# ID FISH Technology, Inc.

# ID-FISH Plasmodium Genus™ and Species Test Kits

Cat. No. 50 tests

## INTENDED USE

**ID-FISH Plasmodium Genus (FISH-PGenus), ID-FISH Plasmodium falciparum (FISH-Pf), ID-FISH Plasmodium vivax (FISH-Pv) are** intended for *in vitro* diagnostic use in the clinical laboratory for detection of *Plasmodium* species in human blood samples from patients of all ages. The assay kits should be used only on samples from patients with a clinical history, signs and symptoms consistent with malaria and is not intended as a screen for asymptomatic patients. The **ID-FISH Plasmodium genus** test is semiquantitative and can replace the thick and thin Giemsa-stained smear microscopy currently used as the standard for detection of Malaria parasites, due to the higher sensitivity of the **ID-FISH Plasmodium genus** test. Positive results should be supplemented with the Plasmodium species specific test kits, **ID-FISH Plasmodium falciparum** and **ID-FISH Plasmodium vivax** test kits for *Plasmodium falciparum* and *Plasmodium vivax,* respectively, to identify the organism to the species level. These kits can also be used to manage patients under treatment, since it detects only live parasitic cells. Negative results should not be used to exclude malarial disease.

Insufficient specimens were available to establish clinical performance for *P. ovale* and *P. malariae*.

For *in vitro* diagnostic use only.

## SUMMARY AND EXPLANATION

Malaria is a serious, often fatal, parasitic disease caused by infection of the red blood cells with protozoan parasites of the *Plasmodium* species. Large areas of Africa, India, South East Asia, Middle East, Central and South America, Hispaniola and Oceania are considered at risk for malaria[1]. World Health Organization estimated that there were 216 million episodes of malaria in 2010, leading to approximately 655,000 deaths.[2] Africa accounted for 81% of all malaria cases, with over 595,000 deaths. Globally, approximately 86% of malaria deaths occurred in children under 5 years of age.[2]

Malaria is diagnosed worldwide either by examination of a stained whole blood smear or through use of rapid diagnostic tests. The latter detect parasite specific antigens or enzymes and have some ability to differentiate among the species of *Plasmodium*.[1, 3] When used by a skilled and careful technician microscopy can detect densities of as low as 5-10 parasites per µl of blood. However, in resource-limited countries, the detection capabilities of a typical microscopist might be more realistically placed at 100 parasites per µl of blood.[4] Microscopy has the advantage that it can be used to assess the parasite load as well as to assess antimalarial treatment. Rapid diagnostic tests offer the advantage of quick diagnosis, but often give false negative results.[5] WHO recommends that antimalarial treatment be limited to test positive cases. However,

treatment based on clinical suspicion should be considered when a parasitological diagnosis is not accessible within 2 hours. [1]

More recently, Giemsa stained smear microscopy has been compared to PCR. The Giemsa stained smear had a sensitivity level of 55% to 65%[5]. Unfortunately, PCR is time-consuming, expensive and requires considerable expertise. Fluorescent *in situ* hybridization (FISH) technology has been used to detect infectious agents in clinical samples.[6-11] For detection of *Plasmodium*, the FISH technology is easy to perform, less likely to have false-positive results, and less-expensive to perform than PCR. It has the advantages of Giemsa smears in that it is rapid and the load of parasites can be determined.

## PRINCIPLES OF THE PROCEDURE

Fluorescent *in situ* hybridization is a microscopic technique in which a DNA probe is labeled with a fluorescent dye and then hybridized on a slide with a target ribosomal RNA of the pathogen in a clinical sample. Whole blood from a patient (EDTA preserved) suspected of malaria is mixed with a specific fixative (smear preparation reagent) and smeared onto a glass slide, air-dried, and preserved with methanol. The smear is then hybridized with a fluorescein-labeled DNA probe complimentary to the rRNA of *Plasmodium* genus, *P. falciparum* or *P. vivax*, depending on the probe used. Following hybridization, the slide is washed to remove excess probe. The processed smear is mounted with mounting medium containing counterstain and covered with a glass cover slip. The slide is then viewed under the fluorescent microscope. *Plasmodium* species, if present, will appear green under a fluorescent filter with the Plasmodium genus probe; P. falciparum will appear green with the *P. falciparum* probe, and *P. vivax* will appear green with the *P. vivax* probe. Non-Plasmodium organisms will not give any green signal when viewed under the florescent filter. In addition, the species-specific probe reagents (*P. falciparum* and *P. vivax*) contain an additional genus probe and, if a red filter is used, parasites of any of the *Plasmodium* species will appear red.

Since ribosomal RNA is present in the cytoplasm of the parasite and is visible within red cell halos, the entire parasite will give a fluorescent signal. Each infected cell can be counted, making the test semiquantitative and allowing the load of parasitemia to be determined. Unlike immunoassays and PCR assays, the assay detects ribosomal RNA within the cell; the parasite must be actively infecting the patient's red blood cells to produce a positive result.

## REAGENTS AND MATERIALS

The following kits and reagents are available and are sold separately:

| **Product name:** | **Part number:** |
|---|---|
| **Smear Preparation Reagent** | **SPR03** |
| **ID-FISH Plasmodium Genus Test Kit** | **PlasGK04** |
| Plasmodium Genus Hyb A | PlasGHA03 |
| Plasmodium Genus Hyb B | PlasGHB03 |
| 2.5x Plasmodium Wash Buffer | PlasWB03 |

| | |
|---|---|
| 10x Plasmodium Rinse Buffer | PlasRB03 |
| Plasmodium Counterstain | PlasC03 |

| **ID-FISH Plasmodium falciparum Test Kit** | **PfalK04** |
|---|---|
| Plasmodium falciparum Hyb A | PfalHA03 |
| Plasmodium falciparum Hyb B | PfalHB03 |
| 2.5x Plasmodium Wash Buffer | PlasWB03 |
| 10x Plasmodium Rinse Buffer | PlasRB03 |
| Plasmodium Counterstain | PlasC03 |

| **ID-FISH Plasmodium vivax Test Kit** | **PvivK04** |
|---|---|
| Plasmodium vivax Hyb A | PvivHA03 |
| Plasmodium vivax Hyb B | PvivHB03 |
| 2.5x Plasmodium Wash Buffer | PlasWB03 |
| 10x Plasmodium Rinse Buffer | PlasRB03 |
| Plasmodium Counterstain | PlasC03 |

Each **ID-FISH Plasmodium Test Kit listed above** contains sufficient materials for 50 tests. The following kit is also available that contains sufficient genus test reagents for 30 tests and 10 each of *P. falciparum* and *P. vivax* species assays.

| **ID-FISH Plasmodium Genus, P. falciparum, P. vivax Test Kit** | **PlasGFVK04** |
|---|---|
| Plasmodium Genus Hyb A-30 | PlasGHA3003 |
| Plasmodium Genus Hyb B | PlasGHB03 |
| Plasmodium falciparum Hyb A-10 | PfalHA1003 |
| Plasmodium falciparum Hyb B | PfalHB03 |
| Plasmodium vivax Hyb A-10 | PvivHA1003 |
| Plasmodium vivax Hyb B | PvivHB03 |
| 2.5x Plasmodium Wash Buffer | PlasWB03 |
| 10x Plasmodium Rinse Buffer | PlasRB03 |
| Plasmodium Counterstain | PlasC03 |

Reagents are supplied ready for use except where indicated. The expiration date of the kit is as indicated on the outer box label. Once reconstituted, reagents must be used within 6 months. Do not use kit beyond its expiration date on the outer box, regardless of when the reagents are reconstituted.

| REAGENT | PART NUMBER | CONTENTS | Volume |
|---|---|---|---|
| **Smear Preparation Reagent** | SPR03 | Phosphate buffered saline with formaldehyde | 200µl |
| **All Hyb A** | NA | Fluorescent-labeled probes | Lyophilized powder |
| **All Hyb B** | NA | Hybridization powder. Contains chaeotropic salt | Lyophilized powder |
| **2.5x Plasmodium Wash Buffer** | PlasWB03 | Buffer with detergent | **50 mL** |
| **10x Plasmodium Rinse Buffer** | PlasRB03 | Buffer with detergent | **6 mL** |
| **Plasmodium Counterstain** | PlasC03 | Glycerol with counterstain | **1.75 mL** |

## Materials not supplied but required to be purchased from ID-FISH

Dual Band Filter Set (Cat. No. IDF004) specific to **ID-FISH** hybridization probes**.**

## Equipment required <u>and</u> may be purchased from ID-FISH

Staining Dish Set – Stainless-steel staining dish with cover and slide holder (Cat. No. IDF005)
Stainless steel Drying Rack (Cat. No. IDF006)
Fluorescent Microscope (Cat. No. IDF001)
100x Oil Objective (Cat No. IDF002)
Mercury bulbs (Cat No. IDF003)

## Consumables required <u>and</u> may be purchased From ID-FISH

Glass cover slips – 100 ea 22x22 mm, thickness 0.15mm (Cat No. IDF008)
Plastic Cover slips – 100 ea (Cat. No. IDF009)
50 Hybridization bags (Cat. No. IDF010)

## Materials required, but not provided

- Pipettor 20 to 200 µl and 1 ml.
- Clean glass microscope slides
- Positive Control Smears (malaria positive EDTA blood less than 3 days old)
- Negative Control Smears (non-malarial EDTA blood less than 3 days old)
- 100% Methanol
- Water, deionized or distilled
- 1.5 ml microcentrifuge tubes

- Immersion oil - Must comply with the microscope objective and be non-fluorescent.
- Incubator at 37±1º C
- Slide warmer at 45-50ºC

# PRECAUTIONS

**For *in vitro* diagnostic use only.**

For professional use only by personnel trained in laboratory techniques and experienced in fluorescent microscopy.

### Safety precautions

The Hyb B contains guanidine thiocyanate and Igepal. May cause eye, skin and respiratory irritation. Harmful if swallowed. In case of eye contact, rinse eyes immediately with water. Seek medical advice. Light sensitive.

SPR03 contains <5% formaldehyde. May cause harm to the unborn child. Keep out of reach of children. Carcinogen. May cause eye, skin and respiratory irritation. May be fatal if inhaled, swallowed or absorbed through skin. Wear gloves and eye protection. Unused reagent should be discarded in hazardous waste.

Material Safety Data Sheets are available upon request.

Establish biosafety precautions in handling human blood specimens and microbiological hazards. Only qualified personnel should perform venipuncture procedures.

Follow standard biological safety precautions. Do not eat, drink, smoke apply cosmetics, insert contact lenses, store or prepare food within the designated work area.

Dispose of reagents in accordance with federal, state and local regulations.

### Technical Precautions

Do not use reagents after the expiration dates printed on labels.

Reagents are provided at fixed concentrations. Assay performance may be affected if the reagents are modified in any way or are not stored under the recommended conditions as specified in "Storage and Preparation of Kit Components". Do not mix reagents between different kits.

Avoid microbial and parasite contamination of reagents.

Avoid any cross-contamination of samples and reagents, as this may give erroneous results.

Do not allow pipet tips to touch the smear, or allow tips to contaminate the reagents.

Do not allow dropper bottle tip to touch the smear as this may cause cross contamination of material between slides

It is important that the microscope is functioning properly. Make sure that the microscope bulb is correctly adjusted and has not aged beyond its specified lifetime.

Do not use filters other than the ID-FISH Dual Band Filter set (**Cat. No. IDF004**)

## STORAGE AND PREPARATION OF KIT COMPONENTS

To ensure optimal kit performance, it is important that kit components are stored and prepared according to the following instructions:

**Storage**
Store kit components at 2-30ºC. If refrigerated, warm kit components to room temperature prior to use.

**Preparation of Reagents**

**Hyb B** reconstitution

- Each Hyb B reagent is reconstituted as follows prior to use:
  (i) Pipette 790 µl of distilled or deionized $H_2O$ into Plasmodium genus Hyb B vial.
  (ii) Pipette 825 µl of distilled or deionized $H_2O$ into Plasmodium falciparum Hyb B vial.
  (iii) Pipette 827 µl of distilled or deionized $H_2O$ into Plasmodium vivax Hyb B vial.
- Use care not to disturb powder or create bubbles.
- Incubate at 37±1°C for 30 –60 minutes or up to 24 h. Assure that powder is completely dissolved.
- Gently pipette up and down to homogenize liquid.
- Immediately use to prepare hybridization mixture or store at 15-30°C for up to 2 days.

**Hybridization Mixture preparation:**

- Ensure **Hyb B** is completely dissolved and homogeneous.
- Pipette the following to prepare mixture:
  (i) For each of the 50 test kits, (PlasGK04, PfalK04, and PvivK04)
    1. Pipet 660 µl from Plasmodium genus Hyb B to Plasmodium Genus Hyb A.
    2. Pipet 660 µl from Plasmodium falciparum Hyb B to Plasmodium falciparum Hyb A.
    3. Pipet 660 µl from Plasmodium vivax Hyb B to Plasmodium vivax Hyb A.
  (ii) For the ID-FISH Plasmodium Genus, P. falciparum, P. vivax Test Kit (PlasGFVK04) use the following dilutions:
    1. Pipet 396 µl from Plasmodium genus Hyb B to Plasmodium Genus Hyb A-30.
    2. Pipet 132 µl from Plasmodium falciparum Hyb B to Plasmodium falciparum Hyb A-10.
    3. Pipet 132 µl from Plasmodium vivax Hyb B to Plasmodium vivax Hyb A-10.

- Use care not to disturb powder or create bubbles.
- Mix well by gently pipetting up and down.
- Allow the tube to sit at room temperature for 15 min before use, to ensure that the solution is homogeneous.
- Store in the dark at 2-30º C.
- Prepared Hyb Mix is stable for up to 6 months.
- Discard remainder of **Hyb** B in hazardous waste.

### Wash Buffer

- Add full content (50ml) of **2.5x Plasmodium Wash Buffer** into a clean wash bottle.
- Add 75ml of distilled or deionized $H_2O$ into the same container to make a total of 125ml of **1x Wash Buffer**.
- Mix well. Avoid creating bubbles.
- Store at 15-30$^{o}$C up to 6 months.

### Rinse Buffer

- Add 54 ml of distilled or deionized $H_2O$ into the bottle containing 6ml of **10x Plasmodium Rinse Buffer**. This makes a total volume of 60ml of **1x Rinse Buffer**.
- Mix well. Avoid creating bubbles.
- Store at 15-30$^{o}$C up to 6 months

## SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE

### Specimen Collection

Blood collection should be performed immediately upon first suspicion of malaria and, preferably, prior to antimalarial drug treatment.

### Specimen Type

Collect venous blood aseptically by standard venipuncture procedure, into EDTA vacutainer tube. Assure that sufficient blood is collected to fill the tube adequately for correct ratio of blood to EDTA. To prevent clotting, mix blood thoroughly with the anticoagulant by inverting the tube several times.

If slides cannot be prepared immediately, store the tubes at 4°C for no longer than 3 days from the date of collection, prior to testing.

Note: If regulatory agencies require submission of smears from positive patients, or if microscopic by Giemsa/ Wright staining is desired, smears for stains should be prepared within one hour of collection without addition of SPR.

## Criteria For Unacceptable Blood Specimens

- Clotted blood.
- Grossly hemolyzed whole blood.
- Inadequately filled tube.
- Improperly labeled specimens or improperly ordered tests.
- Care should be used to avoid contamination of one sample with another sample.
- Any contact with *Plasmodium* rDNA during collection, handling, or storage contaminates the patient sample and must be avoided.

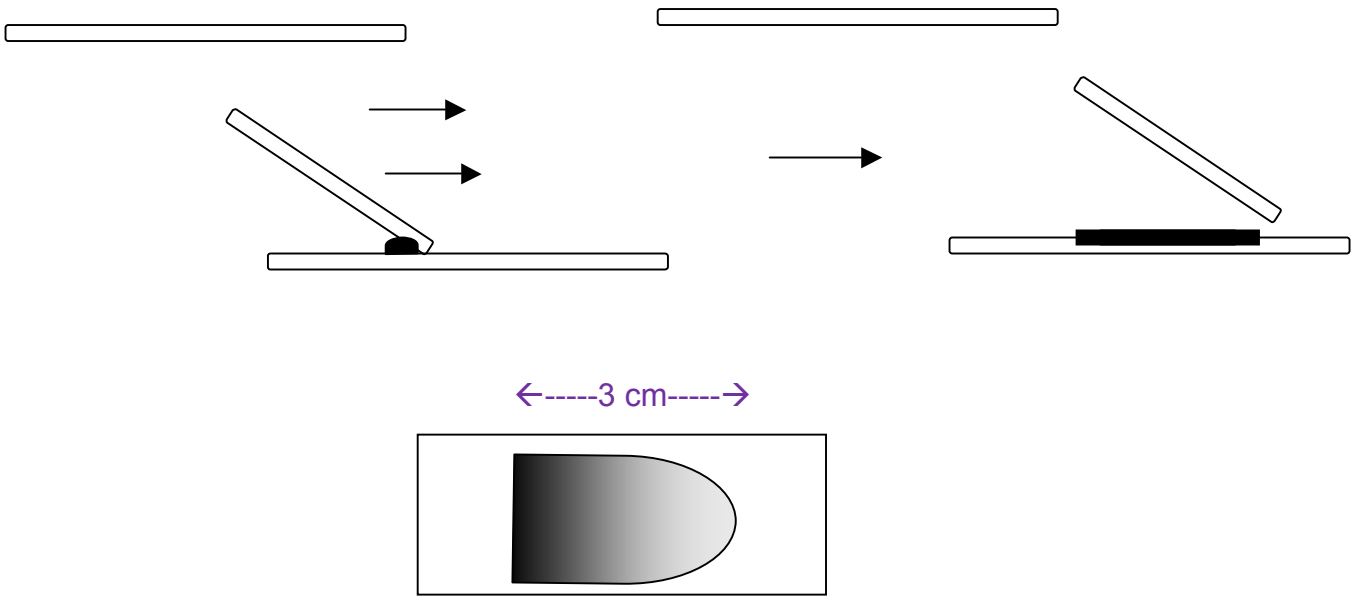
## Preservation of blood

- Add 5 µl of Sample Preservation Reagent (SPR) to a 1.5 ml microcentrifuge tube.
- Using a new pipet tip, add 15 µl of EDTA preserved patient blood to the tube.
- Mix immediately by inverting the tube several times.

## Preparation of Smears

**Prepare at least 3 slides per patient.**
**(Slides prepared for FISH cannot be used for Giemsa staining.)**

- Drop/pipette 4 µl of SPR treated blood onto one end of a glass slide.
- Hold a second slide with one edge at a 40 to 45° angle and immediately draw into the drop of blood. Allow the blood to spread almost to the width of the slide.
- Gently push the second slide at a moderate speed forward with a light pressure, to smear blood into a thin film (~3 cm long). See diagram. The end of the smear should be feathered out, smooth and even.
- Air dry completely at least 1 h. Alternatively place on slide warmer at 45-50°C for 30 min. Let slides cool.
- Add one to two drops of 100% methanol to cover smear completely.
- Air-dry completely




**Criteria For Unacceptable Smears**

- Smears that are poorly made.
- Smears made from frozen blood.
- Smears that are stored frozen.
- Smears made from grossly hemolyzed blood.
- Thick smears are not appropriate for the assay.

**Storage of Smears**

Methanol fixed, air-dried smears are stored at room temperature with desiccant. They are stable for 6 months, based on stability study to-date, if stored in a cool dark place.

## Test Procedure

Place wet paper towels on a tray in an incubator set at 37±1$^{o}$ C.
Assure that all reagents are prepared and warmed to room temperature.
Label slides appropriately for the specific hybridization reagent being tested.

Hybridization reaction:

- Add 12 µl hybridization mixture (in vial labeled **HybA**) onto each sample smear.

- Cover smear with plastic c**o**ver slip. Avoid creating bubbles and disturbing smear.
- Put slide into Hybridization Bag.
- Place Hybridization Bag on wet paper towels in a 37±1 $^{o}$C incubator.
- Incubate slides in the dark for 30 minutes.

Wash step:

One at a time, process each slide as follows:

- Remove slide from humid incubator.
- Remove slide from hybridization bag.
- Gently remove cover slip from slide.
- Place slide on rack of staining dish
- **IMMEDIATELY** add 1ml of Wash Buffer to top of smear. **DO NOT LET SMEAR DRY.**
- **Repeat procedure for all slides**

Once all slides have been removed from incubator and the wash buffer added, allow slides to sit for 2-5 minutes. . **DO NOT LET SMEARS DRY.**

- Tilt slides to remove buffer.
- Add 1ml Wash Buffer to all slides.
- Allow buffer to sit on slides for 2-5 minutes.
- Tilt slides to remove buffer. **DO NOT LET SMEARS DRY.**

Rinse step:

- Add 1 ml of Rinse buffer to each slide.
- Allow buffer to sit on slides for 2-5 min
- Tilt slides to remove buffer
- Air dry slides in the dark for 15-30 min, or until dried.

Mounting

- Once dried, add 30 µl of Counterstain to each smear and cover with glass cover slips. Avoid creating bubbles.
- Store slides in dark
- Slides are ready for microscopic viewing after 15 min but should be viewed within 4 h
- Do not expose the slides to direct sun light or other strong light sources as this may lead to fluorescence quenching.

## QUALITY CONTROL

Control material should be tested in accordance with the guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

To monitor the assay, reagent performance and day-to-day variation, positive controls for *P. falciparum* (PF) or *P. vivax* (PV) along with a negative control (non-malarial blood) must be tested with each run. Negative controls can be prepared from EDTA whole blood collected from normal subjects. Prepare and test negative control blood in the same manner as patient blood specimens.

Positive control slides should be prepared from previously positive patient blood samples. Slides prepared in the same manner as patient samples are stable for 6 months if stored in a dry place at room temperature.

The positive control must show fluorescing parasites (green) within red blood cell halos and the negative control must show no parasites within red cells for the test to be valid. Record control reactions and document control failures. Do not use kit if controls do not perform accurately

## INTERPRETATION AND REPORTING OF RESULTS

Examine each slide with a fluorescence microscope, using oil immersion (100x) objective and dual pass filter.

Positive Result: Viewing the slides processed with the FISH-PGENUS Test Kit will show green fluorescence for *P. falciparum, P. vivax, P. malariae* and *P. ovale* parasites (all forms of parasites including rings, gametocytes, sporozoites, spiral forms ,etc.). If the smear is positive by the FISH-PGENUS Test Kit, further testing can be performed to determine whether the smear is positive for either *P. falciparum* or *P. vivax* , using the FISH-Pf or FISH-Pv Test reagents.

With the FISH-Pf hybridization reagents, *P. falciparum* will fluoresce green and P. vivax*, P. malariae* and *P. ovale* will not fluoresce green with the green filter. However, with the red filter, all species of the genus *Plasmodium* will fluoresce red. If the fluorescence is green, the specimen is positive for *P. falciparum*. If there is no green fluorescence, the specimen is negative for *P. falciparum.*

With the FISH-Pv hybridization reagents, *P. vivax* will fluoresce green and P. *falciparum, P. malariae*, and *P. ovale* will not fluoresce green with the green filter. However, with the red filter, all species of the genus *Plasmodium* will fluoresce red. If the fluorescence is green, the specimen is positive for *P. vivax*. If there is no green fluorescence, the specimen is negative for *P. vivax.*

Negative Result: Viewing the slides processed with the FISH-PGENUS Test Kit will show no green fluorescence for non-malaria parasites and normal blood smears under the green filter. Examine a minimum of 300 fields on each slide using oil immersion (100x) lens before calling a result negative.

**Reading and Grading and Reporting the Malaria Smears**

| Classification | Grading Code | Giemsa or FISH (1000x) |
|---|---|---|
| None | 0 | No parasites /300 fields |
| Doubtful | ± | 1-2 parasites /300 fields |
| Rare | 1 | 1-9 parasites /100 fields |
| Few | 2 | 1-9 parasites /10 fields |
| Moderate | 3 | 1-9 parasites /1 field |
| Numerous | 4 | >9 parasites /1 field |

Alternatively, malaria smears are reported as the percentage of infected RBCs per 100 RBCs.

## LIMITATIONS

1. A positive test does not define the presence of disease. The test detects the presence of malaria parasites in the blood specimens. Results should be used in conjunction with other clinical findings to establish a diagnosis.
2. As with all in vitro diagnostic tests, a negative test result does not exclude the possibility of the presence of malaria parasite. This may occur when the parasite level in the sample is below the detection level of the test. A physician, in light of other laboratory results and clinical findings, should interpret test results.
3. Proper specimen collection and processing are essential to achieve optimal performance of the test. See Specimen Collection, Storage and Transportation section.
4. The type and condition of the instrumentation used will influence the visual appearance of the image obtained. The fluorescence may vary due to the type of microscope employed, the light source and the level of rRNA in the cells. Each laboratory should establish its own criteria for reading the results using appropriate controls.
5. Reading smears with a fluorescence filter other than supplied by ID-FISH can result in erroneous results.
6. The product has not been validated with specimens other than whole blood.
7. Successive films every 8 h for up to three days may be needed for diagnosis.

## EXPECTED VALUES

The expected FISH-PGENUS Test result will vary anywhere from 0.1% to >60%, depending on the area where the samples are being tested. For example, in US, the expected positive rate is <0.1%. Positivity rates are much higher in the tropics and subtropical areas of the world. In endemic areas, the expected positive rate can be as high as 50% or more.

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