Problem	Questions	Cause/Remedy
Decreased fluorescence in controls AND patients	What type of fluorescent bulb is being used? Is bulb past recommended hours of usage?	Check manufacturer recommendations Mercury vapor lamps have finite period of optimal performance. 1. Replace lamp if past this time. 2. Record lamp usage time for future reference. N/A to quartz halogen lamp
	If the fluorescent scope properly aligned?	1. Verify appropriate filters in use. 2. Check bulb and filter alignment. 3. If unable to correct alignment, call scope manufacturer for service. Microscope performance may be verified with use of Optical Standard Slide.

Decreased fluorescence in controls AND patients	How are reagents stored?	1. Verify storage temperature of reagents. 2. Return refrigerated components to correct temperature after use.
	Are all reagents within expiration dates?	1. Check expiration date of all components. 2. Discard expired reagents.
	How recently was PBS prepared?	1. Check PBS expiration date 2. Verify that PBS is not contaminated 3. Prepare a new batch of PBS 4. Verify that all containers are clean and dry before addition of the new buffer. NOTE: PBS contamination is one of the most common causes of decreased fluorescence.

Decreased fluorescence in controls AND patients	Was PBS correctly prepared?	1. Confirm correct reconstitution of PBS. Mix well, equilibrate RT 30 min 2. Distilled water source should be checked periodically for pH, contamination and conductivity. If water quality is suspect, prepare PBS using bottled distilled water.
	What is the pH of the PBS?	1. Check pH of PBS 7.3± 0.1 2. Prepare a new batch, if outside range 3. Verify water source has acceptable pH.
	Are PBS storage bottles washed periodically?	1. Wash PBS storage bottles and rinse squirt bottle periodically with mild soap and water rinse thoroughly and dry. Do not use bleach

Decreased fluorescence in controls AND patients	What is the endpoint titer of the Homogeneous control?	1. See the Quality Control section of the package insert (acceptable range +/- one dilution from the endpoint listed on the QC Report 2. Consider microscope, technique or contamination issues. BR Homogeneous control may be used as a sensitivity control
	What microscope objective was used for reading fluorescent intensity?	1. Determine the specific objective to use for fluorescent intensity (depending on substrate type, light source and optics of microscope). 2. All technologists in the lab must use the appropriate objective. Intensity will vary depending on the magnification.

Decreased fluorescence in controls AND patients	Were reagents brought to room temperature before use?	1. All reagents must come to room temperature before use. 2. Slides must be at room temperature at least 20 minutes before application of controls or samples.
	Were incubation times altered?	1. Verify incubation times Short incubation times = decreased fluorescence
	Was Evans’ Blue Counterstain used?	1. Verify correct volume of counterstain.(2-3 drops of Evans’ Blue/150 mL of PBS) If counterstain concentration is too high, it may cause quenching of positive fluorescence.
	Were slides washed longer than 10 minutes?	1. Use a timer to verify wash times 2. Excessive wash times may result in the loss of antigenic material.

Decreased fluorescence in controls AND patients	Were slides read a day or more following set-up?	1. Read slides as soon as possible 2. Allow slides to come to room temperature before reading. 3. Store processed slides dark in refrigerator
	Were conjugate or reacted slides exposed to light?	1. Protect conjugate from light. 2. Verify that incubation chamber has opaque cover. 3. Store reacted slides in slide holder protected from light. There will be loss of conjugate activity if reagent or reacted slides are exposed to light.
	Was sufficient mounting media used?	1. Apply 4 ± 1 drops mounting media per slide; add coverslip, avoiding air bubbles. Insufficient mounting media may not adequately cover all wells or it may dry out-resulting in decreased fluorescence.

Decreased fluorescence in all patient samples	What starting dilution is being used?	1. Perform normal range study to verify starting dilution for patient population. See BR Technical Bulletin “ANA Normal Range Study” #003008A. Call Technical Service or Product Support for assistance.
	How were patient dilutions made?	1. Verify correct dilution procedure used. 2. Be sure to evenly mix patient dilutions. 3. Verify pipette calibration.
Decreased fluorescence in specific control	How was control stored?	1. Verify controls stored at appropriate temperatures. 2. Liquid controls are stored at 2 to 8 °C. 3. Check for appearance of bacterial growth or sediment in the vial. Discard contaminated controls.

Decreased fluorescence in specific control	How was control diluted?	1. Verify appropriate dilution used and compare positive control endpoint with QC report. 2. BR Positive controls should be tittered in a simple doubling dilution scheme 3. Control titer endpoints are stated on a QC Report that is included with all positive controls.
	What magnification was used for interpretation?	1. Check the magnification used to establish the endpoint titer. 2. The QC Report for a specific lot of control being tested states the magnification and bulb type that was used to establish the expected endpoint.
Decreased fluorescence in specific patient	Is the patient undergoing therapy?	Patients undergoing successful therapy may have a lower titer or be negative for ANA and anti-dsDNA.

Decreased fluorescence in specific patient	How was patient sample stored?	1. Store samples for up to 2 days @2-8°C. 2. Longer storage, freeze samples at -20°C or lower. 3. Avoid repeated freeze/thawing, which may cause deterioration of the specimen. 4. Aliquot and freeze several tubes from specimens that require follow-up testing. 5. Mix thawed samples before pipetting.
	Was titration performed?	1. Titrate the sample Rarely, a high titer antibody may appear as weakly positive or negative due to a prozone reaction.

Problem	**Question**	**Cause/Remedy**
Increased fluorescence in controls <u>and</u> patients	What is the pH of the PBS?	1. Check pH of PBS 7.3± 0.1 2. Prepare a new batch, if outside range 3. Verify water source has acceptable pH.
	What microscope objective was used for reading fluorescent intensity?	1. Determine the specific objective to use for fluorescent intensity (depending on substrate type, light source and optics of microscope). 2. All technologists in the lab must use the appropriate objective. Intensity will vary depending on the magnification.
	Was a new microscope bulb installed or is a new microscope in use?	1. Check type of bulb (mercury vapor bulbs age, the intensity of the bulb decreases and therefore fluorescent reactions will decrease).

Increased fluorescence in controls <u>and</u> patients		2. Verify if a new bulb or microscope was installed 3. Microscope performance may be verified with an Optical Standard Slide. 4. Neutral density filters may be necessary. 5. Consult your microscope manufacturer.
Increased fluorescence in <u>all</u> patients	Is the starting dilution appropriate?	1. Perform normal range study to verify starting dilution for patient population. 2. Positive incidence should not be higher than 5%. 3. See BR Technical Bulletin "ANA Normal Range Study" #003008A. Call Technical Service or Product Support for assistance.

Increased fluorescence in all patients	How were patient dilutions made?	1. Verify correct dilution procedure used. 2. Be sure to evenly mix patient dilutions. 3. Verify pipette calibration.
Increased fluorescence in specific patient	Is the patient receiving therapy?	1. Drugs such as procainamide and hydralazine may induce ANA. 2. ANA and anti-dsDNA titers may rise and fall in response to treatment and disease state.
	Has result been verified with another substrate?	1. Verify positive results using MSK, HEp-2 and Crithidia. 2. Verify ANA patterns using EIA methods for specific ANA antigens or MBIA

Problem	Question	Cause/Remedy
Background fluorescence in all wells	What is the pH of the PBS?	1. Check PBS pH 7.3 ± 0.1 2. Prepare fresh PBS. **3.** Verify distilled water source is free of contamination.
	Is there particulate matter in the PBS?	1. Check water source or storage bottles for contamination 2. Prepare fresh PBS using bottled water, with clean/dry containers, including rinse squirt bottle.
	Were slides washed thoroughly?	1. Verify full 10 minutes wash, using stirring or agitation.
	Was PBS changed after first wash?	1. Change PBS after one use. 2. Do not reuse PBS wash
	Were slides allowed to dry out?	1. Use humid incubation chamber. 2. Set up slides one at a time. 3. Slides should always stay moist until mounted with coverslip.

Background fluorescence in all wells	Is there debris in the background?	1. Powder from latex gloves may be getting into PBS. 2. Use powder-free gloves or rinse outside of gloves in distilled water after donning.
	Are the correct microscope filters in use?	1. Verify microscope filters with manufacturer. 2. Be sure system is set up for FITC staining.
	Is there non-green color on the slides?	1. Marking pen may be flaking off or leaching into wells. 2. Use graphite pencil only for slide labeling. Do not use wax pencils or marking pens.
Background fluorescence in patient samples only	Is there a green film on all patient wells?	1. Try using Specimen Diluent, product #1067. 2. This will absorb non-specific fluorescence, without changing ANA titer. (Do not use for DNA or ANCA testing.)

Background fluorescence in patient samples only	Is there background fluorescence on a specific patient only?	1. Check sample for hemolysis or lipemia, request redraw. 2. Patient may have heterophile antibodies; try serial dilution of sample to dilute heterophile antibodies
Possible non-specific staining of Smooth Muscle layer on MSK slides	Was a PBS control run on the slide?	1. Run a PBS Control and a Negative Control on MSK slides to evaluate the amount of smooth muscle staining due to non-specific binding. 2. Compare patient results to PBS and Negative Controls. 3. Check for staining of the blood vessels of the kidney. Smooth muscle components, especially stomach muscularis, may non-specifically bind fluorescein.

Problem	Question	Cause/Remedy
Varying fluorescence across the well	Did the specimen, conjugate and mounting media cover the whole well?	1. Check technique to insure pipetting of specimen and reagents across the whole well. 2. Air pockets in mounting media will cause variable fluorescence.
	Is there drying of the serum or conjugate?	Nonspecific staining around the edge of the well may be due to drying during incubation. 1. Use a humid, opaque chamber for incubation. 2. Apply conjugate to one slide at a time. 3. Do not extend incubation times.

Varying fluorescence across the well	Is there a strongly positive patient or control on the run?	1. Contamination of wells by positive patient or control due to rinsing or washing technique errors. 2. Rinse stream of PBS should be above the well, with the slide long edge tipped downward. 3. Do not rinse slides across the wells, from the short edge. 4. Varying fluorescence across the well The 24 well slides must be blotted before the first rinse step. 5. Following rinse, place slides in staining tray or Coplin jar with adequate stirring or agitation. 6. Change PBS between washes. 7. **<u>Always</u>** change pipette tips between patient samples

<table>
<tr>
<td rowspan="2">Varying fluorescence across the slide</td>
<td>Is pattern inconsistent across the well?</td>
<td>1. Verify application of 25 μL of sample and incubation in a moist chamber.
2. Do not extend incubation times.

Drying of the specimen changes the salt concentration of the PBS, effecting affinity of the patient antibody, especially Anti-dsDNA.</td>
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<tr>
<td>Is there increased or unexpected Nucleolar staining?</td>
<td>1. Wash container or PBS may be contaminated.
2. Clean wash container periodically with dish soap; rinse well with Distilled water.
3. Use only graphite pencil for labeling slides as chemical based markers can leach on slides and into PBS, causing false Nucleolar staining.</td>
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Varying fluorescence across the slide	Is there increased or unexpected Nucleolar staining?	1. Wash container or PBS may be contaminated. 2. Clean wash container periodically with dish soap; rinse well with Distilled water. 3. Use only graphite pencil for labeling slides as chemical based markers can leach on slides and into PBS, causing false Nucleolar staining.
Varying fluorescence at well edge	Was the whole well used?	1. Reading should be done 2 to 3 fields from the well edge. 2. Edges may have increased intensity due to evaporation or decreased intensity due to failure to apply reagents over the entire well area.

Problem	Question	Cause/Remedy
Difficulty focusing on the slide	How much mounting media was used?	1. Distribute mounting media evenly across the slide. 2. Tap off PBS before adding mounting media. 3. Coverslip should not float on the slide.
	What temperature is mounting media being stored?	1. Store mounting media at room temperature. 2. Mounting media stored @2-8°, can cause hazy results
	Are microscope objectives dirty?	1. Periodically clean microscope lenses and objectives with lens cleaner and lens paper. 2. Do not use tissues or other lab wipes to clean lenses as scratching of lens may result. 3. Clean microscope stage with distilled water, to remove any excess mounting media.

Difficulty focusing on the slide	Is there more than one coverslip on the slide?	Coverslips may stick together and make focusing impossible. 1. Carefully float slide with coverslip(s) in PBS to remove coverslip, then re-mount slide. 2. Do not pry coverslip off the slide.
	Is the coverslip too thick?	1. Use #1 coverslip. Other types are too thick for fluorescent staining.

Problem	**Question**	**Cause/Remedy**
Cut across substrate	Did pipette touch the well?	1. Use care when pipetting sample 2. Do not touch pipette tip to well.
Cells missing	Was rinsing done directly on the well?	1. Direct rinse stream above the slide well. 2. Do not apply rinse directly to the wells.
Edges of the well missing cells	Was blotter applied directly over wells?	1. Use care when blotting, blotters should not touch wells. 2. Do not blot slide with paper towels. 3. Do not scrape slides with pen, pencil or fingers.
Distorted cells, missing nuclei or “ghost” cells	Is there any contamination within the system?	Contamination may distort substrate Verify that all reagents / samples are free of bacterial contamination. Verify all reagent vessels, wash equipment, and **rinse squirt bottles** are periodically cleaned and dried.

Distorted cells, missing nuclei or “ghost” cells	What solution was used for the wash steps?	1. Verify that only Kallestad PBS was used. 2. Washing with water, alcohol, EIA Wash Solution or other reagents will cause distortion of the substrate, including missing nuclei or ghost cells.

Problem	Question	Cause/Remedy
Increased incidence of weak positive patients	Is beginning dilution appropriate for population?	1. Starting dilution may be too low 2. Perform normal range study to determine appropriate starting dilution for patient population. 3. Positive incidence should not be higher than 5%.
	Is an inexperienced technologist reading slides?	1. Personnel should be trained to recognize 1+ reactivity. 2. Determine 1+ endpoint of positive control and use this dilution as reading control on all slides as comparison, until new staff members are fully trained.
More than one pattern is present	Was titration performed?	1. Titrate patient sample (helps view additional patterns) As sample is diluted, second pattern may become easier to detect.

Unable to determine pattern	Is Evans' Blue counterstain being used?	1. Counterstain may obscure weak patterns or small staining structures. 2. Repeat patient without counterstain.
	Is a mixed pattern present?	1. Titration may help dilute patterns, so the second pattern can be determined.
	Are mitotic figures being used to assist in pattern identification?	2. Both interphase and mitotic cells should be used to determine patterns. 3. Consult IFA package Insert and Pattern Plus Auditor™. 4. Call Technical Service for assistance.
	Is there a strongly positive patient or control on the run?	Contamination of wells by positive patient or control due to rinsing or washing technique errors. 1. Rinse stream of PBS should be above the well

Unable to determine pattern	Is there a strongly positive patient or control on the run?	2. Tip slide long edge downward. 3. Do not rinse slides across the wells, from the short edge. 4. The 24 well slides must be blotted before the first rinse step. 5. Following rinse, place slides in staining tray or Coplin jar with adequate stirring or agitation. 6. Change PBS between washes. 7. Be sure to change pipette tips between patients, when making dilutions and applying samples to the slide.
	Has result been verified with another substrate?	1. Use MSK, HEp-2 and Crithidia to verify results. 2. Verify ANA patterns using EIA methods for specific ANA antigens.

Crithidia dsDNA results are negative & discrepant with positive results on other methods and/or manufacturers	What is the salt concentration of the other assay buffers and what avidity levels of dsDNA antibodies do the other assays measure or claim to detect?	dsDNA antibodies produced by patients vary from low avidity to high avidity. High avidity dsDNA is considered to be more diagnostic for the more severe form of SLE with kidney involvement The level of detection by any particular assay (method or manufacturer) varies based on the molarity of the buffer used for dilution and wash steps. 1. Lower salt concentration buffers detect low avidity through high avidity dsDNA antibodies 2. Higher salt concentration buffers detect only higher avidity dsDNA antibodies

Crithidia dsDNA results are negative & discrepant with positive results on other methods and/or manufacturers	What is the salt concentration of the other assay buffers and what avidity levels of dsDNA antibodies do the other assays measure or claim to detect?	3. The buffer in the Kallestad Crithidia assay detects mid to high avidity dsDNA antibodies. It is very possible to obtain a positive result from another method such as EIA or even another manufacturer's Crithidia assay but a negative result using Kallestad Crithidia due to presence of only low avidity dsDNA antibodies.