

ANCA

The international testing consensus, assay differences, and sample interpretation support.

Immuno Concepts produces several diagnostic systems to assist in diagnosis of autoimmune vasculitis. These systems include:

ANCA Ethanol-fixed slides and reagents – This assay system includes substrate slides that contain ethanol-fixed neutrophils and all of the reagents and components that are needed to run the test. This system will detect and help differentiate P-ANCA and C-ANCA patterns.

ANCA-L Ethanol-fixed slides and reagents – This assay system includes substrate slides that contain ethanol-fixed neutrophils that are the same as the above kit, but the substrate also contains an increased number of lymphocytes that will show nuclear staining in the presence of an homogeneous anti-nuclear antibody. The lymphocyte allows the Clinical Laboratory Scientist to report ANA interference without running a separate ANA test.

ANCA Formalin-fixed slides and

reagents – This assay system includes substrate slides that contain formalin-fixed neutrophils and all of the reagents and components that are needed to run the test. The formalin-fixed neutrophils will show staining with a true P-ANCA or a true C-ANCA. No staining is seen with samples that are atypical ANCA.

RELISA® MPO Test System – This assay system is an ELISA test for confirmation of anti-MPO antibody.

RELISA® PR3 Test System – This assay system is an ELISA test for confirmation of anti-PR3 antibody.

Immuno Concepts Technical Support would like to address a few frequently asked questions regarding ANCA (anti-neutrophil cytoplasmic antibody) Test Systems produced by Immuno Concepts and present some interesting samples tested through our incoming samples process.



Immuno Concepts® prides itself on the quality of our product. Achieving this quality means that we strive to be a company with continuous improvement of our products as well as our services to you! Some of those services include educational support, pattern interpretation assistance, and research of literature to support our product. If we can be of any service to you please don't hesitate to contact us at technicalsupport@immuno-concepts.com or call us at 1-800-251-5115.

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NEWSLETTER

What is the recommended testing scheme for ANCA and why?

A method of testing and reporting was published in 1999 as the “International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA)”(1). This paper was the result of a collaboration of the leading rheumatologists, nephrologists, and immunologists in the field of vasculitis. This method recommends screening of all samples with the ethanol-fixed and formalin-fixed indirect immunofluorescent antibody (IFA) slide tests, followed by confirmation of MPO-ANCA and PR3-ANCA by ELISA.

In 2017, a different group published the “Revised 2017 international consensus on testing of ANCAs in granulomatosis with polyangiitis and microscopic polyangiitis” (2). This testing scheme depends on a “gating strategy”, in which a clinician decides if the patient is likely to have

granulomatosis with polyangiitis, microscopic polyangiitis, or another condition. Patients with other conditions, even those who are ANCA positive, are excluded from this testing scheme. The first level of testing in this scheme is ELISA (or another immunoassay) specific for anti-MPO antibodies or anti-PR3 antibodies. Whether this is positive or negative, it is followed with a “second assay”, presumably the immunofluorescent test.

Immuno Concepts recommends following a simplified version of the 1999 consensus.

- 1) Screen all samples on ANCA ethanol-fixed slides. (If using ANCA-L can report “ANA interference – cannot interpret IFA” at this step. See below for details.)
- 2) Test samples that are positive on ethanol-fixed slides

on formalin-fixed slides. If the sample is negative on the formalin-fixed neutrophils, report it as “Atypical ANCA”. Depending on the results of the ethanol-fixed neutrophils it can be reported as “Atypical P-ANCA” or “Atypical C-ANCA.”

- 3) Test samples that are positive on both ethanol-fixed and formalin-fixed neutrophils using RELISA® for anti-MPO and anti-PR3.
- 4) Test P-ANCA positive results on HEp-2000® for ANA interference. (Not applicable for ANCA-L users.)
- 5) Consider all results from ethanol-fixed, formalin-fixed, RELISA® MPO, RELISA® PR3, and ANA (if applicable) in formulating the final report.

Normally, a true P-ANCA confirms as anti-MPO positive and a true C-ANCA confirms as anti-PR3 positive. Known exceptions are:

- 1) One epitope of MPO remains associated with the primary (alpha) granules in the cytoplasm of the neutrophil and does not exhibit a P-ANCA pattern on ethanol-fixed cells. Samples with antibodies to this epitope may show a C-ANCA pattern.
- 2) The Atypical P-ANCA that is associated with Ulcerative Colitis is negative for both anti-MPO and anti-PR3 antibodies.
- 3) The Atypical C-ANCA, which has no known clinical significance, is negative for both anti-MPO and anti-PR3 antibodies.

A comparison of the 1999 Consensus and the 2017 scheme shows us:

How is ANCA-L used?

A homogeneous ANA will stain the nucleus of ethanol-fixed neutrophils with a pattern that strongly resembles a P-ANCA pattern. The 1999 Consensus recommends that laboratories run an ANA using HEp-2 cells for all samples that show a P-ANCA pattern on ethanol-fixed neutrophils. The Immuno Concepts ANCA-L product was designed to eliminate this additional step. P-ANCA and atypical P-ANCA react only with neutrophil and monocyte nuclei, but homogeneous ANA reacts with nuclei of all leukocytes, including lymphocytes (1). In the ANCA-L slides the number of lymphocytes has been increased so they are visible in every microscope field. Figure 1 shows

- 1) The 2017 proposal excludes all patients with non-ANCA Associated Vasculitis diseases as a gating strategy. This excludes potential detection of ANCA antibodies that are associated with inflammatory bowel disease (IBS), Crohn's Disease, Ulcerative Colitis, and autoimmune liver disease (3).
- 2) The 2017 proposal requires laboratories to establish two protocols to run ANCA – one for patients deemed to be at risk for AAV, and one for the other patients.
- 3) The 2017 proposal requires the physician to determine which protocol is to be used for each patient.
- 4) Approximately 10% of serum samples from patients with granulomatosis with polyangiitis or microscopic polyangiitis are only positive on ANCA IFA and do not confirm on ELISA assays (1). These samples will be missed if we screen with an ELISA assay.
- 5) Conversely, approximately 5% of PR3-ANCA and MPO-ANCA positive serum samples are only positive on ELISA assays and do not confirm on ANCA IFA (1). These samples will be missed if we screen with an IFA assay. However, if one system is used to screen for ANCA, IFA has better sensitivity than ELISA methods.
- 6) ELISA tests are more expensive than IFA tests, so it is more cost effective to screen with the IFA.
- 7) The IFA tests can be run using the Image Navigator® automated microscope system, which eliminates much of the tedious work involved in IFA assays.

the positive staining of the neutrophil nuclei and lymphocyte nuclei when a homogeneous ANA is present in the sample. When solid staining is seen in the lymphocyte nucleus we report “ANA Interference – cannot interpret IFA ANCA.” Figure 2a exhibits P-ANCA neutrophil nuclear staining (MPO positive/ PR3 negative) accompanied by a negative lymphocyte. Figure 2b exhibits the C-ANCA pattern on ethanol-fixed neutrophils. ANCA Formalin slides exhibit a C-ANCA pattern with either a true C-ANCA or a true P-ANCA. Atypical ANCA are negative on formalin-fixed slides.

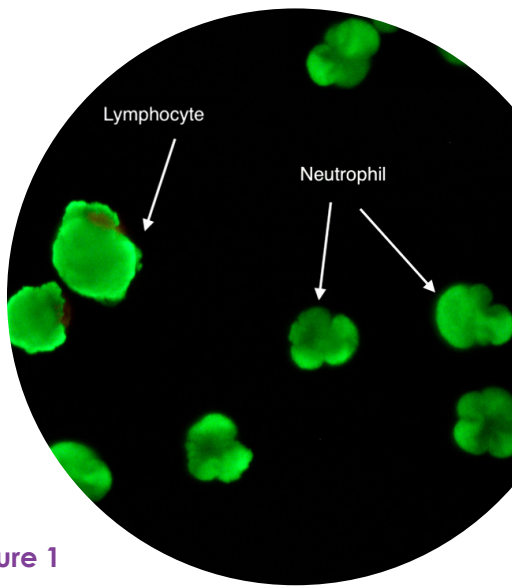


Figure 1

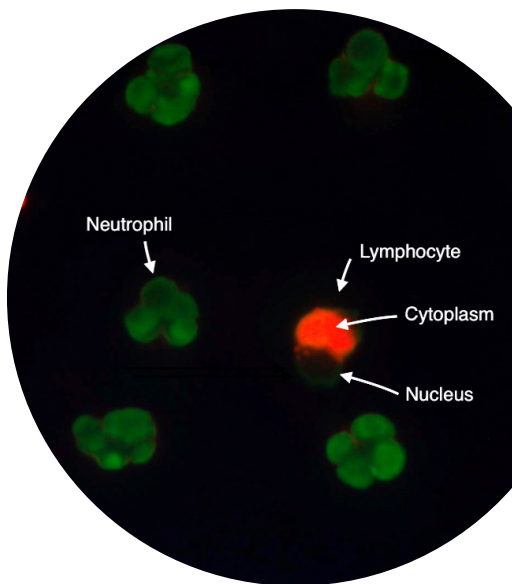


Figure 2a

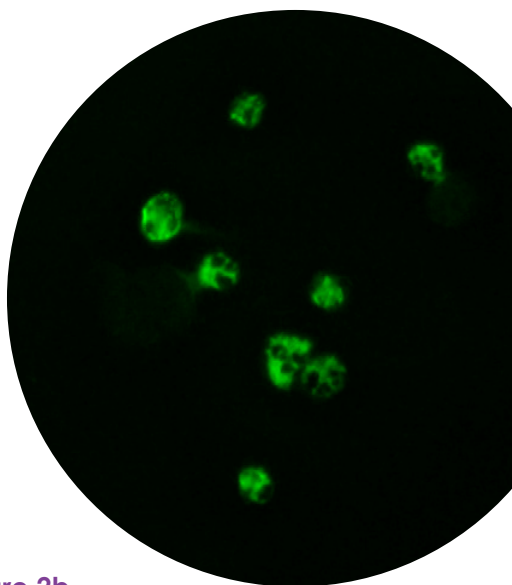


Figure 2b

Figure 3 demonstrates how the recommended testing scheme flows when using ANCA Ethanol-fixed slides. The right side of the flow chart shows the additional testing that is necessary for ruling out ANA interference for a P-ANCA. Figure 4 shows how the additional ANA testing can be eliminated by using ANCA-L. ANA interference is detected by staining of the lymphocytes on the ethanol-fixed slide, so testing an ANA slide for P-ANCA patterns can be eliminated entirely.

Figure 3. ANCA Flow Scheme without ANCA-L (Courtesy of Dr. Eric Hoy)

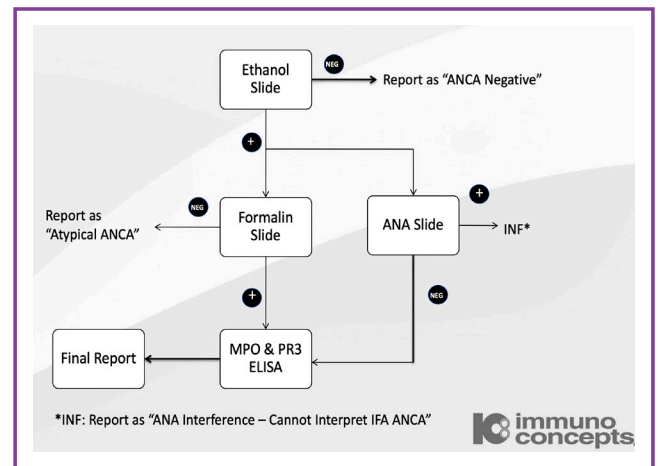
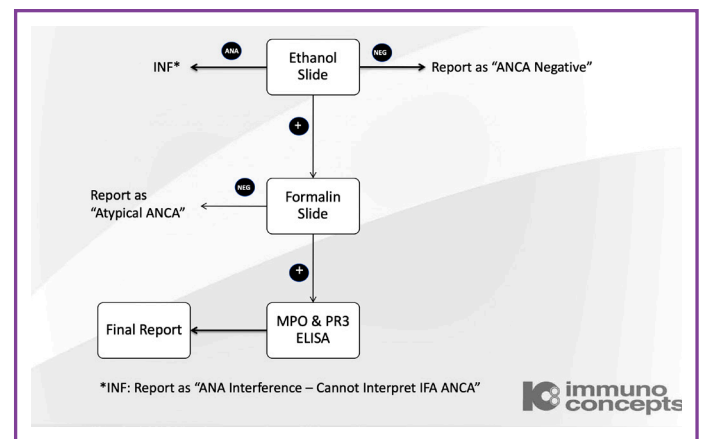


Figure 4. ANCA Flow Scheme with ANCA-L (Courtesy of Dr. Eric Hoy)



Sample Interpretation Assistance

Immuno Concepts offers assistance with pattern interpretation. Our experienced technical support team and our Chief Scientific Officer, Dr. Eric Hoy, are at your disposal. Together this team has a combined experience of 67 years reading and interpreting IFA slides. There are two ways to share your samples for interpretation assistance:

- Send us an image: Using the Image Navigator we can assist you in obtaining an image to send to us to review. Non-automated microscopes often have cameras attached as well. Email the image directly to Technical Support with the appropriate details. IC Technical Support Email Address: technicalsupport@immunoconcepts.com
- Send us your sample: You can also send samples to IC Technical Support. Call or email Immuno Concepts Technical Support to discuss your needs. You will be given a Case Number for your inquiry and the address to send samples.

DISCLAIMER: *Immuno Concepts, N.A. Ltd. is not a licensed clinical laboratory and is not responsible for any use or interpretation of this information. The results are provided only as an aid to interpretation for referring laboratories and physicians. Each laboratory should confirm these results before using this information to aid in patient diagnosis.*

Through this service, Immuno Concepts has assisted customers with all our IFA assay systems, including samples on the ANCA test system. Customers contacted us for assistance in interpretation of the following samples:

Sample A was received from a customer because of an unclear ANCA pattern determination. Results reported by customer were: ANCA-L Ethanol (P-ANCA positive), ANCA Formalin (negative) and ELISA MPO/PR3 testing (negative). The customer was unsure if what they were seeing was an Atypical P-ANCA pattern.

In this case, with P-ANCA positive staining on neutrophils in ANCA-L Ethanol, there is a tendency to want to call an ANCA positive pattern.

Positive P-ANCA on ANCA-L Ethanol (Figure 5) and a negative result for both ANCA Formalin (Figure 6) and MPO/PR3 RELISA®, are suggestive of an Atypical ANCA

pattern. However, these results are also supportive of a potential ANCA negative result due to the presence of ANA interference.

ANCA-L lymphocyte staining alerts the Clinical Laboratory Scientist to ANA Interference. Using regular ANCA Ethanol slides (not ANCA-L) would warrant further testing on ANA slides (Figure 7).

ANCA Ethanol, ANCA Formalin, ANA, and MPO/PR3 RELISA® results should all be considered when reporting.

NOTE: Confirmatory ANA testing of the sample is not required if the presence of lymphocyte staining is seen on an ANCA-L Ethanol slide and is considered to represent ANA interference.

Sample A should be reported as:

ANCA Ethanol: ANA Interference – cannot interpret IFA ANCA

ANCA Formalin: Negative

RELISA®: MPO/PR3 Negative

Similarly, Sample B was received from a customer because of an unclear ANCA pattern determination. Due to results received on ANCA-L Ethanol (positive in Figure 8), ANCA Formalin (negative in Figure 9) and ELISA MPO/PR3 testing (negative), the customer was unsure if what they were seeing was an Atypical P-ANCA pattern or not.

As with the previous case, the tendency is to want to call an ANCA pattern of some description based upon the ANCA-L Ethanol result. With the ANCA Formalin and MPO/PR3 negative test results, the Clinical Laboratory Scientist must decide between reporting an Atypical ANCA pattern or ANCA negative due to ANA interference.

Unlike Sample A, the negative lymphocytes on ANCA-L Ethanol (Figure 8) and negative ANA slide result confirm a lack of ANA interference (Figure 10), which indicates the result is an atypical P-ANCA result.

NOTE: Confirmatory ANA testing of the sample is not required if the presence of lymphocyte staining is seen on an ANCA-L Ethanol slide and is considered to represent ANA interference.

Sample B should be reported as:

ANCA Ethanol: Atypical P-ANCA

ANCA Formalin: Negative

RELISA®: MPO/PR3 Negative

SAMPLE A

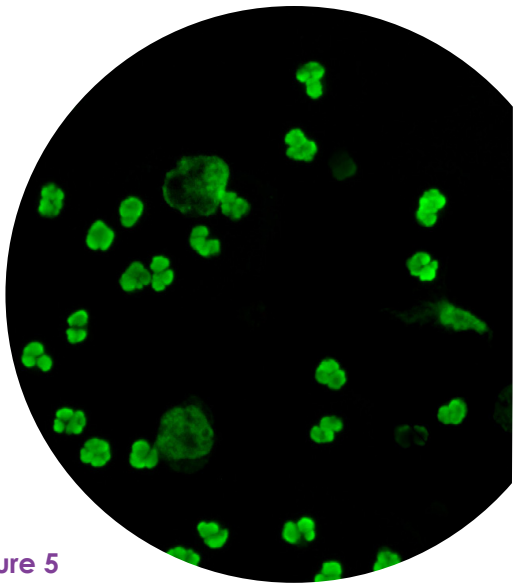


Figure 5

SAMPLE B

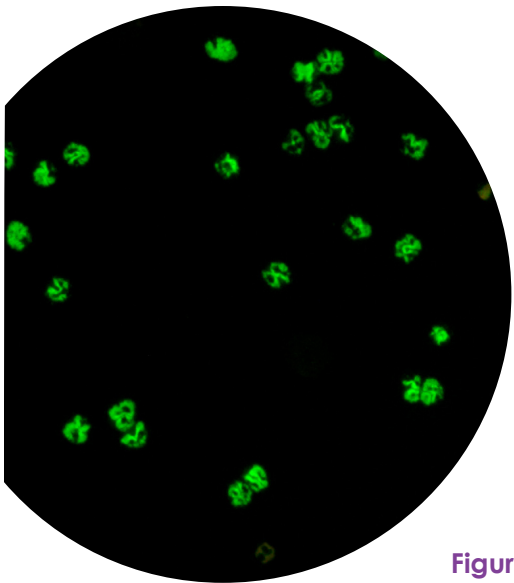


Figure 8

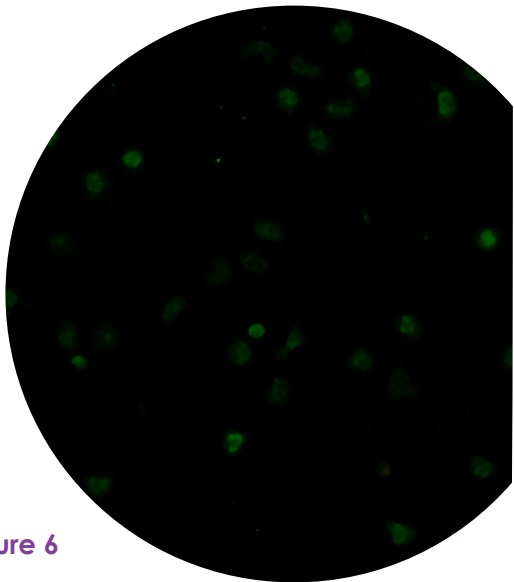


Figure 6

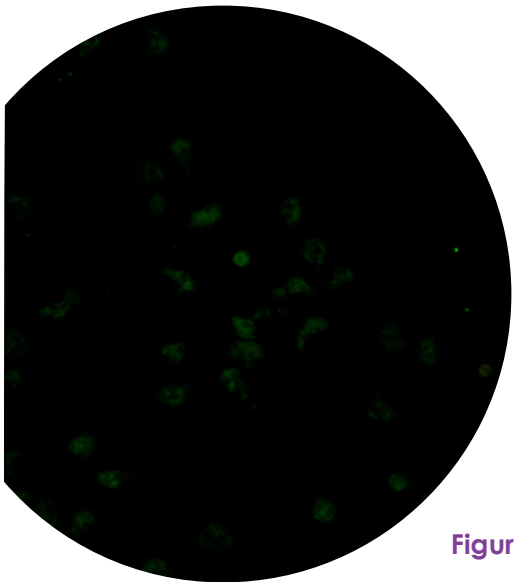


Figure 9

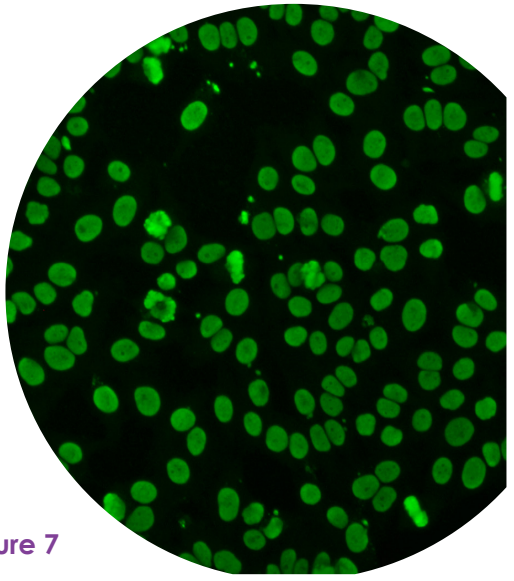


Figure 7

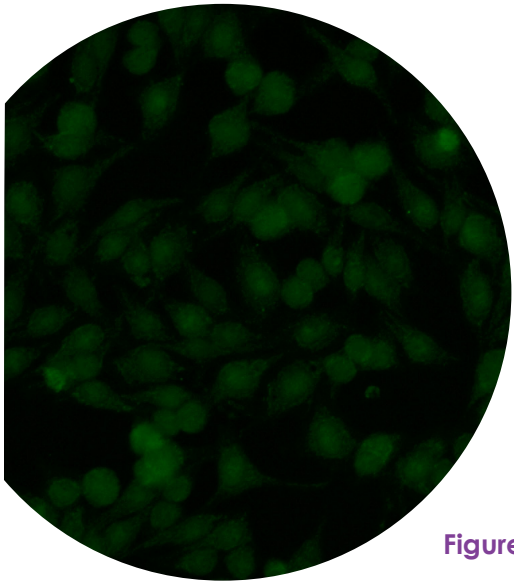


Figure 10

In this final case, Sample C was received from a customer with a very unusual or seemingly contradictory set of results: ANCA-L Ethanol negative (Figure 11), ANCA Formalin negative (Figure 12), ELISA PR3 positive.

Though there might be an expectation of a positive result on ANCA-L and/or Formalin in this case, both were negative. A RELISA® MPO/PR3 positive result in the absence of any other positive result is more indicative of an error in testing. However, testing of the sample at Immuno Concepts confirmed the results obtained by the customer.

NOTE: Though uncommon, it is not unrealistic to expect to see samples such as these. As indicated in the 1999 consensus, approximately 5% of ANCA positive samples test positive on ELISA's and negative on IFA's. Conversely,

approximately 10% of ANCA positive samples test positive on IFA's and negative on ELISA's.

This is one of the reasons why IC supports the 1999 consensus that encourages testing of both RELISA and IFA (as the ideal approach). If the recommended approach is selected, Immuno Concepts recommends first testing with IFA due to sensitivity and cost. (1)

Sample C should be reported as:
ANCA Ethanol: Negative
ANCA Formalin: Negative
RELISA®: PR3 Positive/MPO Negative

Figure 11

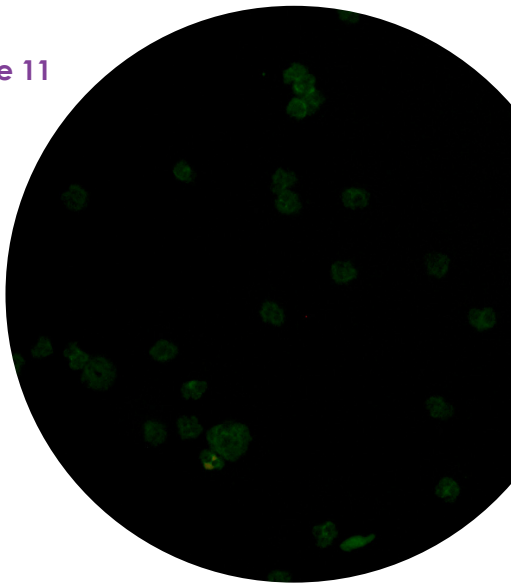
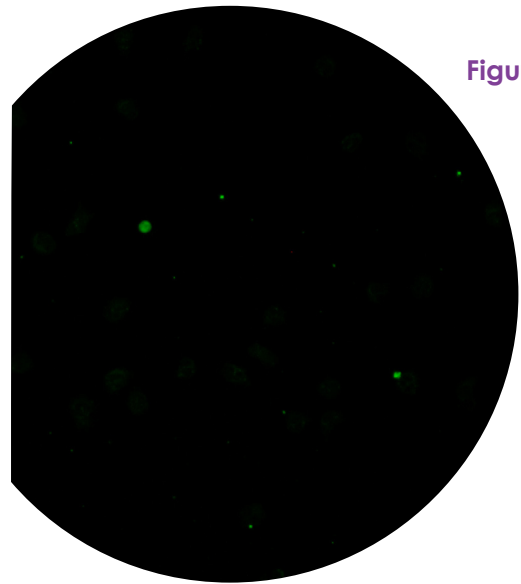


Figure 12



Not doing this assay in your lab?
Contact demo@immunoconcepts.com for a
demonstration and free test kit(s)

References:

- (1) Savage J, Gillis D, Benson E, et al. International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA). Am J Clin Pathol. 1999;111:507-513.
- (2) Bossuyt, X. et al. (2017) Revised 2017 international consensus on testing of ANCAs in granulomatosis with polyangiitis and microscopic polyangiitis Nat. Rev. Rheumatol. doi:10.1038/nrrheum.2017.140
- (3) Savage J, Davies D, Falk R, et al. Perspective in renal medicine. Antineutrophil cytoplasmic antibodies and associated diseases: A review of the clinical and lab-



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