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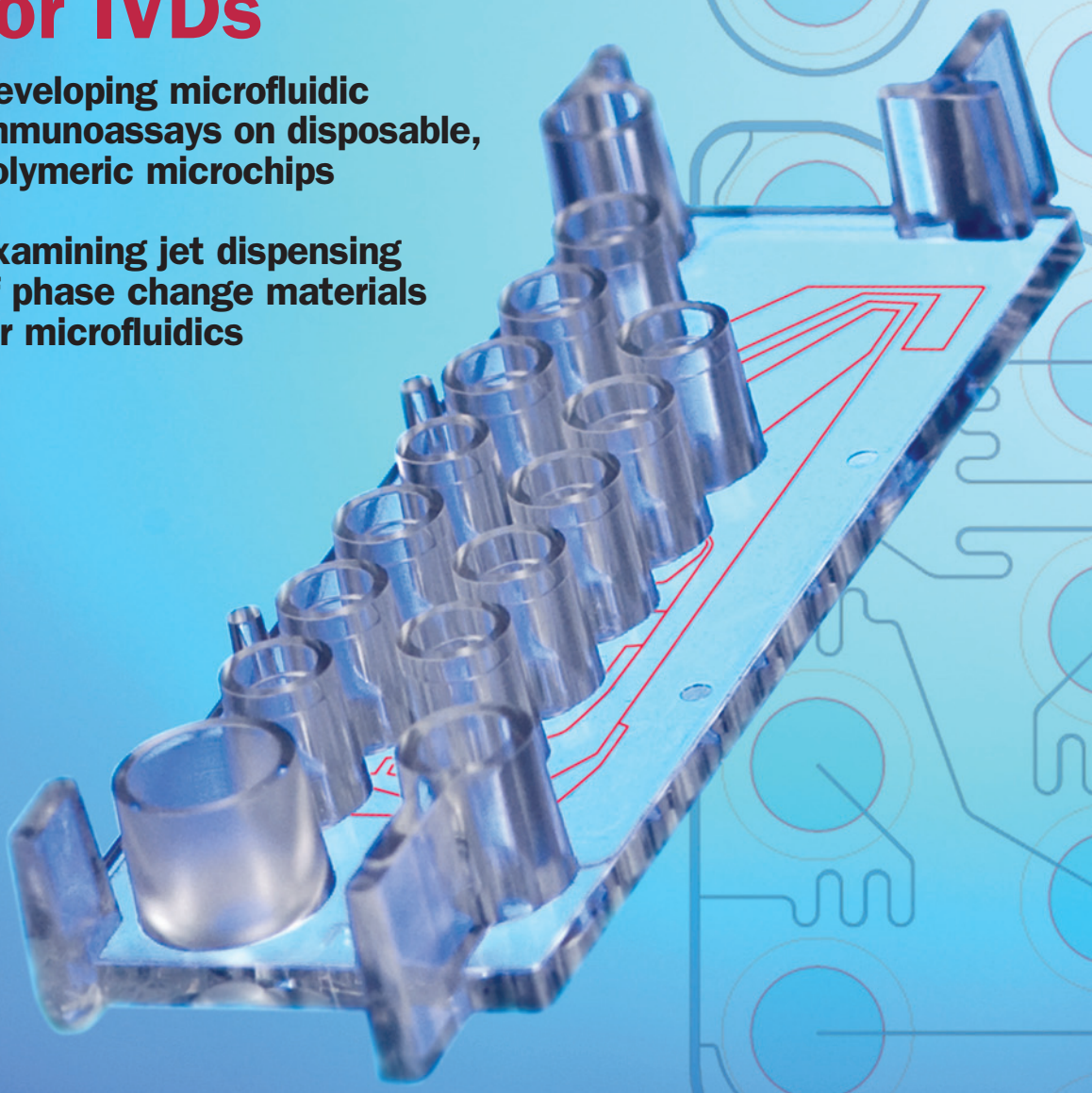
SPRING 2010

Microfluidic Applications for IVDs

Developing microfluidic immunoassays on disposable, polymeric microchips

Examining jet dispensing of phase change materials for microfluidics



PLUS:
The future of microfluidics in the IVD market

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FEATURES

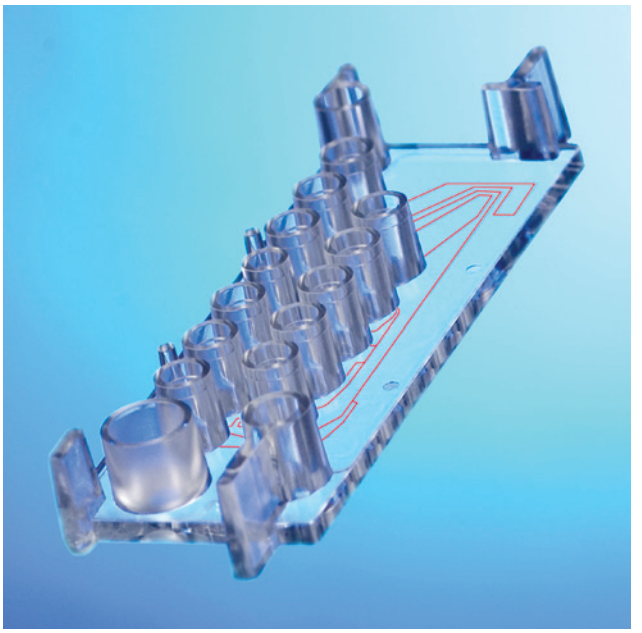
DEPARTMENTS

Microfluidic immunoassays on polymeric microchips

Microfluidics immunoassay on disposable, polymeric microchips for clinical diagnosis has potential for commercialization.

BY WILLIAM W.P. CHANG, CHEN LI, CHIAKI KAGEBAYASHI, AND SHINJI SATOMURA

The first examples of microfabricated electrokinetic devices built onto a planar substrate were demonstrated in the early 1990s.[1,2] At first, they were little more than improvised capillary electrophoresis units etched into silica or glass substrates. Researchers first reported a microfabricated capillary electrophoresis device with multiple channels etched into a glass substrate using electrokinetic sample manipulation and injection.[3] This was quickly followed by many other papers about electrophoresis on microfabricated glass or quartz substrates.[4-6] This concept grew gradually into a new discipline with a wide variety of applications, in the form of many conventional as well as novel analytical methods.[7] Several new names for this nascent field were coined. Some nomenclatures, such as "micro total analysis system" (or μTAS) and "lab on a chip" clearly reflect the much-anticipated miniaturization and integration of multiple routine laboratory processes on a single microdevice. An up-to-date summary of μTAS can be found in a recent review.[8] An even more recent review summarizes many key features of microfluidic devices as well as their projected market growth in both the medical and analytical sectors.[9]



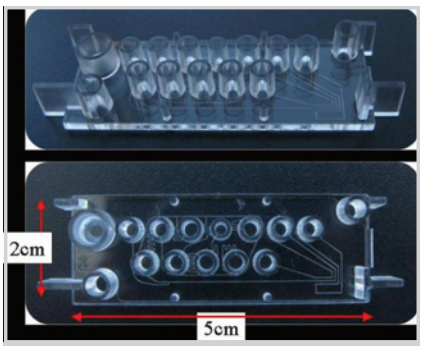


Figure 1. Disposable PMMA microfluidic chip (45-degree-angle side view and top view) made by injection molding. Various wells are used for placing electrolytes, the sample, and conjugated antibodies.

The target markets for microfluidic devices include but are not limited to IVD assays (conventional lab tests, point-of-care tests, and consumer self-administered tests), food and drug safety tests (microbes and contaminants), water and homeland security monitors (pathogens and biowarfare agents), and R&D applications in the pharmaceutical, biotech, and academic sectors. Of these target markets, IVD products are likely to represent a significant growth area for microfluidic devices, because of their potential for miniaturization and automation.

Polymeric Materials for Commercial Devices

A prerequisite for commercialization is the production of microfluidic devices at sufficiently low cost. It became clear that the initial glass or quartz devices could not meet this cost requirement, given the intrinsic cost of the substrates used and the associated fabrication processes.[10] Very quickly, several research groups published a multitude of fabrication methods for microfluidic devices containing channels with micrometer-scale dimensions using polymeric materials. The methods varied from

imprinting or hot embossing[11] to injection molding[12] and laser ablation or laser machining.[13] Another method that has proven to be very popular for research purposes is casting, especially of a silicone rubber such as polydimethylsiloxane (PDMS).[10,14,15] However, casting is considerably slower than injection molding and is most commonly done manually, so it is therefore more difficult to achieve low-cost production of a disposable device with this method. Casting is probably most advantageous for the production of devices that include elastomeric components. Injection molding offers substantial advantages for the mass production of disposable microfluidics devices in thermoplastic polymers. The use of polymeric substrates and injection molding fabrication are therefore likely to be key ingredients of chip manufacturing, from a cost perspective.

In Vitro AFP-L3% Immunoassay on Disposable Microfluidic Chips

A disposable thermoplastic polymeric device exhibiting microfluidics principles shows potential for commercialization. This type of device can be processed in an automated instrument that occupies minimal laboratory space and can be operated easily with little operator intervention. The ability to design and produce microfluidics-based diagnostic thermoplastic polymeric devices provides several desirable characteristics:

1. Thermoplastic polymeric material is low in cost and amenable to precision mass production.
2. The low-cost device is disposable, thus avoiding cross-contamination.
3. Thermoplastic polymeric chips are more robust in terms of handling than comparable devices made of nonpolymeric material, such as quartz or glass.

Using the alpha-fetoprotein L3% (AFP-L3%) analysis, the performance of this technology is demonstrated in terms of separation resolution, assay time, assay sensitivity, specificity, and reproducibility. AFP-L3 is a glycoform of AFP. The accurate determination of AFP-L3% (the percentage of AFP-L3 with respect to total AFP) has been demonstrated to greatly improve the early detection of hepatocellular carcinoma (HCC) by as much as 9 months,[16] compared to conventional medical diagnosis via imaging. These features are clearly beneficial for the care of liver-disorder patients. The speed, accuracy, and reproducibility of this microfluidics-based assay is intended to help clinicians improve clinical diagnosis.

	Migration time(s)		Peak area		
	AFP-L1	AFP-L3	AFP-L1	AFP-L3	AFP-L3
CV	0.97%	0.96%	1.79%	3.04%	0.96%

Table I. Migration times and peak area

Assay Principles

A unique sandwich immunoassay on a thermoplastic polymeric microfluidic chip couples isotachophoresis (ITP) to gel electrophoresis (CGE) to concentrate, separate, and detect AFP-L1 (the noncancerous glycoform) and AFP-L3 (a biomarker for HCC) both quantitatively and reproducibly. This microfluidic immunological/electrophoretic method has been used for the determination of AFP-L3 concentration and percentage in quartz microchips.[17] In this assay, AFP isoforms are recognized by two monoclonal antibodies. One antibody has a mobility modifier (DNA) attached to speed the electrophoretic movement of the immunocomplex, as well as sharpen the immunocomplex peak and improve the resolution;[18] the other antibody is conjugated to a fluorescent label for laser-induced fluorescent (LIF) detection.[19] An immunocomplex between the antibodies and AFP is formed as they migrate toward the anode. The AFP in the serum sample is concentrated through the formation and stacking of immunocomplexes during the isotachophoretic part of the assay, leading to higher detection sensitivity, a process called electrokinetic analyte transport assay (EATA).[19]

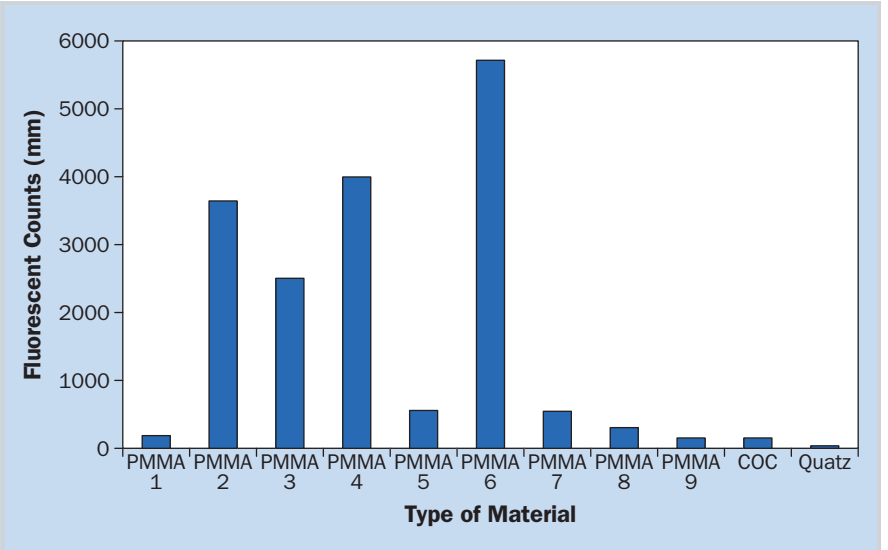


Figure 2. Autofluorescence levels of commercial PMMA materials in comparison to COC and quartz materials.

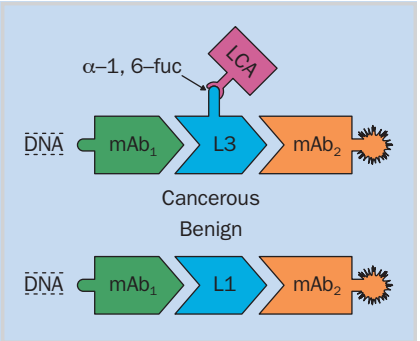


Figure 3. The immunocomplexes between AFP-L1, AFP-L3, and the AFP-specific antibodies conjugated to either a mobility modifier (DNA) or a fluorescent marker for detection. LCA is introduced to enable the electrophoretic separation of immunocomplexes that carry AFP-L3 from those that carry AFP-L1.

As a stacking method, ITP has been demonstrated to enhance analyte concentration by as much as three orders of magnitude, as reported in the literature.[20]

PMMA Polymeric Microchips

Sample loading, antigen-antibody reaction/binding, and immunocomplex concentration, separation, and detection are all carried out on a polymethylmethacrylate (PMMA) disposable chip (see Figure 1). There are a variety of other polymeric materials that can be used as chip substrates for microfluidic devices, such as polydimethylsiloxane (PDMS) and cyclic olefin copolymer (COC). However, PMMA was chosen as the substrate material for microfluidic chip fabrication because this thermoplastic polymeric material is low in cost and has the appropriate optical, molding, and surface properties for certain microfluidic devices.

Using a thermoplastic polymeric material with low autofluorescence background as a chip material is critically important to achieving high sensitivity for target analyte detection. The autofluorescence of quartz, COC, and a variety of commercial PMMA materials were measured using a laser excitation wavelength of 635 nm and an emission wavelength of 670 nm. The results are summarized in Figure 2. As expected, quartz material shows the lowest level of autofluorescence, and COC gives about 5-fold higher fluorescence than quartz. There are considerable variations in the autofluorescence background of PMMA materials, spanning a relatively large range. Some of the PMMA materials (e.g., PMMA 1 and PMMA 9 in Figure 2) are similar to COC, with respect to low autofluorescence.

As a structural component, PMMA has high dielectric strength.[21] PMMA has no ionizable groups. There are at least two advantages of this feature. First, a noncharged substrate minimizes electrostatic interactions between the analytes and the microchannel surfaces. Second, it is commonly known that in the absence of ionizable groups, electroosmotic flow (EOF) as a result of surface charges is minimized. This feature mitigates the need to use either chemical or electrical means to reduce EOF, in contrast to a charged surface such as silica or quartz. It is widely recognized in the literature that the reduction and control of EOF are crucial for achieving high-quality electrophoretic separation in either capillaries or microchannels.

A microliter level of serum sample and reagents are transferred into wells on the chip. A fraction of the sample and reagents in wells are subsequently injected into predetermined zones along the microchannel for precise volume control as determined by the actual dimensions of the microchannel space; this is a critical factor that directly contributes to high assay reproducibility. Following the injection of the sample and the reagents into the microchannel, an electrical field is applied across the microchannel to allow ITP to proceed, leading to analyte concentration through the immunocomplex formation and stacking process by the EATA method.[19] After ITP, the stacked immunocomplex migrates into the CGE separation channel for further separation of AFP-L1 and AFP-L3.

AFP-L3 is very similar to AFP-L1 in terms of molecular weight; in fact, they share the same amino acid sequence, which is immunologically indistinguishable between the two isoforms. AFP-L1 and AFP-L3 are glycoforms as a result of carbohydrate microheterogeneity due to posttranslational modifications. AFP-L3 is a variant of AFP; it has an extra alpha 1-6 fucose residue. Typically, this small size difference

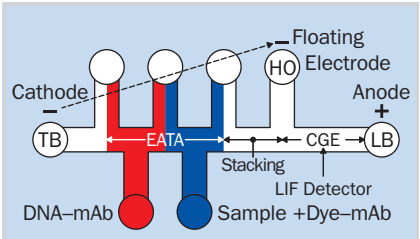


Figure 4. A schematic diagram showing the well and channel layout on the PMMA microchip. LB is the buffer containing the leading ions; TB is the buffer containing the trailing ions. The HO (handoff) well also contains LB. DNA-mAb is anion-bound human anti-AFP antibody; dye-mAb is fluorescent dye-bound human anti-AFP antibody (mouse monoclonal antibodies).

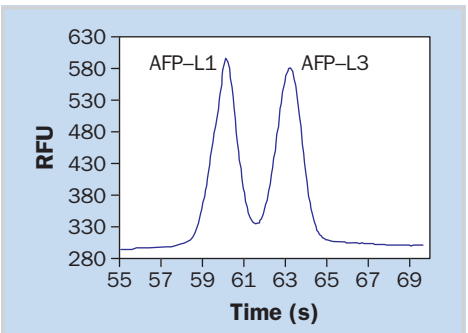


Figure 5. ITP-CE electropherogram showing AFP-L1 and AFP-L3 separation. 500 pM of each isoform was spiked into pooled normal human serum.

cannot be resolved by size separation through gel electrophoresis. Consequently, further modification of the AFP-L3 mobility is required in order to achieve good resolution between the two isoforms.

To effect the separation and detection of immunocomplexes containing AFP-L1 and AFP-L3, a lectin differential binding method was used, as described below. At the end of ITP stacking, immunocomplexes containing either AFP-L1 or AFP-L3 are indistinguishable as they enter the CGE separation channel. Nonetheless, due to differences in their glycan groups, a sugar binding lectin, *Lens culinaris* agglutinin (LCA), was applied to facilitate the resolution of AFP-L1 and AFP-L3 during CGE separation. LCA affinity to fucose residue on N-acetylglucosamine at the reducing end of the carbohydrate group allows it to bind AFP-L3 specifically but not AFP-L1, since the fucosyl residue is only present on AFP-L3. Because LCA has a molecular weight of 49 kDa, which is significant relative to the size of the immunocomplex, the binding of LCA to AFP-L3 segregates the bound and unbound AFP immunocomplexes into two distinct populations based on their relative electrophoretic mobility (see Figure 3). This strategy has produced excellent resolution between the two AFP isoforms that are virtually indistinguishable in terms of size.

Performance of AFP-L3% Immunoassay on Microfluidics PMMA Chips

To demonstrate assay resolution, equal molar concentrations of AFP-L1 and AFP-L3 are spiked into pooled, normal human serum, which is mixed with an AFP-specific antibody conjugated to a fluorescent label for detection. The mixture is then loaded into the microchannel, reacted with monoclonal antibody carrying a mobility modifier (DNA), and stacked by ITP to form AFP immunocomplexes. High voltage is applied across the ITP channel during the ITP process. The cathode is switched to the handoff (HO) position after ITP stacking is completed, and immunocomplexes containing AFP-L3 are separated from those containing AFP-L1 through binding to LCA in the separation channel during the CGE phase (see Figure 4). Size separation between these two populations of immunocomplex leads to the resolution of both isoforms.

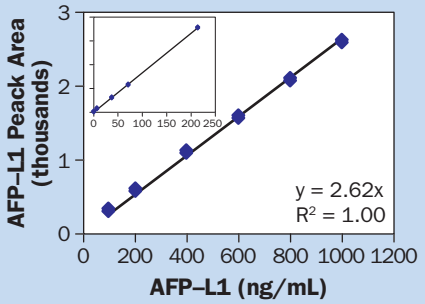


Figure 6. AFP linearity and dynamic range.

Using this method, highly reproducible separation between AFP-L1 and AFP-L3 is obtained. An example of this separation is shown in Figure 5. In addition to the complete separation of AFP-L1 and AFP-L3, the correct ratio of the two isoforms is unequivocally reflected by the electropherogram, which can be calculated based on the ratio of respective peak areas. Starting with sample and reagent loading, followed by mixing, reaction, stacking, and separation, each AFP-L3% assay is completed in no more than two minutes. Comparable AFP immunoassays can take several hours to process a single batch of samples.

The reproducibility for the AFP-L3% assay is excellent, leading to coefficients of variation (CV) at typically less-than-5% levels for all of the parameters tracked (see Table I). The design of chip channels and chip fabrication quality control, as well as reagent development and consistent temperature control, all contribute to the high reproducibility. For example, the volumes of the sample and reagents are fixed by the precise dimensions of the microfluidics channels, as they are first injected into the microchannels at the beginning of the assay (see Figure 4).

The AFP-L3% assay on disposable microfluidic chips provides good assay linearity with respect to AFP quantification. The coefficient of determination, R^2, is 0.999. The measured peak area correlates well with the theoretical AFP concentrations over a wide dynamic range spanning 3 orders of magnitude from 1 to 1000 ng/mL (see Figure 6).

The basis of the AFP-L3% assay is the detection and quantification of AFP-L3 as a percentage of overall AFP amount (AFP-L3% = ([AFP-L3]/[AFP-L1+AFP-L3]) × 100). The ability to accurately measure AFP-L3% contributes to the risk management of chronic liver disease patients with respect to HCC. Different ratios of purified AFP-L1 and AFP-L3 (between 0 and 100 ng/mL) were spiked into normal, pooled serum. These samples were then analyzed by the onchip AFP-L3% immunoassay as described earlier. The AFP-L1 and AFP-L3 peak areas were integrated and compared to calibrators with

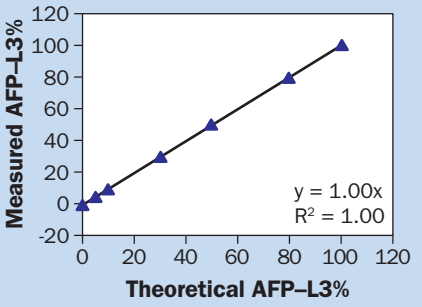


Figure 7. AFP-L3% data provides linear response between measured and expected AFP-L3% values.

known concentrations of AFP-L1 and AFP-L3 to determine their concentrations in the sample. The percentage of AFP-L3 was determined from the ratio of AFP-L3 and total AFP (L1+L3) concentration. The assay was able to accurately determine the AFP-L3% and that the measured values agreed well with the spiked values, leading to a linearly fitted curve (Figure 7). Good correlation between the measured percentage and the theoretical percentage (calculated from spiked analyte concentrations) between 0–100% AFP-L3 is demonstrated by the data set. The recovery for total AFP is 87.3-113.6% and 92.1 to 108.6% for AFP-L3%.

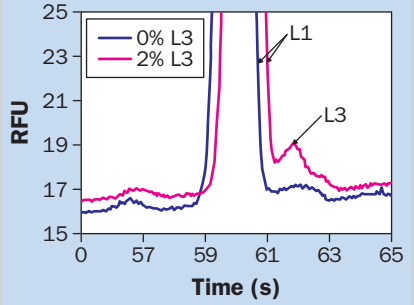


Figure 8. AFP-L3 detection sensitivity. Total AFP concentration is the same for both sets of data (25 ng/mL).

While miniaturization and process integration are the main attributes of microfluidic devices, they present both opportunities as well as challenges. As an example, in microfluidics, sample and reagent/buffer volume requirements are much lower than conventional methods. Low sample-volume requirement, while desirable for situations where the sample amount is limited (such as spinal fluid analysis), inevitably places a large burden on detection sensitivity. With the ITP-CE method, the impact on sensitivity is reduced, since the analytes (AFP-L1 and AFP-L3 in immunocomplex form) are concentrated during the stacking phase by the ITP process. Through this method, the analyte concentration can be increased by 2 orders of magnitude.[19] Using the AFP-L3% assay on a disposable PMMA chip, AFP-L3 down to a level of 2% (0.5 ng/mL) was detected in a sample containing 25 ng/mL of total AFP (see Figure 8). At 10 ng/mL total AFP, as low as 5% AFP-L3 can be detected. The microfluidic AFP-L3% assay has the potential to be more sensitive than existing technologies. With a microfluidics-based device made of disposable PMMA material, it was demonstrated that the application of such a device can provide high-quality data in terms of speed, reproducibility, linearity, and sensitivity. Using AFP-L3% immunoassay as a basis, an automated instrument that can fully realize the key advantages of such a device as reported here is in development (see Figure 9). It is anticipated that such an instrument in a diagnostic setting is capable of providing reliable, rapid test turnaround with a compact footprint, while providing a low-cost structure in terms of both the hardware/instrument and the consumables (thermoplastic polymeric microfluidic chips and buffer reagents). It is expected that the same EATA method on disposable thermoplastic polymeric microfluidic chips can be applied to the development of other assays for which rapid assay time and reliable data are critical. In particular, future embodiments of this microfluidic system with even smaller size and ease-of-use features could be suitable for point-of-care (POC) testing where quick turnaround time is crucial for timely medical evaluation.

Figure 9. An automated instrument that samples, mixes, reacts, concentrates, and separates analytes using PMMA disposable chips by the EATA method.

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