

FROM RESEARCH TO CLINICAL APPLICATION MULTI-PARAMETER TESTING: MARKER PANELS FOR THE EARLY DETECTION OF COMPLEX DISEASES

OD ISTRAŽIVANJA DO KLINIČKE PRIMENE:
PANELI MARKERA ZA RANU DETEKCIJU SLOŽENIH BOLESTI

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Summary: Multi Parameter Analysis can open novel diagnostic opportunities for the early diagnosis and screening of multimodal diseases like cancer. Single proteins have so far failed to describe such complex diseases. Being able to screen with a set of analytes is one promising way to overcome the present limitations. Various marker identification tools including proteomics approaches have been successfully applied to identify new screening markers for early detection of colorectal cancer (CRC). CRC is one of the most incident cancers worldwide and early detection is clearly a key factor in reducing mortality from CRC. Several screening methods are recommended, including colonoscopy, fecal occult blood testing (FOBT) and fecal DNA analysis. Of these annual stool testing with the guaiac based FOBT is most often applied, in spite of limitations such as low sensitivity and dietary influences. Though procedures with improved performance eg. immunological FOBT are available, a screening assay for CRC in serum that could easily be integrated in any health check-up would be highly welcome. A positive result of such a test would trigger a follow-up colonoscopy for an exact diagnosis. In this review we will cover aspects of marker identification strategies and describe a well structured marker validation process that is based on clinically characterized sample materials. Finally the value of analytical multi-parameter platforms enabling the combination of multi markers in routine diagnostics settings is outlined. An appropriate multi-parameter immunochemistry platform concept, currently developed under the working name »IMPACT« will be introduced.

Keywords: colorectal cancer, multi-parameter analysis, early diagnosis, marker combination, multivariate analysis, protein array

Kratak sadržaj: Analiza više parametara (multi-parameter analysis) može doneti nove dijagnostičke mogućnosti za ranu dijagnozu i testiranje multimodalnih bolesti poput raka. Pojedinačni proteini dosad nisu uspjeli da opišu tako složene bolesti. Mogućnost testiranja pomoću seta analita najavljuje prevazilaženje postojećih ograničenja. Različite alatke za identifikaciju markera uključujući proteomske pristupe uspešno se primenjuju za identifikaciju novih markera za rano otkrivanje kolorektalnog kancera. Kolorektalni kancer je jedan od najčešćih kancera u celom svetu a rana detekcija je ključni faktor za smanjenje mortaliteta od te vrste raka. Preporučeno je nekoliko metoda za testiranje, uključujući kolonoskopiju, analizu okultnog krvarenja u stolici i analizu DNK u stolici. Najčešće se primenjuje godišnje testiranje stolice metodom gvajak zasnovano na analizi okultnog krvarenja u stolici, uprkos ograničenjima kao što su niska senzitivnost i uticaj ishrane. Mada postoje postupci s poboljšanim performansama, npr. imunološko testiranje okultnog krvarenja u stolici, skrining test na kolorektalni rak koji bi se lako integrisao u svaki zdravstveni pregled bio bi veoma korisan. Pozitivan rezultat takvog testa ukazao bi na potrebu za kolonoskopijom radi precizne dijagnoze. U ovom radu biće razmotreni aspekti strategija za identifikaciju markera i opisan valjano strukturiran postupak validacije markera zasnovan na klinički okarakterisanim uzorcima materijala. Najzad, biće naznačena vrednost analitičkih platformi s više parametara koje omogućuju kombinovanje multimarkera u rutinskoj dijagnostici. Biće predstavljen i koncept odgovarajuće imunochemijske platforme s više parametara koja se trenutno razvija pod radnim nazivom »IMPACT«.

Ključne reči: kolorektalni kancer, analiza više parametara, rana dijagnoza, kombinovanje markera, multivarijantna analiza, proteinski skup

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Starting a substantial marker identification program, proteomics studies have been the preferred approach to identify promising marker candidates. In addition, there are alternatives that should also be considered. DNA and RNA methods could complement proteomics studies, while extended pathway analysis and information from public domain might identify additional markers.

In the overall perception, proteomics is strongly associated with the discovery phase, that will only be the first step if the whole process of marker discovery is considered. Proteomics aims to identify proteins that are »different« in various states of a biological system classified as diseased and »healthy«. In consequence the discovery phase is overrated if a program in proteomics is limited to the identification of novel validated biomarkers with a clearly defined position in *in vitro* diagnostics. The absolute number of discovery hits cannot be considered the primary goal of proteomic approaches in the *in vitro* diagnostic setting, but the true challenge will be the carefully planned assessment of the selected candidates. Only the subsequent validation phase, not the discovery activities, will reveal whether a marker has the potential to fill the existing sensitivity and/or specificity gaps.

The validation strategies will vary depending on the intended positioning of the marker. A *screening marker* will have to meet completely different performance criteria than, for example, a *monitoring marker* or an *efficacy marker* modulated by the drug being administered. New markers will be used to complement already existent products to significantly advance the specific intended use. This is also an economic necessity because novel replacements are bound to face high »entry barriers« into the market. Currently regulatory agencies give little advice as to the specific benchmark(s) against which the single novel marker must be compared and a real challenge for the future will be gaining approval for marker combinations created with mathematical models (»disease algorithms«) based on multi-parametric testing. Given the intricacy of some diseases, it is quite likely that a single protein on its own will not be sufficient. A »golden bullet approach« seems to be an unrealistic scenario. Thus, it is necessary to combine more than one biomarker in a disease algorithm.

It is our goal that the biomarker should ultimately be detected in serum/plasma using immunological detection (ELISA) formats. If we assume that the marker candidate was identified by comparing sets of tumor and healthy control tissues, the next steps are that selected immunogenic peptides must be synthesized, and full-length recombinant proteins (expressed in different pro- and eukaryotic expression systems) must be purified. In some cases, cDNA (for DNA immunization) complements the family of immunogens used to raise antibodies. For a first screen, the focus lies on throughput and time. As a

consequence polyclonal antibodies are raised in rabbits. Because the immunization will usually be running for 100 days before the final bleeding, several of the above approaches will have to be run in parallel to circumvent extended timelines. However, this will increase the number of animals per marker candidate to up to 12–15. Once the antibody batches are available the IgG fraction has to be purified to be used in extensive pre-validation schemes. In an early verification step, the first round antibodies are used in immunostaining of tissue sections previously used in the discovery phase. Next Western blots with lysates from the same tissues will be applied to confirm the modulation in the expression level of the biomarker candidate. In addition, Western blots using lysates from a variety of tissues are a first check for the disease/organ specificity of the marker candidate. An example from the discovery and pre-validation phase of the colorectal cancer program can be found elsewhere (1).

The detection of the biomarker candidates in serum using ELISA prototypes is only the *first* qualifier hurdle. Next we designed a two-step approach to assess the diagnostic potential of the marker candidates: using optimized ELISA prototypes we measure 50 highly characterized samples from diseased individuals and 50 samples from healthy blood donors. This limited »black and white panel« (panel A) is the *second* qualifier in the process to advance a marker candidate to the status of a validated marker. It should be noted that this particular qualifier principle is restricted to screening scenarios only and that panel A is totally inadequate for classifying markers in monitoring scenarios for example. If a candidate does not correctly classify a preset fraction of the two sample collectives its diagnostic value as a stand-alone marker is limited and might not justify further evaluation. However, eliminating markers solely based on the results of panel A is inherently risky: markers with a low univariate discrimination power might in fact become part of a marker

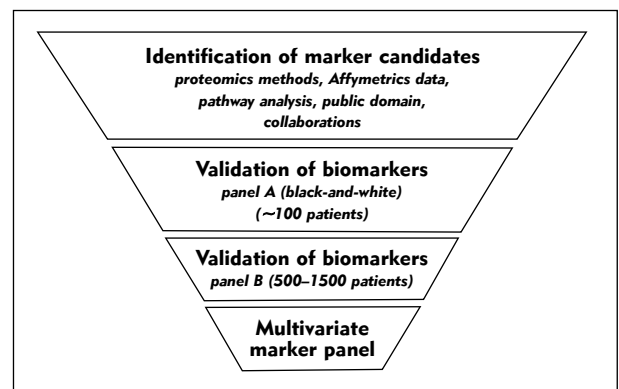


Figure 1 Example for the funnel principle workflow of assessing and validating biomarker candidates in context of multimodal diseases.

combination using mathematical models. Those markers might contribute additional information to an existing set of markers, thus improving the »disease algorithm«. (Figure 1).

The *third* and final qualifier to challenge a marker candidate is a serum/plasma panel of up to 1,500 samples (panel B). Samples are available as both serum and as EDTA-anticoagulated plasma with a minimum volume of 5 mL. This amount guarantees that each potential biomarker for a given disease can be evaluated over the entire validation phase with the same samples. The sample collection itself and the storage of suitable aliquots follow rigorous identical SOPs that are mandatory for all participating collection centers to avoid known variations in measuring the analyte concentration similar to those recently described (2). Each sample is accompanied by extensive case report forms containing all available information on the respective patient. This allows for the eventual formation of subgroups as needed according to previous medication, co-morbidities, disease duration, and other classifiers.

This principle has been for example applied to marker identification programs for the diagnosis of rheumatoid arthritis (3). Out of 131 biomarker considered, 32 were evaluated in a study of which only 7 passed the criteria and were combined by multivariate analysis using four different mathematical models. Anti-CCP as reference showed a sensitivity of 78% at 91.9% specificity. The best marker combination of anti-CCP and interleukin-6 resulted in an increased sensitivity of 85% at a slightly decreased specificity of 90.3% for the diagnosis of rheumatoid arthritis.

Another recent example for this marker identification process is colorectal cancer (CRC). CRC is the third most common cancer worldwide, with an incidence of >400,000 new cases a year and a prevalence of >1.6 million in seven major markets (4). The lifetime risk of developing CRC is about 6%, with a sharp increase over the age of 50, making age the most important risk factor for the disease, ahead of genetic and/or familial predisposition. Of diagnostic relevance is the fact that CRC can be effectively cured through early detection and intervention. Some 37% of all CRC cases are detected in their early localized stages or stages preceding the cancer event (Dukes stage A) or in stages where no invasion of the lymph nodes has yet occurred (Dukes stage B), conditions with a 5-year survival rate of 93% after curative surgery. After infiltration of the lymph nodes (regional stages, Dukes stage C, another 37% of the cases), the survival rate drops to 63%, to fall to only 9% in the distant stages correlated with metastasis (Dukes stage D). This segmentation already underlines the need for early diagnosis in a market segment of important size. The problem of early diagnosis for CRC is compounded by the fact that the available screening methods do have considerable limitations.

Still widely used is the guaiac based test for occult blood (FOBT) in stool. Some nonmalignant conditions (hemorrhoids and ulcers) can lead to false-positive results, as can certain diets and medications (5). Despite the fact that fecal occult blood testing does not reach a satisfactory level of sensitivity and specificity, the test reduces CRC mortality by detecting »true positives« and thus is recommended by many national cancer agencies. In recent years fecal immunological testing (FIT) has increased the sensitivity of CRC screening while at the same time reducing the interference of diet and medications on the test result as compared to FOBT. However, FIT is not yet widely used in national screening programs as there are currently only a limited number of studies that prove the diagnostic value of FIT in a screening setting. As long as the advantages of FIT are not extensively documented national health programs will be reluctant to switch to this more expensive technology.

Colonoscopy is seen as a »gold standard« in CRC testing, but the testing procedure is, by its very nature, highly invasive and thus does not have the wide-spread compliance of the target population (people >55 years of age).

In summary, in CRC screening there is a large diagnostic gap between the established test regimen of stool testing (with all its limitations) on one side and a reliable early detection procedure (which lacks the much needed acceptance) on the other side. Any new screening marker for the early detection of CRC would be a definite improvement likely to be accepted by the patients and the medical community.

Our study results (6) about the identification of novel fecal markers for the diagnosis of colorectal cancer were recently published. In the marker identification program for CRC we evaluated 29 marker candidates including 18 identified by proteomics methods, 7 markers from public domain and four established tumor markers on the Elecsys® system (Roche Diagnostics, Mannheim) that were adapted for stool analysis. 21 of these markers were assessed in panel A to identify marker candidates with a sufficient discriminatory power for a more extensive evaluation in panel B. Eight markers were transferred to Panel B that included 551 samples (186 CRC, 113 advanced adenoma and 252 control patients) and 6 markers appeared to be most promising when inspected alone or in marker combinations. We could show that a combination of hemoglobin-haptoglobin, S100A12 and TIMP-1 had a significantly higher sensitivity for the diagnosis of CRC than FOBT alone with 82 % vs. 73 % sensitivity at 98 % specificity. This marker combination has the potential to identify patients in need of a colonoscopy for a final diagnosis with an improved sensitivity while at the same time reducing the burden for the patients due to its low false positive rate. Clearly the most crucial factor in

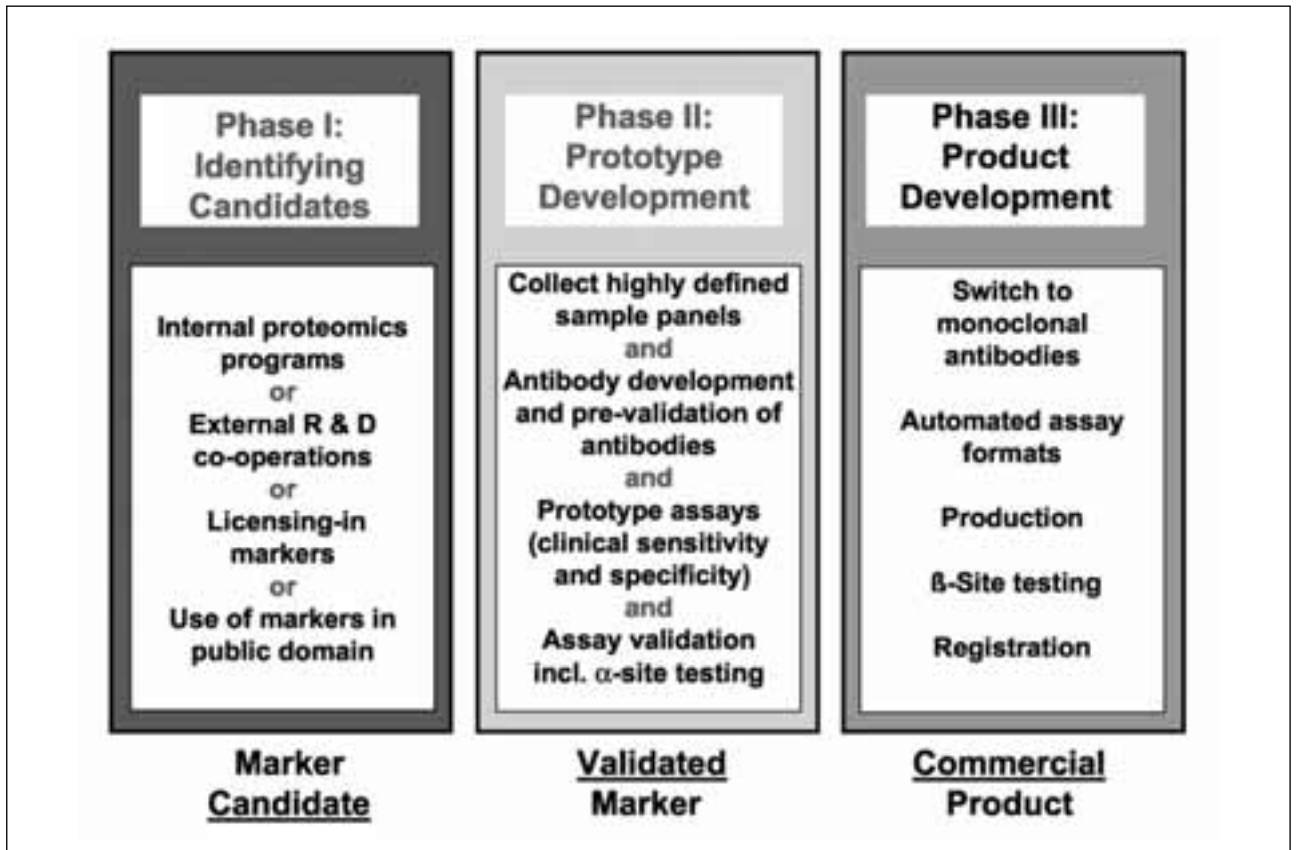


Figure 2 Example for the workflow in industry from discovery phase to a commercial product (8).

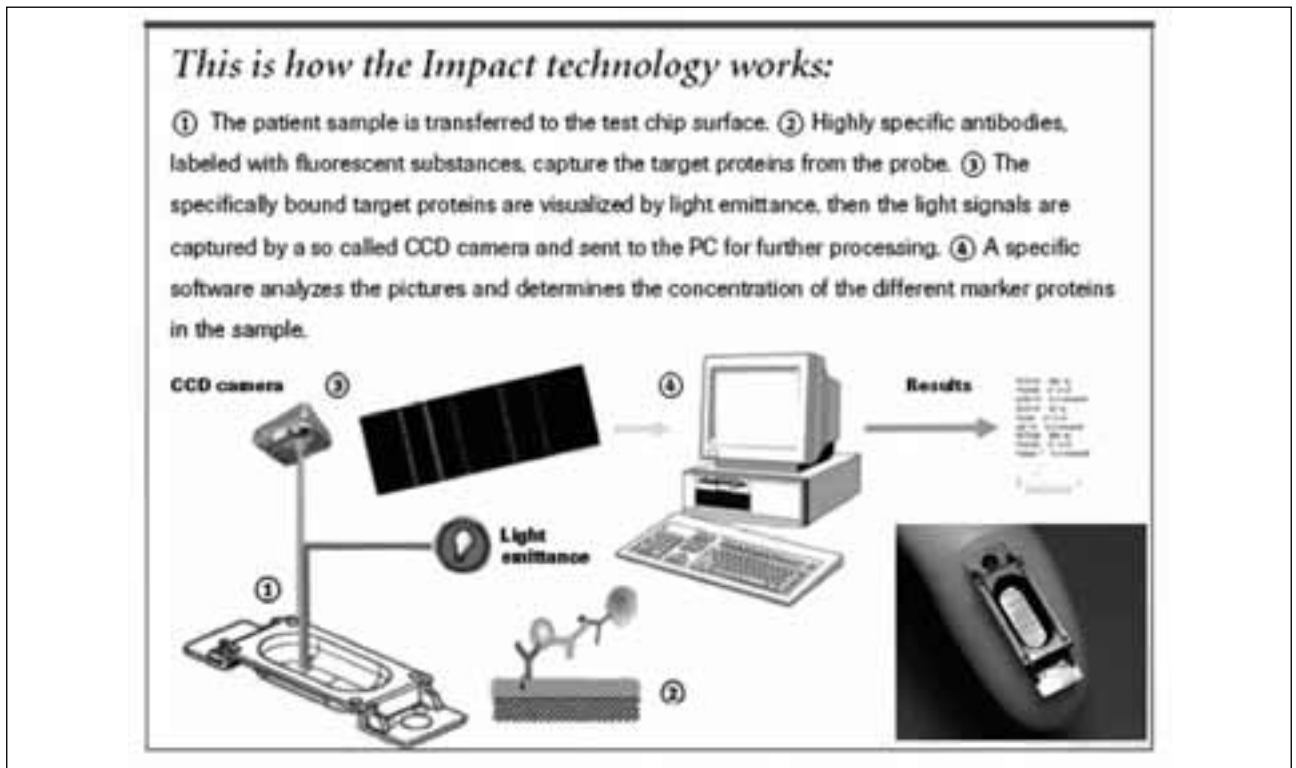


Figure 3 IMPACT Multiparameter Platform.

this study were the elaborate patient cohorts that allowed us a stepwise reduction of the number of potential marker candidates to the meaningful three of the marker combination.

Unfortunately, current CRC screening programs including colonoscopy as well as FOBT suffer from limiting participation rates. It is anticipated that a CRC screening test based on serum as sample material could greatly improve the participation rate because such a test could easily be integrated in any health check-up without the need for additional stool sampling. Therefore we extended the marker identification and validation approach to serum/plasma.

A marker combination of 6 biomarkers was selected by applying penalized logistic regression to 1556 patient samples (manuscript in preparation). The patients included were: 301 CRC, 266 screening controls, 202 adenomas, 144 disease controls, 167 GI-cancer, 261 other cancers and 212 chronic diseases. When the samples were split into a training and a test set the marker combination achieved a sensitivity of 71% and 60% at 95 or 98% specificity, respectively. When the serum panel was compared to FIT in a subset of patients (75 CRC, 234 controls) both tests showed an almost identical performance. The sensitivity was also comparable to a recent study by Morikawa et al. (7) including 21,805 asymptomatic Japanese patients, that reported a sensitivity of iFOBT of 65.8% at a specificity of 95%.

A combination of 6 biomarkers is able to detect CRC from serum with a performance comparable to iFOBT in stool. A serum test will be a valuable tool for CRC screening to increase compliance and to trigger a follow-up colonoscopy for a definite diagnosis.

Finally, after the extensive discovery and evaluation process novel biomarker or combinations thereof enter into a development process for commercialization and clinical applications in a diagnostic setting. Single analytes or small marker panel consisting of <3 analytes are applied to standard analyzer platforms like the Elecsys® system (Roche Diagnostics, Mannheim).

Figure 2 summarizes the workflow process of marker identification, evaluation and commercializing (8).

However, to fully exploit the potential of a marker panel a new multi-analyte platform will be necessary.

Protein array technology has several advantages, including the possibility of simultaneous detection of multiple analytes, potentially higher sensitivity, miniaturization of assay procedures, and reductions in sample and reagent volumes. To date, the most commonly arrayed proteins are antibodies (9), since they are robust molecules that can be easily handled and

immobilized by standard procedures on flat surfaces such as glass slides (chips) or membranes. The most widely adopted strategy consists of a multiplex adaptation of the classical antibody sandwich assay, where a pair of antibodies binds 2 discrete recognition surfaces on each protein (10, 11).

Roche Professional Diagnostics (Roche Diagnostics GmbH) is developing a multi-marker platform under the working name IMPACT (Immunological Multi-Parameter Chip Technology).

The technology is based on a small polystyrene chip manufactured by proprietary and tested procedures. The first step of chip production is coating the chip surface with a streptavidin layer. In the next step biotinylated antibodies are spotted onto the chip. They form a strong bond with the streptavidin on the surface. For each marker of interest, antibody droplets are spotted in straight lines onto the chip. During assay processing, the array is probed with specimen samples containing the specific analytes. The serum volume required per specimen is 4 to 20 microliter. After incubation with digoxigenylated analyte specific antibodies, 40 microliter of antidioxigenin antibody coupled with fluorexcent latex conjugate are added. Using this label, <10 individual binding events in a single spot can be detected, resulting in very high sensitivity down to the fmol/L concentration. Chips are transported into the detection unit, and a charge coupled device (CCD) camera generates an image that is transformed into signal intensities using dedicated software. Individual spots are automatically located at predefined positions and quantified by image analysis. For each marker, lines of 10–12 spots are loaded on the chips to determine the mean concentration of samples. The advantages of the technology are the ability of multiplexing up to 20 parameters in a sandwich or competitive format, low consumption of sample (4–20 µL), and high sensitivity. The throughput of the prototype is 41 patient determinations per hour. In conclusion, the automated protein-array chip showed similar analytical precision, improved analytical sensitivity, and similar concentration results compared to the reference, single assays on the Elecsys® system (Roche Diagnostics, Mannheim) (12).

Conclusion

Multi Parameter Analysis can open novel diagnostic opportunities for the screening and early diagnosis of multimodal diseases like cancer. Single proteins have so far failed to describe such complex diseases. Being able to screen with a set of analytes is one promising way to overcome the present limitations. An appropriate multi-parameter immunochemistry platform concept is a prerequisite to bring multi-marker testing into routine practice.

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