COLORZYME® nDNA TEST SYSTEM

For in vitro Diagnostic Use

For Professional Use

INTENDED USE: This is an indirect enzyme antibody test for the semi-quantitative detection of anti-nDNA antibody in human serum. This test system is to be used as an aid in the diagnosis of systemic lupus erythematosus.

SUMMARY AND EXPLANATION OF THE TEST

Antinuclear antibody (ANA) is a general term used to describe autoantibodies against various cell nuclear proteins. Early studies of these autoantibodies, using immunofluorescent techniques, revealed a select few nuclear protein specificities (1). Because of the high correlation of positive ANA with systemic lupus erythematosus (SLE), a negative ANA essentially ruled out the disease (2).

Although antibodies specific to DNA continue to show a high disease correlation with SLE (3), in the last decade a number of nuclear (4) and cytoplasmic (5-7) macromolecules have been detected and associated with other connective tissue diseases (8-10). Because a number of these antibodies appear to be of diagnostic and/or prognostic use in progressive systemic sclerosis (11-12), mixed connective tissue disease (13-15), Sjögren's syndrome (16-17), polymyositis (18), and rheumatoid arthritis (19), ANA testing is now recognized as a general screening tool for connective tissue disease (20).

SLE patients may produce antibodies to a variety of nuclear antigens, however antibodies directed against Sm (Smith antigen) and nDNA show the highest correlation with disease (20). Antibodies directed against Sm demonstrate a speckled ANA staining pattern while antibodies directed against nDNA generally demonstrate a homogeneous ANA staining pattern. Although low levels of nDNA antibodies are often present in the serum of patients with rheumatoid arthritis, Sjögren's syndrome, progressive systemic sclerosis, dermatomyositis, discoid lupus erythematosus, and mixed connective tissue disease (21), high levels of nDNA antibodies are seen almost exclusively in SLE. Antibodies against nDNA are thought to be involved in the pathogenesis of the most severe variants of SLE when deposited as immune complexes (22). Antibodies to nDNA occur in high titer, and, because they correlate with disease activity (23), their detection is important in the management of SLE patients.

Indirect immunofluorescence has proven to be a problem for some laboratories, due to the small size of the *Crithidia luciliae* organism, unless high quality optics and fluorescent equipment are used. Because of this, and the need to reduce interlaboratory reproducibility problems inherent with fluorescence microscopy, the Immuno Concepts Colorzyme® nDNA test system was developed.

Serum antibody reactive to nDNA is detected by staining of the kinetoplast within the organism *Crithidia luciliae* (24). *C. luciliae* is a parasite of the blowfly and is non-pathogenic to humans. The kinetoplast of these hemoflagellates is part of the large mitochondrion in which the helical nDNA is concentrated (24-25). In electron micrographs, the kinetoplast appears as a slightly concave, disc-shaped structure containing mitochondrial cristae and a fibrous DNA mass (26). The kinetoplast is found between the centrally located nucleus and the basal body of the flagellum. Because the kinetoplast nDNA contains no single-stranded DNA (ssDNA) contaminants, potential problems of ssDNA false-positive reactions, which may occur with calf thymus DNA radioimmunoassay, are virtually eliminated (25, 27-31).

Principle of the Test

The Immuno Concepts Colorzyme® nDNA test uses the indirect enzyme antibody technique. Patient samples are incubated with antigen substrate to allow specific binding of autoantibodies to kinetoplast nDNA. If nDNA antibodies are present, a stable antigen-antibody complex is formed. After washing to remove nonspecifically bound antibodies, the substrate is incubated with an anti-human antibody reagent conjugated to horseradish peroxidase. When results are positive, there is the formation of a stable three-part complex consisting of enzyme labeled antibody bound to human anti-nDNA antibody, which is bound to nDNA antigen. This complex can be visualized by incubating the slide in color reagent, which contains an enzyme specific substrate. The reaction between the enzyme labeled antibody and enzyme specific substrate results in a color reaction on the slide visible by standard light microscopy. In positive samples, the kinetoplast will show a dark blue-purple staining within the *Crithidia luciliae* organisms. If the sample is negative for nDNA, the kinetoplast will show no staining.

System Components - materials provided

Use: All components come ready to use with no aliquoting or reconstitution required (except the PBS buffer and Colorzyme® color reagent 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 reagent should be stored in a closed and stored between 2-25°C. After reconstitution, Colorzyme® color reagent should be stored in a closed container at room temperature for up to 30 days. Depending on the rate of use, 150 ml of Colorzyme® color reagent can be used with up to twenty slides.

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

REACTIVE REAGENTS

Substrate Slides SLIDE**:** nDNA substrate slides using *Crithidia luciliae* stabilized directly on the test wells. The 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.

Positive Control CONTROL| + **:** Catalog No. 3021. Ready-to-use dropper vial containing 1.0 ml positive human control serum with antibody specific to nDNA antigens. This serum demonstrates a positive staining reaction of the kinetoplast or kinetoplast and nucleus on Immuno Concepts' *Crithidia luciliae* substrate.

Titratable Control Serum TC**:** Catalog No. 3026. Ready-to-use vial containing 0.5 ml positive human control serum to be treated as an undiluted patient sample. See vial label for titer value.

Negative Control Serum CONTROL| - **:** Catalog No. 3031. Ready-to-use dropper vial containing 1.0 ml negative human control serum. The negative control serum will not show any specific staining of the kinetoplast on Immuno Concepts' *Crithidia luciliae* substrate.

Enzyme Antibody Reagent CONJ|HRP**:** Catalog No. 5009 (9.0 ml), 5075 (23.0 ml). Anti-human IgG conjugated to horseradish peroxidase (HRP). Reagent comes ready-to-use in precision dropper bottles with 9.0 ml for each 10 slides in complete test kits.

Color Reagent PWDR|CRP**:** Catalog No. 4066. HRP specific enzyme substrate powder, containing 4-chloro-1-naphthol. Each package contains powder to make 150 ml of self-activating Colorzyme® color reagent.

> **Preparation:** Dissolve contents of one pouch in 150 ml of deionized or distilled water. Mix well until completely dissolved. This color reagent is stable for 30 days at room temperature in a closed container. This color reagent may be reused for up to 30 days or until any color change or precipitate is visible. Cloudiness or opalescence, with no visible precipitate upon reuse is normal. Depending on the rate of use, 150 ml of Colorzyme® reagent can be used with up to twenty slides.

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 each 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
Three Coplin jars or slide staining dishes
Squeeze bottle or Pasteur pipettes
Serological pipettes
One-liter screw-cap containers (for PBS buffer)
Closed container for storing Colorzyme® color reagent
Deionized or distilled water
Test tubes to perform serial dilutions
Bibulous paper or paper towels
Chamber for incubation
Disposable gloves
Lab timer
Standard light microscope capable of 200X and 400X magnification

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. In the event hemolyzed or lipemic sera must be used, heat inactivate sera 30 minutes at 56°C for optimal results. Microbially contaminated sera should not be used.
7. 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.
8. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
9. Avoid splashing or generation of aerosols at all times.
10. Incubation times and temperatures other than those specified may give false results.
11. Cross contamination of reagents or samples may give false results.
12. Reusable glassware must be washed and thoroughly rinsed free of detergents prior to use. All glassware must be clean and dry before use.
13. Bring all reagents, slides, and specimens to room temperature (18-25°C) prior to use.
14. Wear disposable gloves when handling specimens and reagents, and wash hands thoroughly afterwards.
15. Microbial contamination of reagents or samples may give false results.
16. 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.
17. Color reagent may be reused up to 30 days or until any color change or precipitate is visible. Cloudiness or opalescence, with no visible precipitate upon reuse, is normal. Depending on the rate of use, 150 ml of Colorzyme® color reagent can be used with up to twenty slides.

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.

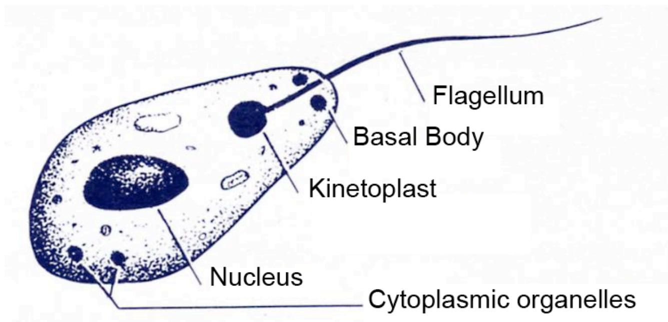
Interfering Substances: Sera exhibiting a high degree of hemolysis, icterus, lipemia, or microbial growth should not be used because these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

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

Proper interpretation of results depends on clear recognition of the various morphologic features of the *Crithidia luciliae* organism.



The outer covering on most protozoa consists of a pellicle layer composed of lipoprotein. Inside the pellicle lies the plasma membrane. The plasma membrane encloses the cytoplasm consisting of a) an outer ectoplasm layer containing the basal body and flagellum and b) the endoplasm, a very fluid inner cytoplasm containing the nucleus, kinetoplast, and other organelles.

The pellicle, plasma membrane, basal body, and flagellum are generally considered permanent fixtures within the organism with little variability in location from cell to cell. Although the kinetoplast is generally located closer to the basal body than the nucleus, the exact location of this organelle may vary from cell to cell due to the fluid nature of the endoplasm.

In order to clearly differentiate the kinetoplast from the nucleus, view the positive control well. The kinetoplast will always be located nearer the flagellum (illustrated above). The negative control well will show no kinetoplast staining while the positive control well will show kinetoplast staining.

READ ONLY SINGLE, WELL-DEFINED ORGANISMS WITHIN EACH FIELD. MORPHOLOGY MAY VARY FROM ORGANISM TO ORGANISM DUE TO FIXATION DURING LOG PHASE GROWTH.

QUALITY CONTROL
Positive, negative, and PBS controls should be run in the wells provided for quality control on each slide. The positive control should show dark blue-purple staining in the kinetoplast of the *Crithidia luciliae*, with or without staining of the nucleus. The negative control will show no staining of the kinetoplast. The PBS control is used to observe non-specific staining by the antibody reagent, and should not exhibit any blue staining. 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 1:10 dilution. The first well in which clearly discernible staining of the kinetoplast is visible is the titer end-point. We recommend this technique for determining titer end-points.

The mean titer and titer range (± one two-fold dilution on either side of the mean) determined for this 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 nDNA 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 Colorzyme® nDNA Test System, 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. Proper alignment of the microscope light path. Refer to your microscope manual for instructions.
2. The numerical aperture of the objective. The numerical aperture is related to the light gathering capability and the resolution of the objective. The numerical aperture is printed on the side of the objective.
3. 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 *Crithidia.*

Negative: A serum is considered negative for antibodies to nDNA if kinetoplast staining is less than or equal to the negative control well. Nuclear staining, without kinetoplast staining, is also considered negative for antibodies to nDNA.

Positive: A serum is considered positive if the kinetoplast shows clearly discernible staining greater than the negative control well.

Titers: When reading titers, many laboratories begin reading with the well that contains the most dilute sample and read “backwards” to the 1:10 dilution. The first well in which clearly discernible staining of the kinetoplast is visible is the titer end-point. We recommend this technique for determining titer end-points.

Enzyme Staining Intensity

The degree of staining intensity has no proven clinical value and only limited value as an indicator of titer (32). To simplify interpretation, record screening results as strongly positive or positive, and titer accordingly.

Strongly Positive Reaction: Dark to very dark blue-purple staining with a clear-cut outline of the kinetoplast.

Positive Reaction: Dim or subdued blue-purple staining with greater variability of staining between organisms. Cell outline may be less well defined in some organisms with a majority of organisms still demonstrating a clearly discernible staining of the kinetoplast.

Reporting of Results

Screening: Results should be reported as strongly positive, positive or negative at the 1:10 dilution.

Titering: Results should be reported as the last serial dilution in which clearly discernible staining of the kinetoplast is seen. Results with a strong reaction at the 1:640 dilution should be reported as greater than 1:640.

STAINING CHARACTERISTICS
Kinetoplast: A smooth or peripheral staining of the kinetoplast located near the flagellar region of the organism.
Result: Positive for antibodies to nDNA.
Antigens: nDNA.
Disease Association: High titers suggestive of active SLE (20) or in the case of previously diagnosed SLE, recurrent disease, or lack of response to therapy (21-23).

Nucleus: A smooth, peripheral, or speckled staining of the nucleus.
Result: Negative for antibodies to nDNA.
Antigens: Nuclear associated antigens (21-23).
Disease Association: Non-specific connective tissue disease may be indicated by the positive nuclear staining.
NOTE: Positive ANA results by HEp-2 or other substrates do not normally give the corresponding nuclear staining on *C. luciliae*, e.g. a speckled ANA by HEp-2 does not demonstrate speckled nuclear staining on *C. luciliae*.

Basal Bodies: A smooth staining of two spheres located where the body of the organism attaches to the flagellum in the ectoplasm.
Synonyms: Basal feet.
Results: Negative for antibodies to nDNA.
Antigens: Basal body associated antigens.
Disease Association: Reported in SLE patients not exhibiting kinetoplast or nucleus staining (33).

Flagellum: Staining of the flagellum of the organism.
Synonyms: Tail region of the organism.
Result: Negative for antibodies to nDNA.
Antigens: Unknown flagella-associated antigens.
Disease Association: Unknown.

Limitations of the Test

1. Diagnosis cannot be made on the basis of anti-nDNA 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 anti-nDNA antibodies. Clinical indications, other laboratory findings, and the physician's clinical impression must be considered before any treatment is initiated.
3. Certain drugs, including procainamide and hydralazine, may induce a lupus erythematosus-like disease. Patients with drug-induced LE may demonstrate positive ANAs commonly directed against nuclear histones, although antibody to nDNA has also been reported (34-35).
4. Although a high-titered nDNA may be highly suggestive of SLE, it should not be considered diagnostic but rather viewed as part of the overall clinical history of a patient. Low titers of nDNA antibodies are often present in the sera of patients with rheumatoid arthritis, Sjögren's syndrome, progressive systemic sclerosis, dermatomyositis, discoid lupus erythematosus, and mixed connective tissue disease (21).
5. Patients undergoing steroid therapy may have negative results for nDNA antibody (36).

Expected Values

The expected value in the normal population is negative at a 1:10 screening dilution. Certain drugs, such as hydralazine, may induce nDNA antibody production (34-35).

Performance Characteristics

DETECTION
The Immuno Concepts Colorzyme® nDNA test system was tested for equivalence with the Immuno Concepts indirect immunofluorescence nDNA test by screening and titering forty-nine serum samples representing the diversity of autoantibodies seen in systemic rheumatic disease. The enzyme results correlated 100% with the Immuno Concepts immunofluorescent nDNA for all sera tested (37).

The Immuno Concepts fluorescent nDNA test system was previously evaluated in comparison with two other fluorescent antibody tests in commercial distribution (37). The study employed 103 serum samples from normal individuals as well as from patients with diagnoses including systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Raynaud's-progressive systemic sclerosis-CREST variant (PSS-CREST), rheumatoid arthritis (RA), juvenile rheumatoid arthritis (JRA), as well as other connective tissue disease. Sera were tested at the recommended screening dilutions for each manufacturer. Study results are summarized in table 1:

TABLE 1

DIAGNOSIS	Number of Patients	Immuno Concepts Positive 1:10	Manufacturer A Positive 1:10	Manufacturer B Positive 1:10
SLE	30	13	13	11
MCTD/ overlap	6	0	0	0
Raynaud's PSS-CREST	17	0	0	0
RA	2	0	0	0
JRA	4	0	0	0
Other connective tissue disease	9	0	0	0
Hospitalized controls	11	1	1	1
Normal controls	24	0	0	0

The hospitalized control, positive on all *Crithidia luciliae* nDNA tests, had immune complex renal disease, which did not meet criteria for diagnosis of SLE.

PRECISION
Ten nDNA positive sera were titered in duplicate on three occasions. In all cases, all titers were reproduced within plus or minus one twofold dilution (37). These results are consistent with the standards of precision established for fluorescent antibody reagents by the Centers for Disease Control in Atlanta, Georgia.

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

	Manufacturer	EC REP	Authorized Representative in the European Community
	Temperature Limitation	Σ	Contains Sufficient for <n> tests
	Consult Instructions for Use	IVD	In Vitro Diagnostic Medical Device

EC REP

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Cat 5000-I, 4.11.02.003.094-En Rev 3.0

COLORZYME® nDNA 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 pouch in one liter of deionized or distilled water. The PBS buffer may be covered and stored at 2-25°C up to four weeks.

2. **RECONSTITUTE COLOR REAGENT**
Dissolve contents of one pouch in 150 ml of deionized or distilled water. Mix well until completely dissolved. This color reagent is stable for 30 days at room temperature in a closed container. This color reagent may be reused for up to 30 days or until any color change or precipitate is visible. Cloudiness or opalescence, with no visible precipitate upon reuse is normal. Depending on the rate of use, 150 ml of Colorzyme® color reagent can be used with up to twenty slides.

3. **DILUTE PATIENT SAMPLES**
Screening: Dilute patient samples to 1:10 by adding 0.1 ml (100 µl) serum to 0.9 ml reconstituted PBS.
Semi-Quantitative Titering: To make two-fold serial dilutions of screening samples (e.g. 1:20, 1:40, 1:80...1:640), remove 0.5 ml of the 1:10 dilution and mix with 0.5 ml of PBS to achieve a 1:20 dilution, and continue serial dilutions in this fashion.

4. **DILUTE OPTIONAL TITRATABLE CONTROL**
Treat the optional titratable control as an undiluted patient sample. Dilute the control 1:10 by adding 0.1 ml (100 µl) of the control serum to 0.9 ml of reconstituted PBS. Make two-fold serial dilutions of the titratable control as outlined above.

5. **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) patient sample to the numbered wells.
CAUTION: DIRECT CONTACT OF DROPPER TIP WITH SLIDE SURFACE MAY RESULT IN DAMAGE TO THE ANTIGEN SUBSTRATE.

6. **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).

7. **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 the slides, direct PBS stream along midline of slide, tilting first toward the upper row of wells followed by tilting toward the lower row of wells.

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

9. **ENZYME ANTIBODY REAGENT** (Cover the wells with 12-14 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 enzyme antibody reagent; begin by placing a drop over each well. Repeat for each slide. Enzyme 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 ENZYME ANTIBODY REAGENT FOR LONGER THAN 15 SECONDS.

10. **INCUBATE SLIDES** (30±5 minutes at room temperature, i.e. 18-25°C)
Place lid on incubation chamber and allow slide(s) to incubate 30 minutes (±5 minutes) at room temperature (18-25°C).

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

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

13. **COLOR REAGENT INCUBATION** (30 minutes at room temperature, i.e. 18-25°C).
Remove one slide at a time from PBS, dip 3-5 times in deionized or distilled water, and tap slide on its side against bibulous paper or paper toweling to remove excess water. Immediately place slide(s) into a Coplin jar containing activated color reagent and incubate for 30 minutes.

14. **PBS RINSE**
Remove one slide at a time from the Coplin jar and rinse each side of slide 4-5 seconds with PBS. Do not squirt buffer directly on the wells. Place each PBS rinsed slide into a Coplin jar filled with deionized or distilled water until all slides have been removed from the color reagent. Immediately proceed to step 15.

15. **MOUNT COVERSLIP**
Remove one slide at a time from the deionized or distilled water and 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 slide may result in a 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. Slides may be read immediately or stored for an extended time at 2-10°C with no loss of reactivity.

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