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Advantages of Multiplex Proteomics in Clinical Immunology

The Case of Rheumatoid Arthritis: Novel IgXPLEX™ Planar Microarray Diagnosis

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Abstract Clinical multiplex diagnostic proteomics is the application of proteomic technologies to improve a patient's clinical outcomes. The future holds impact potential for testing prognosis, diagnosis, and drug therapy, while monitoring efficacious treatment with qualitative and quantitative data. Multiplex clinical diagnostic use of novel biomarkers in body fluids to confirm presence and severity of clinical disease states, holds great promise for clinical use. Challenges for diagnostic clinics include awareness of proteome complexity in clinical samples, the effects of high-abundance proteins, such as albumin, that could mask detection of other and low abundance disease proteins or biomarkers. Standardized approaches to sample collection and preparation, new analytical techniques and novel algorithms for bio-statistical analysis will facilitate release of the great potential of clinical multiplex diagnostic proteomics. A sensitive RA assay has been developed for the simultaneous measurement of the three rheumatoid factors (RFs), RF-IgA, IgG, and IgM, with the option to simultaneously measure anti-cyclic citrullinated peptide (anti-CCP) IgG antibodies using IgXPLEX™ technology. Testing 10-μL serum samples, SQI's multiplex microarray rheumatoid arthritis assay provides both positive/negative as well as qualitative/semi-quantitative results for anti-CCP IgG, RF-IgA, IgG, and IgM in each sample well on a 96-well microtiter-formatted microarray plate. Signal detection uses sensitive fluorescent-tagged markers captured onto planar microarray spots and read in a microarray scanner. Each result is verified with confidence confirmation technology and validating quality controls in every sample well. For an 80-RA positive patient cohort, the 4-PLEX profile sensitivity was determined at 82.5%. The specificity for the 44 RA healthy control cohort was determined at 97.7%. The multiplex data also demonstrated that a patients' severity of disease profile, mild to severe, correlates the status of RA biomarkers to disease status.

Keywords Autoimmune assay · Proteome · Rheumatoid arthritis · Rheumatoid factor · Microarray · Multiplex assay · Multiplex 4-PLEX assay · Anti-CCP antibodies · RA auto-antibody profile

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Introduction

About 3–5% of all human diseases are classified as autoimmune diseases with a clinical need to improve

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diagnosis, outcome prediction, and innovative therapies. Individualized pharmacotherapy that results in sustained remission rather than symptom relief is the goal for long-term management of patients with autoimmune disease. Early biomarker discovery is becoming increasingly important for early diagnosis and treatment of these diseases [1]. Microarray-based investigations have led to the discovery of several candidate markers that are being validated on industry-standard platforms and to the subsequent development of antigen therapeutics for clinical validation [2]. Autoimmune diseases are a family of more than 100 illnesses wherein underlying defects in the immune system lead the body to attack its own tissues, organs, and cells [3]. Clinical proteomics includes identification, characterization, and quantification of the protein content or proteome of whole cells, tissues, or body fluids in reference to specific disease processes, such as rheumatoid arthritis [4]. The potential for proteomic diagnostics is to identify and quantify novel and specific disease-associated gene products, i.e., proteins in the plasma that can function as biomarkers of the presence or severity of clinical disease status [5]. As challenges in identifying and translating recognized disease-specific plasma proteomic biomarkers are overcome, these biomarkers will continue to complete successful transition from verification to routine clinical use [6, 7].

Functional proteomics in the field of rheumatology measures the fluctuation in protein concentration during differentiation and proliferation in both qualitative and quantitative terms [8]. Consecutive steps of proteome study applications in the field of rheumatology may range from proteome analysis of biological fluids of rheumatic disease to identification of possible new diagnostic tools. Proteomics has great potential in the field of rheumatology to impact molecular understanding of these complex diseases [9].

Challenges inherent to plasma proteomics

Of the many sources available for identifying new biomarkers of clinical disease diagnosis and severity, the proteome holds most promise for identifying previously unknown biomarkers that have the potential to be from novel pathways and to be complementary to previously identified biomarkers [7]. Proteomics may exceed genomic approaches because proteins and enzyme are primary activators to determine the diversity of phenotypes that can manifest from a common set of genes. The complement of expressed proteins can change rapidly in response to environmental causes. Consequently, the proteome is highly suited to represent the state of a cell, tissue, or organism at a given time, in the context of a specific stimulus.

One of the barriers to plasma biomarker identification and translation from discovery platforms is the high level of complexity of the proteome. This complexity presents unique analytical challenges that are further magnified when using clinical plasma samples to search for new biomarkers specific for clinical disease. The plasma proteome contains a plethora of unique proteins. The plasma proteome does not result from expression of a single cellular genome, but tends to reflect gene product contributions from the collective expression of multiple cellular genomes. It has been hypothesized that the estimated complement of over 300,000 human polypeptide species arising from variable splicing and post-translational modifications could be present in the plasma proteome [10]. Proteins from all functional classes and cellular localizations are found in the plasma, and a majority of the lower abundance proteins are intracellular or membrane proteins, presumably found in the plasma as a result of cellular turnover. One of the challenges inherent to plasma or serum studies is the issue of high-abundance proteins. Greater than 95% of the serum proteome may contain some 20 high-abundance proteins including albumin and the immunoglobulins. These high-abundance proteins hinder the ability to detect low-abundance proteins. However, it is the low abundance proteins that are most likely to be biologically relevant as markers of a disease state. Concentrations of low-abundance proteins may differ from those of high-abundance proteins by as much as 10 orders of magnitude. In addition, there have been advances in technologies that allow depletion of high abundance proteins. New immuno-depletion strategies efficiently remove as many as 20 of the high-abundance constituents [11]. Techniques for immunoextraction and concentration of targeted biomarker fragments may be more reliable [12–17]. However, the degree to which relevant low-abundance proteins are lost during processing to remove high-abundance proteins is unclear and may be highly variable.

Multiplex immunoassay technologies

There is considerable benefit in multiplex arrays that enable simultaneous quantification of multiple analytes [18]. Single protein measurement can be laborious, time-consuming, and costly, whereas concurrent measurement of multiple biomarkers permits reduced sample consumption, technician time, and reagent volumes and increases sample throughput. The information provided by measurement of a single protein is limited, and multiple markers may be more useful for disease screening and assessing multiple physiological pathways that contribute to disease activity and prognosis.

Enzyme-linked immuno-sorbent assay (ELISA) is a frequently used diagnostic reference method for protein measurement and has in part, been further developed and adapted for multiplex biomarker assessment in both planar microarray multiplex immunoassay formats and bead suspension arrays. For planar arrays, printed analytes are traditionally detected using high-resolution fluorescence or chemiluminescent immunoassay methods wherein immobilized capture antibodies complex with analytes in a biological sample. Detection antibodies linked to fluorescent-labeled reporter molecules bind the captured protein. The signal generated by the reporter molecules is directly proportional to protein concentration in the unknown sample when calibrated using reference standards.

Planar array assays

For planar arrays, capture antibodies are discretely immobilized on a rigid microplate surface using robotic arrayers [19]. Antibodies can be spotted directly onto the plate surface using non-contact arrayers that use piezoelectric elements to transfer material to the microplate surface or by surface contacting pins. Planar array protocols are comparable to traditional ELISAs and typically use a camera to detect fluorescent or chemiluminescent signals. Numeric values are generated based on the density of light image spots, and data are assigned to a specific assay based on the intra-well location of the light-emitting spots. The planar microarray technology of interest for this pertinent methodology is commercially available [20].

Bead suspension assays

In bead suspension assays, capture antibodies are immobilized on polystyrene microsphere beads suspended in buffer. Biological sample is added to mixtures of the beads and a detecting antibody–fluorophore conjugate binds to the captured protein. Flow-cytometric systems identify bead types and captured sample proteins. As beads pass through laser beams housed in the flow cytometer, the reporter fluorophore is excited and emits light that is converted to a numeric signal by internal digital processors. Simultaneous excitation of internal bead dyes allows measurement of bead fluorescent intensity that is unique to each bead type and used to assign fluorophore values to the correct assay. Several bead-based suspension arrays are commercially available [21, 22]. Assay development necessitates in-house assay research and development [23]. A disadvantage is that nonspecific binding of serum proteins directly to the microspheres may result in bead aggregation and nonspecific fluorescent emission, thereby limiting assay sensitivity and accuracy [24].

Comparison of planar microarrays and suspension bead arrays

Capture analytes in both cases are bound to a solid substrate. In the case of planar micro arrays, the capture analytes are covalently bound to the surface of the microarray. They cannot interact with each other in solution, as can capture analytes attached to beads in suspension. The miniaturization of printed microarrays provides a platform where the detecting proteins in solution are in excess concentration over the capture analytes bound to the microarray. Increased concentration means faster binding kinetics and increased sensitivity to provide more sensitive and reproducible results. Planar microarray data quality is checked by careful examination of the raw data. Bead array flow cytometer detection instruments do not provide any raw data. Bead suspensions depend on redundancy in the system to provide data. Bead array platforms cannot use label-free detection methods, whereas microarray spot locations define and identify the capture analytes and only a single image scan is required. Exposure to light damages beads to deliver misleading results. Beads cannot multiplex at both the antigen and antibody level as can only be done by planar microarray. In bead suspension systems, only single beads that are in suspension are counted. All beads aggregated at the bottom of the well or attracted or bound to other beads are not interrogated to contribute data, leading to becoming a false-negative result.

Validation of multiplex array biomarker panels

For validation of diagnostic biomarker panels by planar array technology or bead suspension assay, well-characterized multiplex assay components are needed to ensure that the data derived from multiplex assays are useful in a clinical setting. Capture and detection antibody materials should be well characterized and exhibit minimal inter-lot variability. A sustainable source of antibodies with known specificity is needed. Lack of specific antibodies can be a major impediment to both single-plex and multiplex assays. There is also a need for validated reference standards that allow accurate and consistent quantification of proteins. Multiplex arrays are classified as in vitro diagnostic multivariate index assays by the U.S. Food and Drug Administration (FDA). Formal regulatory guide lines for clinical validation of multiplex assay regulatory requirements are under review [25]. Nevertheless, robustness, sensitivity, reliability, and consistency of proteomic test systems for the detection of changes in analyte expression are crucial parameters, as are test costs and labor expenses for the clinical acceptance of multiplex diagnosis. At present, many proteomic diagnostic techniques still suffer

from insufficient standardization. Only a few have the potential to fulfill essential criteria for practical clinical applications [26].

Clinical specimen considerations

Validation of potential biomarkers for diagnosis or disease severity requires the use of biological fluids from statistically significant numbers of patients with the relevant disease and appropriate controls. Banked specimens that had been collected in conjunction with prior clinical trials or observational studies, have the advantage of immediate availability in conjunction with known phenotype patient populations. However, the use of banked specimens also has significant limitations. Foremost, a clinical trial is usually not designed with the goal of validating a diagnostic or disease severity biomarker. Strict inclusion and exclusion criteria may limit the applicability of findings. Appropriate controls must be included in the study population.

There are a number of pre-analytic variables that can affect the validity of biomarker assays. These variables need to be considered when designing validation studies and assessing the potential utility of banked specimens [27]. Pre-analysis variables that can affect assay validity include the method of sample collection, the type of anticoagulants or preservatives that are used, the procedure used to process the sample, the time between collection and assay. The storage conditions used during this interval, freezing and thawing, especially repetitive freeze–thaw cycles, may be particularly harmful to some protein analytes [28]. Protein degradation can occur at any time from sample collection to time of assay.

The importance of standardization in sample collection and processing for proteomic studies, suggests that the potential limitations of archival samples must be carefully assessed before using these samples for discovery or validation of protein biomarkers.

Analysis of serum and synovial fluids is important in the diagnosis of rheumatic diseases and also in monitoring how patients respond to therapy. As biomarkers, protein interactions and signaling pathways become apparent by proteomic analysis. Understanding mechanisms of disease will lead to open new avenues for drug targets in the field of rheumatology [9].

Multiplex detection of RF isotypes and anti-CCP IgG in a 4-PLEX Micro-array for diagnosis of rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease marked by chronic inflammation of synovial joints affecting 0.5–1% of the population [29]. A number of auto-antibodies are generated in RA including rheumatoid factor (RF) directed against the Fc-region of isotype immunoglobulins. About 70–80% of RA patients are seropositive for RF, as are 1–5% of healthy and about 20% of elderly individuals [30]. Among immunological markers, RF is associated with a more severe radiographic progression in early RA [31]. RF-IgA has been assessed to be the optimal predictor of radiological damage [32], whereas anti-cyclic citrullinated peptide antibodies (anti-CCP antibodies) are good predictors of joint erosion in early RA. The assessment of anti-citrullinated protein/peptide antibodies, in addition to clinical and radiometric outcome indicators, may be important to assess disease prognosis and early therapeutic strategies [33]. This study aims to evaluate sensitivity and specificity of RFs of IgM, IgG and IgA isotype and of anti-CCP IgG antibodies in RA in a cumulative novel, multiplex format.

Validating analysis of multiplex planar proteomic biomarker data sets

This technology provides confidence in that all of the reporter antibodies are binding to the captured antibodies in the same reaction. The value of multiplexed reporters is that all other assay conditions in the sample reaction are the same, thus eliminating potential assay-to-assay variation, when a single patient sample is tested in multiple, independent assays. There also is an inherent economic value in providing multiple results from one sample and therefore the clinician has the benefit of assessing all of the markers at the same time, rather than seeing one and having to request another from a sample which will behave differently due to being a different draw or a frozen aliquot. The data obtained demonstrates that these assays provide accurate and precise clinical data, based on multiple validations, method comparison, sensitivity–specificity, and reproducibility studies.The prospective reproducibility study included three diluted RA samples. One platform was used to perform three runs of 20 replicates of each sample, providing 60 results for each. The resulting panel for the reproducibility analysis was primarily made up of retrospective samples from the precision study.

Significance of anti-CCP antibodies

A lack of specificity and low sensitivity in RA is a significant shortcoming of in-use RF tests [34–36]. The antibody system which combines good sensitivity and specificity for RA targets citrullinated epitopes [37]. A 56% sensitivity of these auto-antibodies in developing RA

has been reported for a cohort of early arthritis patients [38]. Auto-antibodies against citrullinated antigens have been detected and used for diagnostic purposes, using both protein- and peptide-derived epitopes [39]. Diagnostic markers require good sensitivity to detect a high percentage of patients, good specificity to limit false positives and early presence to enable early diagnosis. Anti-CCP antibodies together with RF antibodies were determined to have a specificity of 99.6%, concluding that this combination confirms patients with suspected early RA [40]. Both RF-IgM and anti-CCP have been detected in pre-symptomatic RA patients [41]. RF-IgM has been detected up to 10 years in pre-diagnosed sample and anti-CCP was detected up to 14 years prior to first clinical symptoms. The combination of anti-CCP and RF is highly prognostic for measuring developing RA [42].

Synthetic antigen mimetics are immuno-reactive with rheumatoid arthritis auto-antibodies

SQI Diagnostics' synthetic peptides incorporating immuno-reactive RA epitopes or binding sites are specifically reactive with auto-antibody present in the sera of patients. Peptide epitopes mimic biological activity or specific binding of the anti-citrulline antibody to a citrulline epitope or antigen designed into a relatively small, linear synthetic peptide. These novel, synthetic designer peptides consist of unique amino acid sequences containing a series of citrulline residues at selected locations within a peptide sequence. The combination of citrulline residue spacing is designed into proprietary three-dimensional synthetic antigen mimetic peptide sequences. Sequence for assembly of selected amino acid residues confers optimal, specific binding parameters to enhance selective reactivity with auto-antibodies. The cyclized synthetic antigen mimetic peptides are further assembled in series as multimeric peptides. Selected series of three-dimensional reaction mixtures are conjugated to capture a multiplicity of auto-antibodies (patents pending).

Methods

Multiplex immunoassay

The 4-PLEX, multiplex assay simultaneously measures rheumatoid factors (RF), RF-IgA, IgG, IgM, and anti-CCP IgG antibody. Each test well uses 10 μL of test serum, contains multiplex, quantifiable biomarker test microarrays standardized against multiple in-well calibration microarrays to determine analyte equivalent concentrations (AEC) of biomarkers and calibrator analytes for each microarray spot. The AEC is calibrated against recognized reference standards. Eighty diagnosed rheumatoid arthritis-positive patient samples and 44 healthy controls were tested. All tests were processed in a fully automated SQiDworks™ platform using a IgXPLEX 96-well, RA assay format.

Automated microarray multiplex diagnostic platform

The SQiDworks™ Diagnostics Platform [43] fully automates immunoassay protocols from end-to-end, including sample pipetting, serum dilution, incubation, washing, drying, scanning, and reporting. The instrument integrates an automated pipetting station, a fluorescence scanner, washing, and drying stations, and ancillary hardware components using dedicated instrument control. The software controls scheduling, self-verification, data acquisition, data management, analysis algorithms, and reporting software.

The automated robotic processing of IgXPLEX multiplex diagnostic assays rapidly translates into calibrated patient results. The platform provides advantages of automated assay plate scanning and analysis with internal quality controls; multiple detection and results per patient test well; in-well normalization to provide consistency between lots and data invalidation algorithms to provide internal quality control.

Patient and control cohorts

A first cohort has serum samples from 80 patients with confirmed seropositive erosive RA according to ACR criteria [44] and patient profile was determined by attending rheumatologists. A second cohort has 44 healthy controls (17 males (39%) aged 18–56 years, 27 females (61%) aged 19–53 years; clinically asymptomatic and non-reactive to human immunodeficiency virus, hepatitis B virus surface antigen, and hepatitis C virus (PromedDx LLC). Both cohorts were evaluated to demonstrate the sensitivity and specificity achieved by having combined the three RF isotypes with anti-CCP IgG into a 4-PLEX assay, Tables 1 and 2. A 3-PLEX assay, Table 3, was also compared to confirm the analytical advantage of a 4-PLEX assay. All patients attending the rheumatology clinics were fully informed of the purposes of the study, and all gave their consent. Healthy controls, who were selected from blood donors also gave informed, oral consent.

Results I

Multiplex RF together with anti-CCP IgG makes up a 4-PLEX RA assay

Individual RF results comparing sensitivity of RF-IgA vs. RF-IgG vs. RF-IgM vs. anti-CCP IgG, are shown in Table 1.

Table 1 RA multiplex sensitivity—4-PLEX fluorescent assay combination

	RF-IgA	RF-IgG	RF-IgM	Anti-CCP IgG
Total	80	80	80	80
Seropositive	60	58	64	56
Seronegative	16	20	14	24
Not determined	4	2	2	0
Sensitivity	75.0%	72.5%	80.0%	70.0%

The derived sensitivity for each individual RF isotype supports RF-IgM to have a sensitivity at 80%

Table 2 compares specific combinations of isotypes to report RA 4-PLEX sensitivity at 82.5%.

RA 4-PLEX sensitivity of detection for RA-positive patients

Patients positive for RA, showed a definite increase in 4-PLEX sensitivity; although anti-CCP IgG was detected in only 56/80 RA patients, the 4-PLEX assay of RF-IgA, IgG, IgM plus anti-CCP IgG, detected RA in 66/80 patients. Based on a diagnosis of confirmed seropositive, erosive RA, the study demonstrated that the 4-PLEX RA assay determined 82.5% sensitivity. This performance characteristic surpasses current single and combined RF and anti-CCP tests [39]. The sensitivity was further compared with data published by Berglin [44], who compared the sensitivity for single analytes, i.e., anti-CCP antibodies versus single RF isotypes, as well as against anti-CCP + RF-IgM, or RF-IgG, or RF-IgA, where RF-IgM demonstrated a sensitivity of 79.3% [41].

RA 3-PLEX sensitivity for anti-CCP IgG-positive patients

In any subpopulation of RA patients which are anti-CCP IgG-negative, there is a likelihood for cross-reaction between various biologic drugs (e.g., adalimumab) with the assay for anti-CCP IgG (16). Therefore, we selected the patients which were anti-CCP IgG-positive, i.e., the 56/80 RA patients to analyze the RA 3-PLEX algorithm in Table 3. The sensitivity

Table 2 RA 4-PLEX (multiplex) sensitivity—multiplex fluorescent assay combination

Total final positive based on anti-CCP IgG and RF-IgM	56
Total final negative based on anti-CCP IgG and RF-IgM	14
Total positive when anti-anti-CCP IgG is negative but RF-IgM is positive (with or without others)	10
Total	80
Sensitivity [total identified by 4-PLEX (if Anti-anti-CCP IgG-positive and RF-IgM-positive or RF-IgG/IgA is positive)]	82.5%

Table 3 RA 3-PLEX sensitivity—multiplex fluorescent assay combination

	RF-IgA	RF-IgG	RF-IgM	RA 3-PLEX
Total	56	56	56	56
Seronegative	4	7	3	1
Seropositive	51	49	53	54
Not determined	1	0	0	1
Sensitivity	98.7%	87.5%	94.5%	98.2%

of RF IgA vs. RF IgG vs. RF IgM vs. RA 3-PLEX for the 56 patients who are anti-CCP IgG-positive was at 98.2%.

RA 3-PLEX sensitivity for anti-CCP IgG-negative patients

Using current anti-CCP IgG and total RF assays selected for negative anti-CCP IgG have low sensitivities that do not help to confirm the RA clinical status of this cohort. In the 4-PLEX RA assay, a negative anti-CCP IgG result together with positive RF-IgM identified 43.5% of RA patients. Table 4 shows sensitivity of RF IgA vs. RF IgG vs. RF IgM vs. RA 3-PLEX for the 24 patients who are anti-CCP IgG-negative. The RF-IgM is directly comparable to the RA 3-PLEX assay.

Comparison of RA multiplex (2-PLEX, 3-PLEX, 4-PLEX) combinations sensitivity

With multiplex determination, when anti-CCP IgG is positive combined with RA 3-PLEX results, sensitivity of 98.2% was achieved and when anti-CCP IgG is negative, sensitivity of 54.2% was obtained as shown in Table 5. These combined results provide confidence in confirming RA diagnosis.

RA 4-PLEX specificity of detection

Table 6 illustrates specificity of RF-IgA vs. RF-IgG vs. RF-IgM vs. anti-CCP IgG and RF-IgM + anti-CCP IgG. If each of these tests is performed on a stand-alone basis, the

Table 4 RA 3-PLEX sensitivity—multiplex fluorescent assay combinations

	RF-IgA	RF-IgG	RF-IgM	RA 3-PLEX
Total	24	24	24	24
Seronegative	16	15	13	13
Seropositive	5	7	10	10
Not determined	3	2	2	0
Sensitivity	23.8%	31.2%	43.5%	43.5%

Table 5 RA multiplex combined sensitivity—multiplex fluorescent assay combinations

	Anti-CCP IgG + RF-IgM-positive	Anti-CCP IgG + RF-IgG/IgA-positive	Anti-CCP IgG-positive + 3-PLEX RA	Anti-CCP IgG-negative + RF-IgM	Anti-CCP IgG-negative + RF-IgG/IgA	Anti-CCP IgG-negative + RF-IgM/IgG + IgA
Total	80	80	56	24	24	24
Seropositive	53	49	55	10	3	13
Seronegative	3	5	1	14	21	11
Sensitivity	66.3%	61.3%	98.2%	41.7%	12.5%	54.2%

individual results have to be combined to confirm negative disease status. If only one of these tests is performed, the test result may not be sufficient to confirm negative disease status. The results in Table 6, confirm that the RA 4-PLEX assay specificity of RF-IgM when combined with anti-CCP IgG has specificity at 97.7%.

Correlation of patient RA severity profile to RA 4-PLEX assay results

Patient disease profile (mild to severe) was compared to RA 4-PLEX with negative or positive results for RF-IgA, IgG, IgM, and anti-CCP IgG, to evaluate if a combination of RA panels would be useful in predicting RA severity as shown in Table 7. 4-PLEX reports only 5% of severe cases as negative and up to 75% of positive cases. For this cohort, the patients had been graded as mild, moderate, or severe based on the overall clinical assessment of each patient for: (1) speed of disease progression; (2) level of radiographic destruction; i.e., amount of bone destruction as determined by X-ray; and (3) level of disease-modifying anti-rheumatic drug (DMARD) treatment required; i.e., number of DMARDs or biologics required over time to control the disease process.

Patient disease profiles (mild to severe) have also been compared to RA multiplex when anti-CCP IgG is both positive and negative. Table 8 shows correlation of RA markers to disease status when anti-CCP IgG is positive and Table 9 shows correlation when anti-CCP IgG is negative. When anti-CCP IgG is positive, moderate and severe disease status report higher percentage of positive results for RF compared to mild status. However, when anti-CCP IgG is negative for mild status, RF is also negative.

Patient and control cohorts II

A second cohort consisted of serum samples obtained from commercial suppliers. 358 samples were from donors with confirmed RA, 385 donors with other autoimmune diseases, and 150 healthy controls.

Results II

Overall sensitivity and specificity of RF IgA was 85.5% and 80.6%, respectively. When all other diseases are excluded, the specificity increased to 92.7%. In this study, IgA antibodies to RF were found with the highest frequency in Sjogren's syndrome, vasculitis and progressive systemic sclerosis; whereas the lowest frequency of these autoantibodies were found in arthropathies, osteoarthritis, myalgia, and myositis (Table 10).

Overall sensitivity and specificity of RF IgG was 71.2% and 71.5%, respectively. When all other diseases were excluded, the specificity was 74%. IgG antibodies to RF were found with the highest frequencies in Sjogren's syndrome, progressive systemic sclerosis and MCTD; whereas the lowest frequencies were found in osteoarthritis, arthropathies, myalgia, and myositis patient groups (Table 11).

Overall sensitivity and specificity of RF IgM was 93.3% and 77.9%, respectively. The increase to 94% when all other diseases are excluded, shows a 16% cross reactivity with other disease conditions. IgM antibodies to RF were found with the highest frequencies in Sjogren's syndrome, vasculitis, progressive systemic sclerosis, and MCTD, whereas the lowest frequencies were found in joint pain, CTS, myalgia, myositis, and arthropathies (Table 12).

Table 6 RA 4-PLEX specificity—multiplex fluorescent assay combination

	RF-IgA	RF-IgG	RF-IgM	Anti-CCP IgG	RF-IgM + anti-CCP IgG
Total	44	44	44	44	44
Seropositive	2	13	1	1	1
Seronegative	42	31	43	43	43
Specificity	95.5%	70.5%	97.7%	97.7%	97.7%

Table 7 RA 4-PLEX patient disease severity profile—multiplex fluorescent assay combination

Disease status	Mild	Moderate	Severe
Total	24	36	20
Negative for (all) 4-PLEX	6 (25%)	4 (11%)	1 (5%)
Positive for (all) 4-PLEX	11 (46%)	22 (61%)	15 (75%)

Overall sensitivity and specificity of anti-CCP IgG was 77.7% and 89.9%, respectively. After exclusion of other disease conditions, the specificity increased to 96.0%. This suggests that CCP is very robust in distinguishing other conditions from RA-positive samples. CCP demonstrated the highest overall specificity against other conditions, with the lowest specificity of 79.1% against MCTDs, and the highest against vasculitis and osteoarthritis. The consistently high specificity for CCP attests to its utility as a tool for the positive diagnosis of RA patients.

Discussion

Evidence indicates that early, accurate, and quantifiable measurement of RA biomarkers, to initiate early therapeutic intervention, improves disease outcome [45]. Implementation of diagnostic models into practice requires validation ideally leading to probabilities of outcomes that are valuable to practicing physicians. The prevalence and predictive value of anti-CCP antibodies in developing RA and the relationship to rheumatoid factor (RF) isotypes has shown that sensitivities for detecting these auto-antibodies, before appearance of RA symptoms, were 52% for anti-CCP; 30% for IgM-RF; 27% for IgG-RF; and 39% for IgA-RF. Anti-CCP antibody and IgA-RF were found to be significant predictors of RA, with anti-CCP antibody having the highest predictive value [46]. In a comparative prospective study, baseline levels of anti-CCP and RFs were measured by ELISA in 104 patients with RA disease duration of less than 2 years. The data confirmed that measurement of anti-CCP and individual RF-isotypes in combination analysis provided the most accurate prediction of structural damage [47].

Current methods in clinical laboratories for detecting multiple analytes determine each autoantibody separately for screening and diagnosis of rheumatoid disease [12], e.g., Specialty Laboratories Rheumatoid Arthritis EvaluatR™ Kit. Emphasis remains to derive a strong link between clinical statistics and evidence-based laboratory medicine [48]. Due to a lack of true quantitative calibration, the respective clinical cutoff value has to be defined with each assay system. To measure signal for auto-antibodies when using microarrays, fluorescent intensity continues to be used [49]. Although ELISA for IgM-RF (highest sensitivity found was 66.4%) has use as a screening marker, anti-CCP ELISA has comparable sensitivity (64.4%) and has proven to be the most specific marker for the disease with a specificity of 97.1% [50]. Anti-CCP proved to be decisive in ambiguous cases or RF-negative patients [51]. RF as an accepted laboratory test for RA is part of the revised ACR classification for RA. More recent diagnostic markers are anti-citrullinated antibodies with a specificity of 89–100% and sensitivity of 41–80% [52]. Antigen microarray profiling of auto-antibodies in RA, i.e., proteomic analysis of reactivity, has allowed stratification of patients with early RA into clinically relevant disease subsets [53]. Citrullinated fibrinogen ELISA determined sensitivity at 60.9% with a specificity of 98.7% [54]. Anti-CCP has been approved by the FDA for diagnosis of RA. Current evidence supports testing for both markers. i.e., anti-CCP and RF. Positive tests for both markers yields the highest positive predictive value for evolution of erosion in recent onset RA [55]. Possible environmental risk factors for serological subtypes of RA, as defined by the presence or absence of auto-antibodies to CCP with distinct etiologies for anti-CCP-positive and -negative RA, include tobacco smoking, alcohol consumption, coffee consumption, and

Table 8 RA multi-PLEX patient profile—multiplex fluorescent assay combination

Disease status		RF-IgA	RF-IgG	RF-IgM	Anti-CCP IgG
Mild	Seropositive	11 (78.6%)	12 (85.7%)	12 (85.7%)	14 (100%)
	Seronegative	3	2	2	0
	Not determined	0	0	0	0
Moderate	Seropositive	24 (92.3%)	22 (84.6%)	25 (96.2%)	26 (100%)
	Seronegative	1	4	1	0
	Not determined	1	0	0	0
Severe	Seropositive	15 (93.8%)	15 (93.8%)	16 (100%)	16 (100%)
	Seronegative	0	1	0	0
	Not determined	1	0	0	0

Table 9 RA multi-PLEX patient profile—multiplex fluorescent assay combination

Disease status		RF IgA	RF IgG	RF IgM	CCP IgG
Mild	Seropositive	1 (10.0%)	2 (20.0%)	2 (20.0%)	0
	Seronegative	7	6	7	10
	Not determined	2	2	1	0
Moderate	Seropositive	3 (30.0%)	4 (40.0%)	5 (50.0%)	0
	Seronegative	6	6	5	10
	Not determined	1	0	0	0
Severe	Seropositive	1 (25.0%)	1 (25.0%)	2 (50.0%)	0
	Seronegative	3	3	2	4
	Not determined	0	0	0	0

obesity [56]. The impetus towards early treatment for patients with RA needs more confirmatory markers than only RF-IgM, which has been considered a non-specific immunoglobulin. However, combining results of anti-CCP antibodies and RF-IgM did not increase the diagnostic accuracy for RA [57]. Anti-CCP antibodies were reported as more specific than RF for diagnosing RA [58]. IgG in combination with IgA improved specificity over single tests [59]. Another assay strategy [60] combined a multiplexed cytofluorimetric RF test with anti-CCP antibody testing. The number of false-positives due to low specificity of RF was markedly reduced by use of the multiplex RF test combined with the anti-CCP antibody.

The clinical benefit of erythrocyte sedimentation rate introduced in 1897 remains limited to monitoring the response to therapy in certain inflammatory diseases and rheumatoid arthritis [61]. In contrast, the utility of anti-citrullinated protein antibodies in rheumatoid arthritis has been confirmed [62], wherein anti-citrullinated protein–peptide antibodies have proven to be highly specific for RA. They are detected in developing disease and implicated in more severe joint erosion. The objective of multiplex testing is based on data that the quantified detection of panels of auto-antibodies against citrullinated proteins will increase the sensitivity and specificity for RA diagnosis.

Expressed autoantibody reflects immune-mediated inflammation and act as surrogate markers of prognosis, diagnosis, and clinical outcome in disease assays. An appropriate strategy when autoantibody testing is to determine a differential diagnosis before ordering clinically relevant autoantibody screening tests. Different algorithms need to be planned, as both the possibility to obtain an initial, tentative diagnosis by the clinician and an alternate strategy to be adopted when the clinical information cannot be obtained. Consequently, assay strategy guidelines are best formulated by close collaboration between clinical and laboratory experts that reach agreement on testing and reporting strategies, while also considering the closest possible compliance with local and international recommendations for diagnostics [63].

Array technology and proteomics are about to launch the era of multiplex analysis, to allow simultaneous detection

Table 10 Clinical sensitivity and specificity of RF IgA in a 4-PLEX assay

Sample group	Sample disease classification	# of samples	Sensitivity (%)	±95% CI	Specificity (%)	±95% CI
RA	Rheumatoid arthritis	358	85.5	83.6–87.3	N/A	N/A
Other autoimmune diseases	SLE	43	N/A	N/A	72.1	65.3–78.9
	Sjogren's syndrome	26	N/A	N/A	42.3	32.6–52.0
	MCTD	43	N/A	N/A	65.1	57.8–72.4
	Progressive Systemic Sclerosis	53	N/A	N/A	62.3	55.6–68.9
	Arthropathies	26	N/A	N/A	96.2	92.4–99.9
	Osteoarthritis	43	N/A	N/A	93.0	89.1–96.9
	Joint pain, CTS	37	N/A	N/A	81.1	74.6–87.5
	Myalgia and myositis	38	N/A	N/A	94.7	91.1–98.4
	Vasculitis	17	N/A	N/A	58.8	46.9–70.8
	Other autoimmune diseases	61	N/A	N/A	82.0	77.0–86.9
Other autoimmune diseases		387	N/A	N/A	76.0	74.1–77.8
Healthy controls		150	N/A	N/A	92.7	90.5–94.8
Healthy controls + other autoimmune diseases		537	N/A	N/A	80.6	78.9–82.3

Table 11 Clinical sensitivity and specificity of RF IgG in a 4-PLEX assay

Sample group	Sample disease classification	# of samples	Sensitivity (%)	±95% CI	Specificity (%)	±95% CI
RA	Rheumatoid arthritis	358	71.2	68.8–73.6	N/A	N/A
Other autoimmune diseases	SLE	43	N/A	N/A	79.1	72.9–85.3
	Sjogren's syndrome	26	N/A	N/A	30.8	21.7–39.8
	MCTD	43	N/A	N/A	58.1	50.6–65.7
	Progressive systemic sclerosis	53	N/A	N/A	50.9	44.1–57.8
	Arthropathies	26	N/A	N/A	84.6	77.5–91.7
	Osteoarthritis	43	N/A	N/A	86.0	80.8–91.3
	Joint pain, CTS	37	N/A	N/A	75.7	68.6–82.7
	Myalgia and myositis	38	N/A	N/A	84.2	78.3–90.1
	Vasculitis	17	N/A	N/A	76.5	66.2–86.8
	Other autoimmune diseases	61	N/A	N/A	77.0	71.7–82.4
Other autoimmune diseases		387	N/A	N/A	70.5	68.6–72.5
Healthy controls		150	N/A	N/A	74.0	70.4–77.6
Healthy controls + other autoimmune diseases		537	N/A	N/A	71.5	69.6–73.5

of numerous autoantibody specificities and the possibility of defining broad autoantibody profiles. This will improve disease staging, risk stratification, prognosis, and treatment. However, although this technology is very promising, it remains to undergo strict analytical and clinical validation processes. The latter should involve clinicians and pathologists in prospective, multi-centric studies conducted on large numbers of patients to define the specific significance of the various autoantibody profiles. Establishing common standards for publication and sharing of microarray-generated data will be important. Only when clinical validation has been confirmed will this advanced technology find application in clinical diagnostics. The next decade will see a radical advance in the diagnostic approach to autoimmune diseases, as sufficient knowledge to apply proteomic technologies on a large scale is implemented. For the time being, therefore, it is advisable to continue using well-established approaches and diagnostic algorithms such as those reported in the international guidelines, which have been prepared in accordance with the principles of appropriateness and evidence-based medicine [64].

Proteomics are also important as discovery tools in basic biological sciences and as diagnostic and rational therapeutic aids in clinical use. Protein microarrays are leading the forefront of this biochip revolution and promise the parallel examination of large numbers of proteins. These miniaturized arrays are being developed to facilitate high analytical resolution, detection sensitivity, and sample throughput.

Table 12 Clinical sensitivity and specificity of RF IgM in a 4-PLEX assay

Sample group	Sample disease classification	# of samples	Sensitivity (%)	±95% CI	Specificity (%)	±95% CI
RA	Rheumatoid arthritis	358	93.3	92.0–94.6	N/A	N/A
Other autoimmune diseases	SLE	43	N/A	N/A	74.4	67.8–81.1
	Sjogren's syndrome	26	N/A	N/A	42.3	32.6–52.0
	MCTD	43	N/A	N/A	58.1	50.6–65.7
	Progressive systemic sclerosis	54	N/A	N/A	51.9	45.1–58.7
	Arthropathies	26	N/A	N/A	88.5	82.2–94.7
	Osteoarthritis	43	N/A	N/A	86.0	80.8–91.3
	Joint pain, CTS	37	N/A	N/A	86.5	80.9–92.1
	Myalgia and myositis	38	N/A	N/A	86.8	81.4–92.3
	Vasculitis	17	N/A	N/A	47.1	35.0–59.2
	Other autoimmune diseases	61	N/A	N/A	80.3	75.2–85.4
Other autoimmune diseases		388	N/A	N/A	71.6	69.7–73.6
Healthy controls		150	N/A	N/A	94.0	92.1–95.9
Healthy controls + other autoimmune diseases		538	N/A	N/A	77.9	76.1–79.7

Many challenges to develop methodological equivalent to gene chip [65]. Recent reports confirm that proteome investigations are discovering and confirming multiple novel disease biomarkers in plasma and serum. Validation and adaptation in clinics for early and correct diagnosis will be applied for many diseases to improve patient benefits. Commitment is to a functioning diagnostic pipeline, research to develop biomarkers for future advances for comprehensive early diagnosis, using large panels of protein biomarkers [66]. Proteomics promises to provide an efficient solution to the emerging crisis in healthcare cost. Proteomic arrays for testing autoreactivity in autoimmune diseases have led to development of autoantigen microarrays for multiplex autoantibody profiling and to define specificity and sensitivity in a variety of autoimmune diseases. Array analysis of RA and control patients for reactivity against deiminated proteins and peptides determined a sensitivity of 45% and specificity of 95% for RA. The data confirms the specific and sensitive detection of autoimmune responses against citrulline-modified peptides [67]. Application of immunoproteomic diagnostic assays depends on diagnostic antigens specific for certain diseases. Some 90% of B-cell produced antibodies recognize non-linear epitopes, even if distortion of native structures may affect assay outcome. Linear natural and synthetic epitopes are more stable but make up only about 10% of an immunoproteome. Clinical utility includes monitoring of vaccines and therapeutic drugs, diagnosis of autoimmune diseases, early detection of cancer, diagnosis of infection, and cancer-associated infections, gastric cancer, hematological malignancies, and colon cancer. Published results continue to demonstrate that antigen profiling is destined to become a valuable clinical tools for screening, diagnosis, prognosis, and monitoring of therapeutic intervention in disease, infection, and cancer [68], and autoimmune, e.g., celiac disease [69]. The concurrent detection and quantification of analytes that vary widely in concentration is a challenge in multiplex assay systems. By combining competitive and sandwich immunoassays, both abundant as well as low concentrations of analytes are to be quantified within the same assay. For these multiplex assays to provide diagnostically relevant results, standardization and validation will be mandatory for use in clinical diagnostics [70]. Multiple determination systems play a crucial role in identification of autoantibody signatures in a patient. Autoantibody profiling provides advantages of reduced sample and reagent volume, shorter turn-around time and reduced costs. Markers are multiplexed to capture the disease profile. Benefits include rapid screening for autoantibody to facilitate early diagnosis and treatment, diversity of epitope autoantibody response, isotype subclass identity, selection of antigen-specific therapy and discovery of novel epitopes and autoantigens [71]. As diagnostic multiplex planar microarray immunoassays are applied in clinical settings for diagnosis of patients, establishing robust quality control markers and algorithms has become mandatory, especially for commercial assays. The FDA has classified multiplex arrays as in vitro diagnostic multivariate index assays (IVDMIAs), but no formal validation guidelines are available for multiplex assays [72]. Multiplex testing has specific analytical and quality control challenges. Present day commercial multiplex assays include various performance controls to monitor consistent analytical results and to safeguard analytical data. Typical controls include positive and negative controls, normalization controls, non-specific binding, to confirm sample addition, cross-reactivity controls, and confirmation of assay reagents such as detection of conjugates [73].

In compliance with these standards, SQI's multiplex planar microarrays and methods for quantification of analytes, incorporates improved methods to standardize analyte concentrations against internal reference standards. The present method also incorporates algorithms and checks for simultaneous measurements, confirming normalization standards and controls in each test well. The platform provides advantages of automated assay plate scanning and analysis with internal quality controls; multiple detection and results per patient test well; in-well normalization to provide consistency between lots, and data invalidation algorithms to provide internal quality control.

Lipid microarrays

Assays for lipid microarrays include the biomarkers cardiolipin, phosphotidyl serine, and beta-2 Glycoprotein I. The development and manufacture of lipid multiplex microarrays presents technical challenges for lipid attachment to substrates to let polar regions be accessible to protein binding. Since lipids can exist as bilayers or membrane like structures, microarrays that incorporate these structures need extensive development [74].

Clinical laboratory testing for antiphospholipid syndrome (APS) using anti-cardiolipin (aCL) was introduced in 1983. Limitations of this assay have led to the development of validated ELISA kits, which, by incorporation of standards and calibrators, have produced more consistent semiquantitative test results. These newer assays, including β2-Glycoprotein I (β2GPI) ELISA and the *APhL* ® ELISA kit, using a phospholipids mixture, provide reliable diagnosis of APS with good sensitivity. Other ELISA assays with annexinV and pro-thrombin antibodies are confirmed as standardized. The anti-β2GPI has proven to be more specific than the aCL assay which is very sensitive. In concert with the lupus anticoagulant assay, this combination identifies APS patients. In some

cases, aCL IgA and anti-β2GPI positive serum is sparse but is associated with clinical APS symptoms. Based on these results, the inclusion of both assays for aCL IgA plus IgG, as well as anti-β2GPI IgM plus IgA confirms APS diagnosis [75]. More recent assays use phosphatidylserine, 77 negatively charged phospholipids [76] or β2GPI anti-β2GPI and or *APhL* ® ELISA kits to confirm APS. The symptoms associated with antiphospholipid antibodies have broadened to include thrombocytopenia, non-thrombotic neurological syndromes, psychiatric manifestations, livedo reticularis, skin ulcers, hemolytic anemia, pulmonary hypertension, cardiac valve abnormality, and atherosclerosis [77]. As a consequence, consensus guidelines for anti-β2GPI testing have been developed to provide more consistent reporting of assay results, including minimum and optional recommendations for testing and reporting of isotype tested, specimen, controls and assay precision, calibrators, patient samples, rheumatoid and anti-β2GPI IgM testing, reporting of results, cutoff values, and data interpretation [78].

The conclusive demonstration [79] of the data presented, illustrates that affinity maturation processes of natural autoantibody can lead to pathogenicity in human disease. Following the introduction of an antigen, affinity maturation of the antibodies normally occurs in the germinal center of secondary lymphoid organs. This process is under the control of tolerance mechanisms to avoid pathologic autoimmunity.

Bead suspension assays

Multiplex technologies using beads or microspheres for the solid phase as an alternative to planar immunoassays, when applied in combination with flow cytometry, have provided simultaneous detection results in several autoimmune diseases [80–82]. Antibodies to extractable nuclear antigens (ENA) are found in collagen vascular diseases. Using a multiplexed fluorescent microsphere (bead) immunoassay, ENAs were detected in 249 serum samples, including 56 patients with lupus erythematosus (SLE). Results when compared with ELISA single analyte confirmed that both methods provided comparable results, making the bead assay as sensitive and specific for semiquantitation as regular ELISA assays for ENA antibodies in human sera [83]. A follow-up study [84] concluded likelihood ratios to be considered more useful results than sensitivity and specificity because likelihood ratios provide better indication of the influence of a negative or positive test on clinical outcome decisions [85]. For example, the BioRad BioPlex™ 2200 autoimmune vasculitis kit has demonstrated similar relative sensitivity and relative specificity for semi-quantitative detection of IgG autoantibodies to MPO, PR3, and GBM. The bead platform simultaneously detects three analytes in a single tube. The automated platform maybe also be of value in differential diagnosis of glomerulonephritis [86]. This suspended array platform may offer advantages over other bead-based platforms and is similar in sensitivity and specificity agreement with pertinent ELISA kits.

Infectious agents had been linked to pathogenesis of vasculitides, likely due to direct microbial invasion of endothelial cells, immune-complex-mediated vessel wall damage, and stimulation of auto-reactive lymphocytes using molecular mimicry and superantigens. Wegener's granulomatosis (WG) in patients is suspected tied to the presence of nasal *Staphylococcus aureus*, with increased risk of relapse being reduced by applied antibiotics [87]. It is important to consider increasing susceptibility of a

Table 13 Clinical sensitivity and specificity of anti-CCP IgG in a 4-PLEX assay

Sample group	Sample disease classification	# of samples	Sensitivity (%)	±95% CI	Specificity (%)	±95% CI
RA	Rheumatoid arthritis	358	77.7	75.5–79.9	N/A	N/A
Other autoimmune diseases	SLE	43	N/A	N/A	88.4	83.5–93.3
	Sjogren's syndrome	26	N/A	N/A	88.5	82.2–94.7
	MCTD	43	N/A	N/A	79.1	72.9–85.3
	Progressive systemic sclerosis	54	N/A	N/A	86.2	80.4–90.0
	Arthropathies	26	N/A	N/A	80.8	73.0–88.5
	Osteoarthritis	43	N/A	N/A	90.7	86.3–95.1
	Joint pain, CTS	37	N/A	N/A	89.2	84.1–94.3
	Myalgia and myositis	38	N/A	N/A	89.5	84.5–94.5
	Vasculitis	17	N/A	N/A	94.1	88.4–99.8
	Other autoimmune diseases	61	N/A	N/A	90.2	86.4–94.0
Other autoimmune diseases		388	N/A	N/A	87.4	85.9–88.8
Healthy controls		150	N/A	N/A	96.0	94.4–97.6
Healthy controls + other autoimmune diseases		538	N/A	N/A	89.9	88.5–91.1

patient with autoimmune disease to pathogen infection resulting from immunosuppressive treatment and also immune dysregulation. The results of the bead-based test support novel associations between WG and infectious agents including HCV, *Helicobacter pylori*, *T. gondii*, CMV, and EBV. These infectious agents also appear to have a clinical phenotype-modulating effect on the disease. Five different infectious agents were titered using the BioPlex 2200 multiplexed immunoassay method. HCV, HBV, and *H. pylori* were ELISA-tested in the sera of 179 individuals.

Future directions

With the explosion of genetic and genomic studies of human disease, including the growing number of genome-wide association studies, there is a critical need for complementary proteomic technologies for proteomic surveillance of autoimmunity. The potential for plasma proteomic analysis to identify and quantify novel proteins that can function as plasma biomarkers of the presence or severity of clinical disease, continues to hold great promise for clinical use as shown by the results obtained in this 4-PLEX multiplex planar microarray rheumatoid arthritis assay. Indeed, although this manuscript is focused on rheumatoid arthritis, we will cite as examples recent literature that would greatly benefit from a proteonomic approach, including diseases such as systemic lupus erythematosus, scleroderma, Wegener's granulomatosis, osteoarthritis, autoimmune myocarditis, and the spondyloarthropathies, amongst others. This can include analysis of both individual cell populations as well as broad analysis of autoantibodies [88–104].

The IgXPLEX RA 4-PLEX multiplex assay, when run in conjunction with the SQiDworks™ platform, measured a sensitivity of 82.5% and a specificity of 97.7%. When anti-CCP IgG results are negative, RA 3-PLEX still identifies 54.2% of RA patients. However, RF IgG has low significance whereas RF IgM is prominent in increasing sensitivity as more positives are identified. The 4-PLEX RA assay provides confidence in confirming negative RA disease status. The RA 4-PLEX assay provides results to help predict the severity of RA and further benefits advanced patient management and profiling.

Overall sensitivity and specificity in Table 11, of RF IgM was 93.3% and 77.9%, respectively. The increase to 94% when all other diseases are excluded, indicates a likely 16% cross reactivity with other disease conditions. IgM antibodies to RF were found with the highest frequencies in Sjogren's syndrome, vasculitis, progressive systemic sclerosis and MCTD, whereas the lowest frequencies were found in joint pain, CTS, myalgia, myositis, and arthropathies.

After exclusion of other disease conditions in Table 13, the specificity increase to 96.0% indicates that CCP is very robust in distinguishing other conditions from RA positive samples. CCP demonstrated the highest overall specificity against other conditions, with the lowest specificity of 79.1% against MCTDs, and the highest against vasculitis and osteoarthritis. The consistently high specificity for CCP attests to its utility as a tool for the positive diagnosis of RA patients.

In compliance with quality assurance and confidence in the observed assay results, SQI's multiplex planar microarrays methods for qualitative and quantitative measurement of analytes standardizes analyte concentrations against multiple internal reference standards. The method also incorporates checks for simultaneous measurements confirming normalization standards and controls. The platform provides advantages of automated assay plate scanning and analysis with internal quality controls; multiple detection and results per patient test well; in-well normalization to provide consistency between lots and data invalidation algorithms to provide internal quality control.

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