

QUANTA Plex™ ANCA Profile: anti-MPO and anti-PR3 Autoantibody Detection on the Luminex™ Flow Cytometer

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ABSTRACT

Objective: Develop a fluorescent immuno-assay (FIA) for the simultaneous semi-quantitative detection of IgG autoantibodies to myeloperoxidase (MPO) and serine proteinase 3 (PR3) in human serum. This QUANTA Plex™ ANCA Profile is to be used as follow-up testing to screening for anti-neutrophil cytoplasmic antibodies (ANCA) by indirect immunofluorescent assay (IFA). A control bead will be included to detect technical errors. In conjunction with other clinical findings this FIA will aid in the diagnosis of the autoimmune vasculitides microscopic polyarteritis, crescentic glomerulonephritis, and Wegener's granulomatosis (WG). This multiplex technology is also useful for interpreting IFA samples with "difficult" patterns such as those that exhibit several antibodies simultaneously or those with high background fluorescence.

Methods: Purified MPO and PR3 are each bound to different, fluorescently "colored" beads. The two different antigen coated beads are mixed together and put into wells of a microwell plate under conditions that preserve the antigens in their native state. An IgG-coated control bead is included in each microwell to ensure that false negative results due to operational errors are detected. Pre-diluted controls and diluted patient sera are added to separate microwells, allowing any anti-MPO and anti-PR3 autoantibodies present to bind to the immobilized antigen. Then an anti-human IgG conjugated to a fluorescent probe is added to each microwell. A second incubation allows the anti-human IgG fluorescent conjugate to bind to any patient autoantibodies that have become attached to the antigen on the beads. The samples are then measured in the Luminex™ flow cytometer. Each antibody can be semi-quantitated by comparing the fluorescent intensity of the patient sample with the fluorescence of the corresponding Low Positive.

Results: The two Luminex™ tests in the ANCA Profile, anti-MPO and anti-PR3, compared very well to the QUANTA Lite™ MPO and PR3 ELISAs. Only 1.8% of the tests were discrepant (21 of 1166 tests) between the two types of assays. When these discrepant samples were tested by IFA on ethanol and formalin fixed neutrophils, the resulting patterns agreed approximately one third of the time with the Luminex result, one third with the ELISA result and one third had an indeterminate result.

Conclusion: The FIA technique employed by the QUANTA Plex™ ANCA Profile is highly sensitive and specific to both anti-MPO and anti-PR3 autoantibodies. Following ANCA screening by IFA, the QUANTA Plex™ ANCA Profile is objective, semi-quantitative, and can be conveniently used to simultaneously test large numbers of patients on each of these antigens.

INTRODUCTION

ANCA IFA testing has revolutionized the diagnosis and treatment of the various autoimmune mediated vasculitides.¹⁻⁴ The pANCA and cANCA autoantibodies have proven to be especially useful clinically; anti-MPO is the main component of pANCA while anti-PR3 is the main component of cANCA pattern. Solid phase methods to detect anti-MPO and anti-PR3 cannot replace the standard IFA using fixed human neutrophils for detecting ANCA, as there are too many other specificities that are important. This is especially true in the case of atypical or xANCA patterns found in patients with inflammatory bowel disease (IBD) and in patients with sclerosing cholangitis.⁵ The QUANTA Plex™ ANCA Profile provides an important confirmatory result for the two most important identified antigens, MPO and PR3. This FIA is also useful for interpreting "difficult" IFA samples such as those which exhibit several antibodies simultaneously or those with high background fluorescence.

The QUANTA Plex™ ANCA Profile consists of two ½ hour incubations and no wash steps, similar to other QUANTA Plex™ assays.⁶ Each test results in the semi-quantitative determination of anti-MPO and anti-PR3 antibodies, as well as an indication from the IgG bead of possible technical error. After the incubations the three different beads are measured in the Luminex™ flow cytometer. This flow cytometer can discriminate the color of each bead from the others as well as measure the fluorescent intensity of the conjugate on each bead.⁷ The conjugate's fluorescent intensity is proportional to the amount of labeled anti-human IgG bound to the patient autoantibodies on the bead. Calculations using a prediluted control result in semi-quantitative values for both anti-MPO and anti-PR3 autoantibodies found in the patient sample.

METHODOLOGY

The ability of the QUANTA Plex™ ANCA Profile to detect antibodies against MPO and PR3 was evaluated by comparison to INOVA QUANTA Lite™ ELISA kits measuring the same antibodies. The reference interval consisted of 256 average blood donors. Another 90 samples consisted of various disease groups including 67 with atypical ANCA IFA. No samples from clinically defined patients with microscopic polyarteritis, crescentic glomerulonephritis and Wegener's granulomatosis were included as positive samples because these are very rare diseases and clinically defined sera were not available. However, in our in-house library we found 172 samples that had been sent to the technical service department at INOVA over the last several years for ANCA testing. Many of these samples were sent to technical services because they had given confusing results to INOVA's customers. These included a large number of samples that were positive for anti-MPO or anti-PR3, as well as samples that were positive by immunofluorescence on neutrophils, but negative for antibodies reactive with MPO and PR3. Thus, these sera presented a stringent test of the Luminex™ assay. Samples that gave discrepant results between comparable tests were retested on both the Luminex™ and ELISA. Samples that were still discrepant were then tested for ANCA pattern by IFA on both ethanol- and formalin-fixed neutrophils, as well as HEp-2 cells. The slides were read by someone blinded as to the ELISA or Luminex™ results.

RESULTS - MPO

Blood Donors N=256	QUANTA Plex ANCA Profile (MPO)		
MPO ELISA	Negative	Positive	
99.6% agreement	Negative 255	Positive 1*	
	Positive 0	0	

*This sample was a low positive (41 LU) on the MPO bead, as well as moderately positive (64 LU) on the PR3 bead. The cutoff for both Luminex™ and ELISA is 20 units.

Tech Serv ANCA N=237	QUANTA Plex ANCA Profile (MPO)		
MPO ELISA	Negative	Positive	
96.6% agreement	Negative 158	Positive 5**	
	Positive 3*	71	

*One sample was low positive by ELISA and yielded a cANCA pattern on IFA that is more typical of anti-PR3 than anti-MPO. The other two samples were both low positive by ELISA, high negative (17 LU) by Luminex™, and yielded the pANCA pattern that is typical of anti-MPO.

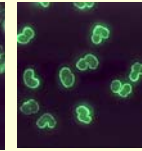
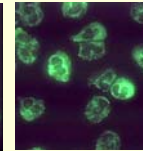
**Four samples were all low positive by Luminex™, high negative by ELISA, and gave the pANCA pattern that is typical of anti-MPO. Another sample was low positive by Luminex, high negative (14 units) by ELISA and gives an atypical ANCA pattern.

Other Controls N=90	QUANTA Plex ANCA Profile (MPO)		
MPO ELISA	Negative	Positive	
98.8% agreement	Negative 89	Positive 1*	
	Positive 0	0	

*This sample was negative for ANCA by IFA.

ANCA IFA SCREENING

pANCA	cANCA	atypical (x)ANCA
Follow-up test: MPO Positive PR3 Negative	Follow-up test: MPO Negative PR3 Positive	Follow-up test: MPO Negative PR3 Negative



RESULTS – PR3

Blood Donors N=256	QUANTA Plex ANCA Profile (PR3)		
PR3 ELISA	Negative	Positive	
99.2% agreement	Negative 253	Positive 1**	
	Positive 1*	1	

*This sample was low positive on ELISA, 22 U, and negative on the PR3 bead for Luminex™ and negative by ANCA IFA.

**This sample was moderately positive on the PR3 bead for Luminex™, 65 LU, but negative on the PR3 ELISA and the ANCA IFA.

Tech Serv ANCA N=237	QUANTA Plex ANCA Profile (PR3)		
PR3 ELISA	Negative	Positive	
97.5% agreement	Negative 131	Positive 5**	
	Positive 1*	100	

*This sample was moderate by ELISA (42 units), high negative by Luminex™, and negative on ANCA IFA.

**Two samples were moderately positive by Luminex™, high negative on ELISA, and negative on ANCA IFA. Two other samples were low or moderately positive on Luminex™, high negative on ELISA, and pANCA positive by IFA, more consistent with anti-MPO than anti-PR3. The fifth sample was high positive by Luminex™, negative by ELISA, and cANCA by IFA, consistent with anti-PR3.

Other Controls N=90	QUANTA Plex ANCA Profile (PR3)		
PR3 ELISA	Negative	Positive	
96.7% agreement	Negative 81	Positive 3*	
	Positive 0	0	

*One sample was 19 U on ELISA and cANCA by IFA, so was likely a true positive. Two other samples were 11 U by ELISA and atypical ANCA by IFA.

SENSITIVITY & SPECIFICITY

Clinical Specificity for the MPO and PR3 ELISAs and the QUANTA Plex™ ANCA Profile.

All	ELISA	ELISA	Clinical	QUANTA	QUANTA	Clinical
Negative	Neg.	Pos.	Specificity	Plex	Plex	Specificity
Controls			ELISA	Negative	Positive	QUANTA
N=279						Plex
MPO	279	0	100%	278	1	99.6%
PR3	277	2	99.3%	277	2	99.3%

Relative Sensitivity and Specificity for the QUANTA Plex™ ANCA Profile.

All Samples	Both	Both	EIA Neg.	EIA Pos.	Relative	Relative	Percent
N=583	Neg.	Pos.	QP Pos.	QP Neg.	Sensitivity	Specificity	Agreement
MPO	502	71	7	3	95.90%	98.60%	98.30%
PR3	465	107	9	2	98.20%	98.10%	98.10%

CONCLUSIONS

The QUANTA Plex™ ANCA Profile is an objective, convenient multiplex based FIA for the semi-quantitative detection of anti-MPO and anti-PR3 autoantibodies. It provides clinically relevant data that is both sensitive and specific. The two autoantibody tests in the QUANTA Plex™ ANCA Profile, anti-MPO and anti-PR3, compared very well with the corresponding ELISAs. Only 1.8% of the tests were discrepant (21 of 1166 tests) between the two types of assays. When these discrepant samples were tested by IFA on ethanol and formalin fixed neutrophils, the resulting patterns agreed approximately one third of the time with the ANCA Profile result, one third with the ELISA result and one third had an indeterminate result.

In addition, the QUANTA Plex™ ANCA Profile has several advantages over other solid phase methods. With only two ½ hour incubations, and no wash steps, results are obtained quickly and easily, and the procedure can be automated. The multiplex platform allows for simultaneous detection of multiple autoantibodies, as well as detection of possible technical error using the IgG control bead.

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