freshly drawn sample is preferred.

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Scrub Typhus *Detect* IgM ELISA

Quick Instruction Card

Procedure:

1. Allow reagents to reach room temperature (RT).
2. Using Sample Dilution Buffer for Scrub Typhus, dilute samples and controls to 1:100. Positive and negative controls should be assayed in duplicate. Test samples may be assayed in singlet. Use at least 4µL serum from samples and controls when making dilutions.
3. Apply 100µL of 1:100 diluted samples and controls per well according to Sample Application chart below, or any preferred arrangement.

Example for Sera Application, 1:100 Diluted Samples, 100µl per well

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative Control	Negative Control	S# 13	S# 21	S# 29	S# 37	S# 45	S# 53	S# 61	S# 69	S# 77	S# 85
B	Positive Control	Positive Control	S# 14	S# 22	S# 30	S# 38	S# 46	S# 54	S# 62	S# 70	S# 78	S# 86
C	S# 1	S# 7	S# 15	S# 23	S# 31	S# 39	S# 47	S# 55	S# 63	S# 71	S# 79	S# 87
D	S# 2	S# 8	S# 16	S# 24	S# 32	S# 40	S# 48	S# 56	S# 64	S# 72	S# 80	S# 88
E	S# 3	S# 9	S# 17	S# 25	S# 33	S# 41	S# 49	S# 57	S# 65	S# 73	S# 81	S# 89
F	S# 4	S# 10	S# 18	S# 26	S# 34	S# 42	S# 50	S# 58	S# 66	S# 74	S# 82	S# 90
G	S# 5	S# 11	S# 19	S# 27	S# 35	S# 43	S# 51	S# 59	S# 67	S# 75	S# 83	S# 91
H	S# 6	S# 12	S# 20	S# 28	S# 36	S# 44	S# 52	S# 60	S# 68	S# 76	S# 84	S# 92

4. Cover plate with parafilm or platecover, incubate plate at 37°C in an incubator for 30 minutes.

   NOTE: When covering plate, be careful not to touch bottom of wells since that could interfere with the reading of the plate.

5. Wash plate six times with 1X Wash Buffer: 300µL per well (prepared from 10X Wash Buffer, ensuring all salt crystals are dissolved).
6. Apply 100µL of ready to use Enzyme-HRP Conjugate per well. Cover (same as step 4) and incubate plate at 37°C in an incubator for 30 minutes.
7. Wash plate six (6) times with 1X wash buffer.
8. Apply 150µL EnWash to each well, incubate at RT for 5 minutes.
9. Wash plate six (6) times with 1X wash buffer.
10. Apply 100µL Liquid TMB Substrate to each well. Incubate at RT, in a dark place, for ten (10) minutes.
11. Apply 50µL Stop Solution to each well in the same order and at the same speed as Liquid TMB Substrate.
12. After one (1) minute read OD 450 nm values with a Microtiter plate reader. Do not subtract any blank background.

Data Analysis:

Results are determined by strength of average OD values for a given sample.

*Cut-off: Calculation of cut-off OD requires running normal, confirmed scrub typhus and unrelated disease specimens from relevant endemic and non-endemic areas. See the Results section for more information

*For statistically valid results %CV <20%.

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IVD

TROUBLESHOOTING

Problem	Possible Cause	Possible Resolution
High Control/Sample OD Absorbances	Incorrect component used	Do not combine controls or reagents between different lots of the ELISA kits.
	Samples incorrectly diluted	Sera should be diluted 1:100 in kit's sample dilution buffer.
	Cross contamination of wells	A new tip must be used for every test or control sera.
	Incomplete washing of wells	Wells must be completely filled and emptied 6 times during each wash cycle.
	Incubation times too long	Incubation times vary, please refer to the "Test Procedure" section for correct times.
	Humidity	Do not use a humidified chamber for incubations.
	Conjugate contamination with TMB	It is recommended to use a new pipette/ pipette tip each time to dispense conjugate and TMB.
	Incorrect wavelength filter	The optical density readings must be read with **only** a 450nm filter. There must not be any background subtraction.
Low Control/Sample OD Absorbances	Samples incorrectly diluted	Sera should be diluted 1:100 in kit's sample dilution buffer.
	Kit expiration date and storage	Verify that the kit is not expired and that components were properly stored
	Incorrect component used	Do not combine controls or reagents between different lots of the ELISA kits.
	Component temperatures	All kit components must be equilibrated at room temperature for optimal performance.
	Incubation times too short	Incubation times vary, please refer to the "Test Procedure" section for correct times.
	Incubation temperature too low	Verify that incubators are calibrated and that the temperatures are monitored.
	Conjugate contamination	The conjugate is very susceptible to contamination. It is recommended to use a new pipet/pipette tip each time to dispense conjugate. Keep the lid on the conjugate unless in use. When possible, dispense conjugate in a clean laminar flow hood or biological safety cabinet.
	TMB contamination with Stop solution	It is recommended to use a new pipet/ pipette tip each time to dispense TMB and stop solution.
	Use of reagents in the wrong sequence, or omission of step(s)	Check the "Test Procedure" section and component labels prior to use.
	Incorrect wavelength filter	The optical density readings must be read with **only** a 450nm filter. There must not be any background subtraction.