ANCA TEST SYSTEM

For in vitro Diagnostic Use
For Professional Use

INTENDED USE: This is an indirect fluorescent antibody test for the semi-quantitative detection of antineutrophil cytoplasmic autoantibodies (ANCA) in human serum. The test system is to be used as an aid in the detection of antibodies associated with autoimmune vasculitis.

Summary and Explanation of the Test

Antineutrophil cytoplasmic autoantibodies (ANCA) are a group of antibodies that react with cytoplasmic antigens in human neutrophils. Although these antibodies were originally reported in 1964 (1), the first report linking these antibodies to disease was in 1982, when Davies et al reported the antibodies in eight patients with segmental necrotizing glomerulonephritis (2). In 1984, four more patients with vasculitis and glomerulonephritis were reported. In 1985, van der Woude et al showed that ANCA had a high association with granulomatosis with polyangiitis, and that antibody titer correlated with disease activity (3). In 1988, Falk and Jennette reported that ANCA have more than one antigen specificity (4). A subsequent report showed that the specificity of ANCA correlated with the pathologic features of vasculitides (5).

In the immunofluorescent test for ANCA, several patterns of cellular staining may be seen. Two major patterns of staining have been described and well characterized when ethanol-fixed neutrophils are used in the immunofluorescent ANCA test. Autoantibodies that show a fine granular cytoplasmic pattern, called C-ANCA, are usually directed against a serine protease, Proteinase 3 (PR3). These autoantibodies have been shown to have a high association with granulomatosis with polyangiitis. The other major pattern of staining, the perinuclear, or P-ANCA pattern, which is usually due to antibodies directed against myeloperoxidase (MPO), has been associated with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis (4). The P-ANCA pattern is an artifact induced by the use of ethanol as a fixative (4). If the neutrophils are fixed in formalin, myeloperoxidase (the major antigen responsible for the P-ANCA pattern in ethanol fixed cells) remains associated with the primary (alpha) granules, and shows a granular cytoplasmic distribution. Proteinase 3 (PR3) remains associated with the primary (alpha) granules in either ethanol or formalin fixatives.

Principle of the Test

The Immuno Concepts antineutrophil cytoplasmic autoantibodies (ANCA) test uses the indirect fluorescent antibody (IFA) technique first described by Weller and Coons (6). Diluted patient samples are incubated with human neutrophils, which are fixed on glass microscope slides, to allow specific binding of ANCA. If ANCA are present, the autoantibodies bind to neutrophilic antigens. After washing to remove non-specific antibodies, the substrate is incubated with anti-human IgG conjugated to fluorescein. When results are positive there is the formation of a stable three-part complex consisting of fluorescent anti-human antibody bound to human ANCA, which are bound to antigen located in the cells. This complex can be visualized with the aid of a fluorescent microscope. Samples that are positive for C-ANCA will show a distinctive granular cytoplasmic staining of the neutrophils on both ethanol and formalin fixed slides. In samples that are positive for P-ANCA, a diffuse or peripheral nuclear staining of the neutrophils will be seen on ethanol fixed cells, and a granular staining of the cytoplasm will be seen on formalin fixed cells. If the sample is negative for ANCA, specific staining of the neutrophils will not be seen.

System Components - materials provided

Use: All components come ready to use with no aliquoting or reconstitution required (except the PBS buffer which must be dissolved in deionized or distilled water before use).

Storage: All components can be stored under refrigeration at 2-10°C. After reconstitution, PBS buffer should be stored in screw cap containers and store between 2-25°C for up to four weeks or until signs of contamination or other visible changes occur.

Stability: All components remain stable at least 12 months from date of manufacture. Do not use any component beyond its expiration date.

REACTIVE REAGENTS

ANCA Substrate Slides SLIDE: Slides containing human neutrophils stabilized and fixed directly on the test wells. Unique moat slide design minimizes cross contamination of wells during testing. The slide pouch is filled with an inert non-toxic gas that contributes to the stability of the cells.

ANCA Sample Diluent SOLN|DIL: Catalog number 10100 (100 ml). Proprietary buffered sample diluent used to dilute patient samples.

C-ANCA Positive Control CONTROL| +: Catalog number 10021-12. Ready to use dropper vial containing 1.0 ml of C-ANCA positive human control serum. This serum demonstrates granular staining of the cytoplasm between the nuclear segments of the neutrophils on both ethanol and formalin fixed slides.

P-ANCA Positive Control CONTROL| +: Catalog number 10021-11. Ready to use dropper vial containing 1.0 ml of P-ANCA positive human control serum. This serum demonstrates diffuse or peripheral nuclear staining of the neutrophils on ethanol fixed slides and granular cytoplasmic fluorescence on formalin fixed slides.

C-ANCA Titratable Control TC: Catalog number 10026-12. Ready to use vial containing 0.25 ml of liquid stable C-ANCA positive human control serum. This control should be treated as an undiluted patient sample. See label for mean titer information.

P-ANCA Titratable Control TC: Catalog number 10026-11. Ready to use vial containing 0.25 ml of liquid stable P-ANCA positive human control serum. This control should be treated as an undiluted patient sample. See label for mean titer information.

Negative Control CONTROL| -: Catalog number 10031. Ready to use dropper vial containing 1.0 ml of ANCA negative human control serum. This serum demonstrates low intensity, nonspecific dull green fluorescence of the neutrophils.

Fluorescent Antibody Reagent-IgG Specific CONJ|FITC: Catalog number 10009 (9ml). Anti-human IgG conjugated to fluorescein isothiocyanate (FITC). This reagent contains Evans Blue as a counterstain. Reagent comes ready to use in precision dropper bottles.

NON-REACTIVE COMPONENTS

PBS Buffer Powder PWDR|PBS: Catalog No. 1011. Phosphate-buffered saline powder (0.01 M, pH 7.4 ± 0.2). Each pouch contains sufficient buffer powder to make 1 liter. (One pouch of buffer powder is supplied for every five slides in complete test kits.)

> **Preparation:** Dissolve one pouch of buffer powder in 1 liter of deionized or distilled water, cover, and store between 2-25°C for up to four weeks or until signs of contamination or other visible changes occur.

Semi-Permanent Mounting Medium SOLN|MM: Catalog No. 1111. Ready-to-use dropper vial containing 5.0 ml glycerol-based mounting medium.

Coverslips CVSLP: Catalog No. 1042. Each packet contains ten 24 x 64 mm No. 1 glass coverslips.

Additional Materials Required - but not provided

Volumetric pipettes to deliver 20-25 µl volumes
Coplin jars or staining dishes
Squeeze bottle or Pasteur pipettes
Serological pipettes
One-liter containers (for PBS buffer)

Deionized or distilled water
Test tubes to prepare optional serum dilutions
Bibulous paper or paper towels
Chamber for incubation
Disposable gloves
Lab timer
Fluorescent microscope equipped with 495 nm exciter filter and 515 nm barrier filter

Precautions

1. All human source material used in the preparation of controls for this product has been tested and found to be negative (not repeatedly reactive) for antibody to human immunodeficiency virus-1 and human immunodeficiency virus-2 (HIV-1 & HIV-2), antibody to hepatitis C virus (HCV), and for hepatitis B surface antigen (HBsAg) by an FDA approved method. No test method can offer complete assurance that HIV-1, HIV-2, hepatitis C virus, hepatitis B virus, or other infectious agents are absent. Thus, all control sera should be handled in the same manner as potentially infectious materials.
2. All patient samples should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health Manual: *Biosafety in Microbiological and Biomedical Laboratories, 1999 Edition.*
3. Dilution of the components or substitution of components other than those provided in this system may yield inconsistent results.
4. Sodium azide (0.09%) is used as a preservative. Sodium azide may react with lead or copper plumbing and form explosive metal azide salts. When disposing of reagents, flush with ample volumes of tap water to prevent potential residues in plumbing. Sodium azide is a poison and may be toxic if ingested.
5. This kit is for *in vitro* diagnostic use.
6. The titratable control serum is intended for use in monitoring lot-to-lot and run-to-run reproducibility. It is not intended as a measurement of overall sensitivity or specificity of the assay.
7. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
8. Avoid splashing or generation of aerosols at all times.
9. Incubation times and temperatures other than those specified may give erroneous results.
10. Cross contamination of reagents or samples may give false results.
11. Reusable glassware must be washed and thoroughly rinsed free of detergents prior to use. All glassware must be clean and dry before use.
12. Bring all reagents, slides, and specimens to room temperature (18-25°C) prior to use.
13. Wear disposable gloves when handling specimens and reagents, and wash hands thoroughly afterwards.
14. Microbial contamination of reagents or samples may give false results.
15. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with a germicidal soap and copious amounts of water.

Specimen Collection

Collection: Serum is the preferred specimen. Approximately 5 ml of whole blood should be collected aseptically by venipuncture using a sterile vacuum collection tube or other suitable collection system. Allow blood to clot at room temperature (18-25°C). Serum should be separated from the clot by centrifugation as soon as possible to minimize hemolysis.

Interfering Substances: Sera exhibiting a high degree of hemolysis, icterus, lipemia, or microbial growth should not be used because a decrease in antibody titer may occur on positive samples. Specimens with very high levels of lipid may cause a non-specific fluorescent film to form over the cell substrate. Use of fasting specimens or clearing specimens by ultracentrifugation may eliminate this problem. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Storage: Sera may be stored at 2-10°C up to one week. If testing is further delayed, sera should be stored frozen at –20°C or lower. Serum should not be stored in a self-defrosting refrigerator or freezer.

CAUTION: Repeated freeze/thawing of patient samples may yield false positive or false negative results.

Interpretation of Results

QUALITY CONTROL

Positive, negative and blank controls should be run in the wells provided for quality control on each slide. The C–ANCA positive control should show bright apple-green fluorescent granular cytoplasmic staining of the neutrophils, between the nuclear segments on either ethanol or formalin fixed slides.

The P-ANCA positive control should show bright apple-green diffuse or peripheral nuclear staining of the neutrophils on ethanol fixed slides, and granular cytoplasmic fluorescence on formalin fixed slides. The negative control should not exhibit any bright green fluorescence. Low intensity, nonspecific dull green fluorescence may be observed in the negative control. The blank control is used to observe nonspecific fluorescence of the antibody reagent, and should not exhibit any green fluorescent staining. The counterstain provided in the conjugate may stain the cells a dull red color. If the controls do not appear as described, the test is invalid and should be repeated.

OPTIONAL TITRATABLE CONTROL
When reading titers, many laboratories begin reading with the well that contains the most dilute sample and read "backwards" to the 1:20 dilution. The first well in which clearly discernible cytoplasmic staining is visible is the titer end-point. We recommend this technique for determining titer end-points.

The mean titer and titer range (± one dilution on either side of the mean) determined for each lot number were established in our laboratory and are stated as a guide. This control is provided to allow each laboratory to assess the reproducibility (precision) of its ANCA testing. Since this control is not intended to be an indicator of titer accuracy, each laboratory should establish its own mean titer end-point for this sample, and should use this information to assess run-to-run reproducibility (precision).

Through multiple testing of this titratable control, using the Immuno Concepts Fluorescent ANCA Test Systems, a mean titer value has been established for each lot number. The lot number, mean titer and titer range (± one twofold dilution on either side of the mean) are stated on the vial label and should be used as a guide for the test system performance.

The values obtained in our laboratory may differ from your values. Some of the many factors that can affect your results may include, but are not limited to:

1. The type of light source used. Mercury light sources will produce greater excitation energy at 495 nm than Quartz/Halogen. The 50-watt, 100-watt, and 200-watt mercury light sources differ little in excitation energy at 495 nm. The 100-watt Quartz/Halogen light sources will produce greater excitation energy at 495 nm than 50-watt Quartz/Halogen.
2. The condition and age of the light source. This is particularly true for mercury light sources, which generally exhibit a gradual reduction in excitation energy at 495 nm prior to burning out. This gradual reduction in excitation energy can result in a significant loss in sensitivity over several weeks. This problem can be avoided by keeping a time log. For best results, replace 50-watt mercury bulbs at 100 hours, and 100 or 200-watt mercury bulbs at 200 hours. Quartz/Halogen light sources generally do not exhibit a gradual reduction in excitation energy prior to burning out.
3. The type of exciter filter used. Interference exciter filters provide greater sensitivity over a much narrower wavelength than absorption exciter filters. Refer to your fluorescent microscope manual or sales representative for more information.
4. Proper alignment of the microscope light path. Refer to your fluorescent microscope manual for instructions.
5. The numerical aperture of the objective. With incident light fluorescence (Epi), fluorescence is increased exponentially as the numerical aperture (NA) of the objective is increased additively. This may cause a 40X objective with a NA of 0.65 to read one or more dilutions lower than a 40X objective with a NA of 0.85. The numerical aperture is printed on the side of the objective.
6. Suppression filters. Suppression filters reduce specific wavelengths of excitation and may be used in reducing sensitivity. Refer to your fluorescent microscope manual or sales representative for more information.
7. Precision and accuracy of dilution technique, equipment, and performance of the test procedures.

INTERPRETATION OF PATIENT RESULTS
400X total magnification is recommended for viewing the neutrophils.

Negative: A serum is considered negative for ANCA if, at a dilution of 1:20, green fluorescent staining of the cells is less than or equal to the negative control well. Non-specific background staining due to heterophile antibodies or autoantibodies may be observed in the neutrophils.

Positive: A serum is considered positive for ANCA if, at a dilution of 1:20, the cells in each field demonstrate granular cytoplasmic fluorescence similar to that seen with the C-ANCA control on either ethanol or formalin fixed slides. Alternatively, a serum is considered positive for ANCA if, at a dilution of 1:20, the cells demonstrate diffuse or peripheral nuclear fluorescence similar to that seen with the P-ANCA control on ethanol fixed slides.

Reporting of Results

Screening: Results should be reported as positive or negative at the 1:20 dilution.

Staining Patterns: Many autoantibodies can cause staining of the cytoplasm and/or nucleus of the neutrophils. There are two major patterns of specific staining:

C-ANCA (classical or cytoplasmic staining): Staining of the alpha (primary) granules in the cytoplasm shows a consistent speckled cytoplasmic staining pattern, often with a concentration of staining between the lobes of the nucleus. The cytoplasmic speckling will be seen with either ethanol fixed or formalin fixed neutrophils.

P-ANCA (perinuclear staining): Smooth or homogeneous staining of the multi-lobed nucleus, often with marked peripheral staining of the nuclear lobes on ethanol fixed neutrophils. On formalin fixed neutrophils, these antibodies show a granular cytoplasmic staining.

Antinuclear and other autoantibodies can react with the neutrophils, so all positive samples should be tested for ANA using a HEp-2 cell substrate before reporting the presence of ANCA.

Optional titering: Results should be reported as the reciprocal of the dilution of the last well in which a positive reaction is seen.

Limitations of the Test

1. Diagnosis cannot be made on the basis of antineutrophil cytoplasmic antibody detection alone. The physician must interpret these results in conjunction with the patient's history and symptoms, the physical findings, and other diagnostic procedures.
2. Treatment should not be initiated on the sole basis of a positive test for antineutrophil cytoplasmic antibodies. Clinical indications, other laboratory findings, and the physician's clinical impression must be considered before any treatment is initiated.
3. Antinuclear antibodies present in patient samples may react with the cell substrate. If antinuclear antibodies are present, the ANCA results can often be interpreted because of the different character of the specific ANCA staining, differing titers, and/or intensity of reactions. If the fluorescent antinuclear antibody reaction is strong enough to obscure the ANCA staining, the test should be reported as "uninterpretable", and an alternative method must be used for determination of the patient's ANCA status.
4. Because of the many options available in fluorescent microscopes, it is recommended that light sources, filters, and optics be standardized when comparing patient titers between laboratories.
5. The results of this test should be used in conjunction with information available from the clinical evaluation and other diagnostic procedures to determine the patient's clinical status.

Expected Values

The expected value in the normal population is negative at a 1:20 screening dilution. In patients with disease, titer values as high as 1:640 have been reported (7).

Performance Characteristics

NORMAL SAMPLES

Serum samples from 497 blood donors (247 males and 250 females) were tested in parallel using the Immuno Concepts ANCA kit and another kit in commercial distribution. All of the samples that were positive on ethanol fixed slides in this population were also tested for Antinuclear Antibodies (ANA) using Immuno Concepts HEp-2 ANA Test System.

Among the normal samples there were 22 samples that were positive for ANA, and were considered uninterpretable for ANCA. The remaining discrepant samples were tested for antibodies to MPO and PR3 using ELISA tests to determine the true antibody status.

SERA PREVIOUSLY DETERMINED TO BE POSITIVE FOR ANCA BY INDIRECT IMMUNOFLUORESCENCE

Serum samples, which had been judged to be positive for ANCA using in-house IFA assays, were obtained from reference laboratories in the USA, the United Kingdom, and Australia. A total of 383 sera, which we expected to be positive for ANCA, were examined in this part of the study. These samples were tested in parallel using the Immuno Concepts ANCA kit and another kit in commercial distribution. Discrepant samples were tested for ANA using Immuno Concepts HEp-2 ANA Test System, and for antibodies to MPO and PR3 using ELISA tests.

Combining the results of the normal population and the abnormal population, we obtained the following data in the initial comparison for the Immuno Concepts ANCA Test System with Ethanol Fixed Human Neutrophils (Table 1):

		Comparison Method	
		Positive	Negative
IC Ethanol ANCA	Positive	324	94
	Negative	124	316

These data yield the following comparative statistics:
- Relative sensitivity: 72.3%
- Relative specificity: 77.1%
- Overall agreement: 74.6%

In the initial comparison for the Immuno Concepts ANCA Test System with Formalin Fixed Human Neutrophils we obtained the following data (Table 2):

		Comparison Method	
		Positive	Negative
IC Formalin ANCA	Positive	255	52
	Negative	45	528

These data yield the following comparative statistics:
- Relative sensitivity: 85.0%
- Relative specificity: 91.0%
- Overall agreement: 89.0%

Many autoantibodies, other than those associated with autoimmune vasculitis, can react with human neutrophils (8). In order to confirm that the antibodies detected by any immunofluorescent ANCA test are clinically significant, confirmatory tests using ELISA assays for myeloperoxidase (MPO) and proteinase 3 (PR3) are strongly recommended (9).

All of the discrepant samples in the above tables were tested for antinuclear antibodies and for antibodies to MPO and PR3. Taking the results of these tests into account, we see the overall results for the Immuno Concepts ANCA Test System with Ethanol Fixed Human Neutrophils in Table 3:

		Comparison Method	
		Positive	Negative
IC Ethanol ANCA	Positive	380	32
	Negative	6	434

These data yield the following comparative statistics:
- Relative sensitivity: 98.4%
- Relative specificity: 93.1%
- Overall agreement: 95.5%

The overall results for the Immuno Concepts ANCA Test System with Formalin Fixed Human Neutrophils are shown in Table 4:

		Comparison Method	
		Positive	Negative
IC Formalin ANCA	Positive	292	13
	Negative	5	548

These data yield the following comparative statistics:
- Relative sensitivity: 98.3%
- Relative specificity: 97.7%
- Overall agreement: 98.6%

SERUM SAMPLES FROM PATIENTS WITH KNOWN VASCULITIDES

Samples obtained from 102 patients with clinically characterized vasculitides were tested using the Immuno Concepts ethanol fixed neutrophils and the Immuno Concepts formalin fixed neutrophils. The results of this comparison are shown in Table 5:

Table 5

Clinical Diagnosis	Number	Positive	Staining Pattern (Ethanol Fixed)
Granulomatosis with polyangiitis	30	26 (86.7%)	All C-ANCA
Polyarteritis nodosa	12	8 (66.7%)	All P-ANCA
Microscopic polyarteritis	20	18 (90.0%)	All P-ANCA
Eosinophilic granulomatosis with polyangiitis	3	2 (66.7%)	One P-ANCA; one both P-ANCA and C-ANCA
Immune Complex Crescentic Glomerulonephritis	15	10 (66.7%)	All P-ANCA
Inflammatory Bowel Disease	22	17 (77.3%)	All atypical P-ANCA

CROSS REACTIVITY
Sera from 57 patients with various autoimmune disorders were tested on both the Immuno Concepts ANCA Test System with Ethanol Fixed Human Neutrophils and the Immuno Concepts ANCA Test System with Formalin Fixed Human Neutrophils. Three of these samples showed an atypical type of nuclear staining, which resembled the P–ANCA pattern, on the ethanol fixed neutrophils. All three of these samples were from patients with SLE, were positive for anti-DNA antibodies and showed a positive ANA with a homogeneous pattern. One additional sample showed cytoplasmic speckling on the ethanol fixed neutrophils, but was negative on the formalin fixed neutrophils and on the ANA test. This sample was judged to be a non-specific reaction. All other sera in this population were negative on both the ethanol fixed and formalin fixed neutrophils. Since many autoantibodies can react non-specifically with human neutrophils (8), positive immunofluorescent tests should always be confirmed using specific assays for the ANCA-associated antibodies.

REPRODUCIBILITY
Intra-run, inter-day and inter-lot studies were performed to demonstrate the reproducibility of the Immuno Concepts ANCA Test System with Ethanol Fixed Human Neutrophils and the Immuno Concepts ANCA Test System with Formalin Fixed Human Neutrophils. The sera used in this study included three P-ANCA positive samples (one that exhibited strong staining, one that exhibited moderate staining and one that exhibited weak staining), and three C–ANCA positive samples (one each that showed strong, moderate or weak staining). Six samples, which were negative for ANCA, were also used. In the intra-run study these 12 sera were tested in replicates of six wells each. For inter-day and inter-lot reproducibility, these 12 sera were each run on three lot numbers of kits on three separate occasions. The six negative samples were negative on all slides tested, and the positive samples were positive, with consistent fluorescent intensities, on all slides tested.

BIBLIOGRAPHY

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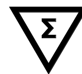
In the event of damage to the protective packaging, please contact Immuno Concepts prior to use.

Manufacturer

Authorized Representative in the European Community

Temperature Limitation



Contains Sufficient for <n> tests

Consult Instructions for Use

In Vitro Diagnostic Medical Device

MDSS GmbH
Schiffgraben 41
D-30175 Hannover, Germany

Immuno Concepts, N.A. Ltd. 9825 Goethe Road, Suite 350 Sacramento, CA. 95827
Technical Support USA: 1.800.251.5115 Outside USA: 1.916.363.2649
Email: technicalsupport@immunoconcepts.com

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ANCA Test Procedure

NOTE: If the laboratory is using an automated sample processing system, the procedure and recommendations of the manufacturer of the processor should be followed. The slide processing system should be programmed for the appropriate sample dilutions, dispense volumes, and incubation times as outlined below.

1. **RECONSTITUTE BUFFER (PBS)**
   Dissolve contents of one buffer package in 1 liter of deionized or distilled water. Cover and store at 2-25°C for up to four weeks or until signs of contamination or other visible changes occur.

2. **DILUTE PATIENT SAMPLES**
   Screening: Dilute patient samples to 1:20 by adding 0.05 ml (50 µl) of serum to 0.95 ml (950 µl) of Sample Diluent.
   Semi-Quantitative Titering: To make two-fold serial dilutions of screening samples (e.g. 1:40, 1:80, 1:160...1:640), remove 0.5 ml of the 1:20 dilution and mix with 0.5 ml of sample diluent to achieve a 1:40 dilution, and continue serial dilutions in this fashion.

3. **DILUTE OPTIONAL TITRATABLE CONTROL**
   Treat the optional titratable control as an undiluted patient sample. Dilute the control 1:20 by adding 0.05 ml (50 µl) of the control serum to 0.95 ml of Sample Diluent. Make two-fold serial dilutions of the titratable control as outlined above.

4. **PREPARE SUBSTRATE SLIDES** (20-25 µl/well)
   Remove slide(s) from pouch(es) and place control sera on control wells as follows: Invert control dropper bottle and squeeze gently until drop is visible at the tip. Gently touch the drop to appropriate control well while avoiding direct contact of dropper tip with slide surface. Add 1 drop (20-25 µl) of diluted patient sample to numbered wells.
   CAUTION: DIRECT CONTACT OF DROPPER TIP WITH SLIDE SURFACE MAY RESULT IN DAMAGE TO THE ANTIGEN SUBSTRATE.

5. **INCUBATE SLIDES** (30 ± 5 minutes at room temperature, i.e. 18-25°C)
   Place slide(s) into a moist covered chamber (a petri dish with moistened paper toweling will be adequate). Incubate, with lid in place, for 30 minutes (± 5 minutes) at room temperature (18-25°C).

6. **PBS RINSE**
   Remove slide(s) from incubator tray and rinse briefly with PBS using a squirt bottle, Pasteur, or serological pipette. Do not squirt buffer directly on the wells.
   NOTE: To avoid cross contamination on 10-well slides, direct PBS stream along midline of slide, tilting first toward wells 1-3 followed by tilting toward wells 4-6.

7. **PBS WASH** (10 minutes)
   Wash slide(s) 10 minutes with PBS in a slide staining dish or Coplin jar. This wash may be extended up to 30 minutes with no variability in final test results. Discard PBS wash solution after use.

8. **FLUORESCENT ANTIBODY REAGENT** (Cover the wells with 10-12 drops)
   Remove one slide at a time from PBS and dip 3-5 times in deionized or distilled water. Tap slide on its side against bibulous paper or paper toweling to remove excess water. Immediately return slide to the incubation chamber and cover the wells completely using fluorescent antibody reagent; begin by placing a drop over each well. Repeat for each slide. Fluorescent antibody reagent has been titered to compensate for residual deionized or distilled water remaining on the slide after rinsing.
   NOTE: It is important that slide wells do not dry during this procedure or damage to the substrate may occur.
   DO NOT BLOT OR DRY THE SLIDE IN ANY MANNER OR ALLOW SLIDE TO SIT WITHOUT FLUORESCENT ANTIBODY REAGENT FOR LONGER THAN 15 SECONDS.

9. **INCUBATE SLIDES** (30 ± 5 minutes at room temperature, i.e. 18-25°C)
   Place lid on incubation chamber and cover with a paper towel to prevent exposure to light if the chamber is not opaque. Allow slide(s) to incubate 30 minutes (± 5 minutes) at room temperature (18-25°C).

10. **PBS RINSE**
    Remove slide(s) from incubator tray and rinse briefly with PBS. Do not squirt buffer directly on the wells.

11. **PBS WASH** (10 minutes)
    Wash slide(s) 10 minutes with PBS in a slide staining dish or Coplin jar. This wash may be extended up to 30 minutes with no variability in final test results.

12. **MOUNT COVERSLIP**
    Remove one slide at a time from PBS and dip 3-5 times in deionized or distilled water (Optional). Tap slide on its side against bibulous paper or paper toweling to remove excess water.
    DO NOT BLOT OR DRY THE SLIDE IN ANY MANNER OR ALLOW TO SIT WITHOUT COVERSLIP FOR LONGER THAN 15 SECONDS. Add 4-5 drops of semi-permanent mounting medium along midline of each slide. Carefully place coverslip in position, avoiding air pockets, by gently lowering coverslip from one end of the slide to the other.
    NOTE: Excess mounting medium on the slide may result in high background fluorescence due to light scattering, or lack of clear resolution of cells (blurred image). Excess mounting medium may be removed from slide by gently blotting coverslip with bibulous or lens paper while avoiding any direct movement of the coverslip.

FOR TECHNICAL ASSISTANCE:
USA: 1-800-251-5115 Outside USA: 1-916-363-2649
Email: technicalsupport@immunoconcepts.com