Asp84Glu variant of the melanocortin 1 receptor (MC1R) is associated with melanoma. Hum Mol Genet 1996;5:1663–6.


DOI: 10.1373/clinchem.2005.056820

Inflammation has been identified as the underlying cause of atherosclerosis, a condition associated with the deposition of lipids in the lining of arteries, which progressively leads to acute myocardial infarction (AMI) or heart attack. Serum concentrations of markers of inflammation, including C-reactive protein (CRP), and the leukocyte count are powerful predictors for the development of coronary heart disease (1–7). In addition, AMI patients with increased CRP concentrations or leukocyte counts are at higher risk of mortality and recurrent AMI (8–12). The combination of these 2 biomarkers provides additive and powerful diagnostic information. In fact, people with both high leukocyte counts and high CRP concentrations exhibit a 7-fold higher risk for heart disease (7).

Although tests targeting CRP concentrations and leukocyte counts are widely available in clinical settings, they are performed separately on different instruments. Consequently, these tests require large sample volumes, additional sample preparation steps, and longer assay times. In addition, the clinical instruments and methodologies currently used to perform these tests are not suitable for point-of-care testing, such as in a doctor’s office and in the (more relevant to a heart attack setting) emergency room or ambulance. Clearly, the diagnostic and prognostic value of these biomarkers would be increased if these 2 tests could be performed concurrently on the same instrument, in a convenient, accurate, and highly accessible manner.

Previously we described studies of the design, fabrication, and testing of lab-on-a-chip (LOC) structures composed of chemically sensitized beads that are populated into etched silicon wafers with integrated fluid-handling and optical detection capabilities (13–19). These miniaturized systems have been used for the identification and quantification of electrolytes, sugars, proteins, antibodies, toxins, and biological cofactors as well as for determination of pH. In independent studies, we have defined and developed miniaturized microfluidic systems that support cellular analysis applications. These membrane-based microsystems have been demonstrated as suitable screening tools in the bioterrorism sector, as well as for measurement of CD4 cell counts for use in immune function monitoring of HIV-positive patients (20, 21).

In this study, we describe an integrated LOC assay
method suitable for the concurrent measurement of CRP concentrations and leukocyte counts. This dual-function microchip system uses both bead- and membrane-based assay platforms, each of which is packaged within an integrated flow cell system. The membrane-equipped structure is dedicated to the capture and detection of blood cells, whereas the bead-equipped structure is dedicated to the capture and measurement of blood proteins. The 2 assay platforms share a common computer-controlled fluidics system and optical components, which support the fluid delivery requirements for the samples and reagents as well as enabling microscopic evaluation of the signals generated by the 2 microanalysis systems, respectively. Fluorescence signals generated on the bead and the membrane structures are visualized by a charge-coupled device (CCD) video chip along with the use of transfer optics, and specialized software provides image capture and automated data analysis capabilities (13–21).

Bead-based immunoassays are performed on porous agarose microspheres positioned in a microetched array of wells on a silicon wafer microchip. Depending on the application, the ~1-cm² microchip can accommodate 3 × 3, 3 × 4, 5 × 7, and 10 × 10 array sizes. Each ~280-μm bead within the array serves as its own independent microreactor sensor, with its selectivity determined by the specificity of the capture antibody coupled to the bead. For the CRP-specific immunoassays, typically a 3 × 4 bead array is used, with 3 beads coated with antibodies irrelevant to CRP used as negative controls and 9 beads dedicated to CRP capture and detection. This bead redundancy increases the precision of the CRP measurements. A schematic illustration of the CRP immunoassay developed on the bead sensor is shown in Fig. 1A. In this assay, a rabbit CRP-specific antibody coupled to the bead captures the CRP antigen, and an AlexaFluor-488–conjugated detection antibody is used to visualize the bead-captured protein. Each assay step is followed by a wash with phosphate-buffered saline (PBS; 0.008 mol/L sodium phosphate, 0.002 mol/L potassium phosphate, 0.14 mol/L sodium chloride, 0.01 mol/L potassium chloride, pH 7.4). The low internal volumes of each bead (~20–30 nL per bead), used in conjunction with high effective flow rates (1–5 mL/min), allow the completion of highly stringent washes (>5000 effective washes per minute) that reduce nonspecific binding of antigens and detection antibody reagents. After the final wash, an image of the bead array is acquired (Fig. 1B). Using the standard epi-illumination geometry, white light from a 100-W mercury lamp is collimated, passed through a filter to select the excitation wavelengths (centered at 480 nm with 40 nm spectral bandwidth), reflected by a dichroic mirror (505 nm long pass), and focused on the bead array by use of a 4× microscope objective (numerical aperture ~0.13). The fluorescence from the beads is collected by the microscope objective, transmitted through the dichroic mirror, passed through an emission filter (centered at 535 nm, with 50 nm spectral width), and detected by the CCD camera. The image is digitally processed and analyzed, and the signal intensity for each bead is converted into a quantitative CRP measurement with the aid of a calibration curve. The time required to process each sample as described above is ~12 min.

For analysis of the leukocyte content of whole blood, a supported 13-mm track-etched polycarbonate membrane is used. Whole blood is processed and analyzed by this minianalysis system in the following manner: A sample of anticoagulated blood is fixed with 40 g/L paraformaldehyde and then incubated for 5 min with an AlexaFluor-488–conjugated anti-CD45 antibody specific for leukocytes. The mixture is then diluted with PBS and introduced into the membrane chamber by an external peristaltic pump equipped with an injection valve. Image acquisition is performed as described above for the bead-based platform. The basic methodology for this membrane separation and filtration of immunolabeled leukocytes is shown in Fig. 1C. Analysis of the scanning electron micrographs of the filtered whole blood reveals that the erythrocytes, with roughly the same diameter as the leukocytes, deform and pass through the 3.0-μm pores of the membrane, whereas leukocytes are efficiently captured on the membrane. Therefore, despite the overabundance of erythrocytes in whole blood, with the appropriate selection of membrane structure, as used here, it is possible to separate and isolate the leukocytes from whole blood. The removal of the erythrocytes and the use of a fluorescent antibody stain specific for leukocytes allow efficient detection of captured leukocytes with minimal background (Fig. 1D). Interestingly, the anti-CD45 antibody used to stain leukocytes identifies 2 main populations of cells captured on the membrane. This result is consistent with the expected pattern of leukocyte staining, in which CD45 expression on lymphocytes is stronger than that observed on granulocytes (22).

Having defined LOC methods suitable for the quantification of protein and cells, we next aimed to demonstrate the efficacy of these approaches on real human samples with values that spanned the physiologically relevant ranges for these 2 types of tests. The results of experiments with the bead-based CRP assay demonstrated a wide detection range suitable for the measurement of CRP (1–10 000 ng/L). With the appropriate choice of assay conditions and sample dilution or use of beads coated with various concentrations of capture antibody, this range can be extended to >100 000 ng/L, which is relevant to its utility in patients with AMI (23). We compared this CRP assay with a commercial high-sensitivity CRP ELISA (Fig. 1E). CRP values from 9 human blood samples evaluated in parallel by ELISA and the bead-array method showed excellent agreement (r = 0.995).

To evaluate the linearity and analytical range of the membrane leukocyte assay, we delivered increasing volumes of a CD45-stained whole blood suspension to a membrane-equipped LOC structure. After a thorough rinse with PBS, images of leukocytes on the membrane were captured for 3 different fields of view (FOV). A custom pixel analysis algorithm was used to identify and count individual leukocytes based on size, shape, and
fluorescence intensity thresholding within the Image J environment (24). As shown in Fig. 1F, leukocyte counts increased in a linear fashion with increasing volume of blood delivered to the flow cell [$R^2 = 0.999$; data shown as the mean (SD) counts from 3 FOV]. The intraassay CV of the counts measured in different FOV was 5%–15% and was strongly dependent on the volume of blood delivered on the membrane. Optimum precision with the chosen flow cell structure was achieved for volumes of blood between 0.81 and 14.3 μL, with too many cells on the...

Fig. 1. CRP and leukocyte dual-function assay: Principle and characteristics. (A), scanning electron micrograph of the bead platform and relevant immunoschematic of the CRP assay, shown for a 3 × 3 bead array. (B), same type of bead array as in A, imaged with fluorescence microscopy. This system is suitable for a variety of diagnostic applications through the appropriate selection of capture antibodies. (C), scanning electron micrograph showing the membrane element after the processing of a whole blood sample along with the relevant immunoschematic of labeled membrane-captured leukocytes. Captured on the membrane structure is a collection of leukocytes. The much more numerous erythrocytes are absent for the most part, except for a few fragments, which are shown spanning 2 membrane pores. (D), membrane-processed whole blood sample visualized by fluorescence microscopy. The leukocytes are stained with the relevant labeled antibody. (E), correlation between the bead-array LOC structure and the standard ELISA method for CRP measurements. The dashed lines indicate the 95% confidence interval. (F), analysis range for the cell-counting application, evaluated by plotting the cell count as measured by the pixel analysis macro vs added blood volume. The leukocyte counts (WBC counts; 1 to 300 cells/FOV) increase linearly with the volume of blood delivered to the membrane. Error bars, SD. (G), inter- and intraassay precision of the cell-counting application, evaluated for 5 different flow cells. The counts obtained from the membrane method (numbers inside columns) are also correlated with flow cytometry as the comparison method. Error bars, SD.
membrane contributing to counting errors because of cell overlap and too few cells reducing the precision of the measured counts.

To evaluate the interassay precision of the leukocyte assay, the equivalent of 2.1 μL of stained whole blood was delivered to a membrane-equipped flow cell. For healthy donors with 5000–11 000 leukocytes/μL, this volume of blood includes 10 500–23 100 leukocytes. With the optical instrumentation used in the membrane microchip system described here, 1 FOV represents an area of 0.60 mm². Given that the total surface area of the membrane used for cell capture is 78.54 mm², the current membrane element yields ~130 FOV. Consequently, although the entire sample volume yields 10 500–23 100 leukocytes, the single FOV collects the fluorescence signature of ~80–176 cells. This analysis, of course, assumes that the cells become evenly distributed across the entire membrane. Images from 5 nonoverlapping FOV are captured to obtain the preliminary mean leukocyte count. This preliminary count is converted to an absolute count after application of a scaling factor that incorporates the volume of blood delivered to the flow cell as well as the number of FOV covering the membrane region onto which leukocytes are captured. We repeated this experiment 5 times, using different flow cells. The interassay CV of the counts from flow cell to flow cell was 4.3%. Importantly, as shown in Fig. 1G, the leukocyte counts achieved by the membrane counting method were in excellent agreement (95%) with those determined by flow cytometry, which requires a larger blood sample (100 μL) and an additional processing step to lyse the erythrocytes. The excellent agreement between the 2 methods strongly suggests that the above assumption on even cell distribution is accurate with respect to the conditions and the microchip design used here.

In summary, recent reports have concluded that the combined measurement of CRP concentrations and leukocyte counts provides one of the most accurate methods available, to date, to assess an individual’s risk for heart disease. This information is useful both in the context of future risk profiling for apparently healthy individuals as well as for prognosis of individuals with recent occurrences of AMI. Likewise, the availability of convenient methods to measure these 2 disparate quantities in the same setting would be of great importance in the management of cardiac disease, the foremost health issue in developed countries. In this report, we demonstrate the capacity of miniaturized LOC methods to perform both protein and cellular analyses of bodily fluids. Although further work is required to produce practical instrumentation to support these tests, the basic methodologies described here are suitable for full automation, and they allow for multiplexing of related groups of analytes, thus promoting a more comprehensive approach to disease risk assessment and diagnosis. The use of bead- and membrane-based assay platforms and the implementation of dual-function LOC structures may aid in the development of a flexible miniaturized total analysis system to be used in point-of-care settings. Larger scale studies are currently in progress using the newly fashioned miniaturized analysis system to test the inflammatory marker CRP and leukocyte counts in healthy and heart disease patients.

The research described here was supported by the National Institutes of Health (Grant U01 DE015017-03), the Welch Foundation (Grant F-1193), Philip Morris USA Inc., and Philip Morris International.

References

22. Dalchau R, Kirkley J, Fabre JW. Monoclonal-antibody to a human leukocyte-
Quantitative Assessment of Human β-Globin Gene Expression In Vitro by TaqMan Real-Time Reverse Transcription-PCR: Comparison with Competitive Reverse Transcription-PCR and Application to Mutations or Deletions in Noncoding Regions, Leonid M. Irenge,1 Annie Robert,2 and Jean-Luc Gala1,3 (1 Laboratory of Applied Molecular Technologies, Center for Human Genetics, Université Catholique de Louvain, Brussels, Belgium; 2 Epidemiology and Biostatistics, Clos-Chapelle-aux-Champs, Université Catholique de Louvain, Brussels, Belgium; 3 Defense Laboratories Department, Belgian Armed Forces, Brussels, Belgium; *address correspondence to this author at: Applied Molecular Technologies, Center for Human Genetics, Clos Chapelle-aux-Champs, 30, UCL/30.46, B-1200 Brussels, Belgium; fax 32-2-764-39-59, e-mail gala@lbcm.ucl.ac.be)

β-Thalassemia is a genetic hemolytic disorder characterized by diminished production of β-globin chains, attributable to alterations within coding and noncoding sequences of the β-globin gene (1). Whether noncoding mutations are involved in the decrease of β-globin chain production or are simple polymorphisms remains a difficult issue (2). Although stable transfection with constructs bearing key regulatory β-locus control region elements upstream of the promoter are commonly used to assess the transcriptional effect of noncoding mutations in β-globin (3–7), discrepant results are still reported, as evidenced recently by the research team that used Northern blotting or ribonuclease protection assay to assess the functional impact of the +10 (−T) deletion (3, 5, 8). Although highly accurate, the competitive reverse transcription-PCR (RT-PCR) technique that we recently developed to quantify the transcriptional effect of genetic alterations in any part of the human β-globin gene is tedious and time-consuming (6). There is a need, therefore, for an expression method that is accurate, rapid, easier to perform, and well controlled quantitatively.

In our current study, we developed a TaqMan® real-time quantitative RT-PCR assay (QRT-PCR), using the same in vitro expression model and the following human β-globin variants: −223T→C, −101C→T, −30T→A, +20C→T, IVS-1-108T→C, +10 (−T), and +15653→15777 (6). An additional codon 39 stop-mutation variant construct was included as a control that markedly affects human β-globin mRNA concentrations (4). The β-globin expression for each construct was quantified by real-time QRT-PCR, and the results were compared with those originally obtained with the competitive RT-PCR (6), except for the wild-type, the +10 (−T), and the +40→+43 (−AAAC) 5′-untranslated region deletion variant constructs, for which both quantitative methods were performed in parallel on the same total RNA. RNA extraction, reverse transcription, and competitive RT-PCR were carried out as described previously (6).

Gene-specific PCR primers and TaqMan probes for human β-globin (GenBank accession no. AF007546) and the mouse housekeeping gene glyceraldehyde phosphate dehydrogenase (mGAPDH; GenBank accession no. BC083149) were designed by use of Primer Express™ Software (Ver. 1.5; PE Applied Biosystems). The primers and probe for human β-globin were as follows: sense primer, 5′-TGCACGTGGATCCTGAGAAACT-3′; antisense primer, 5′-AAATTCCTTGGCAAAATGTAGGG-3′; probe, 5′-CAGCACGGTCACCCAGGAGCCTG-3′. The primers and probe for mGAPDH were as follows: sense primer, 5′-CAACAGGTAGAAGAACCCCTGGA-3′; antisense primer, 5′-CGAGTGGATAGGGCCCT-3′; probe, 5′-CACCCACCCACGAAGGACACTG-3′. Each probe was labeled with a fluorescent 5′ reporter dye [6-carboxyfluorescein (FAM)] and a 3′ quencher [6-carboxytetramethylrhodamine (TAMRA)]. mRNA was reverse-transcribed as described previously (6). For each sample of total RNA, 3 separate reverse transcriptions were carried out. For each cDNA, a triplicate amplification was carried out using 2.5 μL of cDNA, 12.5 μL of Universal PCR Master Mix (2X; Applied Biosystems), 300 nM each of the primers, and 100 nM probe in a total reaction volume of 25 μL. TaqMan PCR was performed in a 25-μL total volume containing 12.5 μL of Universal PCR Master Mix (PE Applied Biosystems), 300 nM each of the primers, and 100 nM fluorescent probe. The human β-globin and mGAPDH genes were amplified in parallel, and the reaction was carried out as described previously (9).

Data were recorded as cycle threshold (Ct) on a TaqMan 7700 Sequence Detection System (Applied Biosystems), using the analytical software from the same manufacturer. The mean Ct value for mGAPDH was subtracted from the mean Ct value for the human wild-type β-globin. This ΔCt value obtained with a mutated construct was then subtracted from the ΔCt value obtained with the wild-type construct, giving a 2−ΔΔCt value. As amplification efficiencies of the human β-globin and mGAPDH were comparable (data not shown), the amount of human β-globin mRNA, normalized to mGAPDH, was given by the relationship 2−ΔΔCt. Two other mouse housekeeping genes were compared with mGAPDH: cyclophilin A (CypA; accession no. X52803) and hypoxanthine guanine phosphoribosyltransferase gene (HPRT1; accession no. BC083415). The mGAPDH mean Ct values obtained with the human wild-type β-globin was normalized alternatively with either housekeeping gene. Data were reported as the mean (SD). For each construct, the β-globin expression values, as given by the 2−ΔΔCt values, were compared by use of the