Multiplex Assays with Fluorescent Microbead Readout: A Powerful Tool for Mutation Detection

After the completion of the Human Genome Project, the goal for biomedical research is to apply genomic approaches for the improvement of human health (1). The study of human DNA variation promises to have a great impact on understanding how genetic factors contribute to human disease, conferring susceptibility or resistance; it is also expected to help uncover the reasons that individuals respond differentially to therapeutics.

Technologies for mutation detection can be classified into methods for the detection of unknown mutations and methods for the detection of known mutations (2). Methods for the detection of unknown mutations (3–8), also known as screening or scanning methods, are used during the discovery phase. Once a new mutation associated with a genetic disease has been characterized and validated, it is usually detected over and over in many DNA samples by specific, cost-efficient, and easy-to-use methods (9–13), also known as “diagnostic methods” (14).

Typically, genotyping assays for known mutations start with sample preparation followed by amplification of the target DNA sequence containing the mutation to be tested. This latter step is most commonly accomplished by use of the PCR, but other amplification strategies are available (15–17). Subsequently, the presence or absence of the mutation is determined by allele-specific biochemical reactions. The selected allele-specific method largely determines the specificity, accuracy, and reproducibility of the genotyping assay. The discriminating power of DNA ligases (13, 15) and DNA polymerases (10–12), as well as the difference in thermodynamics between matched and mismatched DNA duplexes (9), has been extensively exploited for mutation detection. The final step of a genotyping approach is the detection of the signal resulting from the allele-specific assay and genotype assignment. Although several detection technologies have been described (18–20), fluorescent technology is the most commonly used detection technology for high-throughput genotyping. Several fluorophores became commercially available that facilitated the development of multiplexing strategies. For mutation detection, the use of fluorescent dyes has been coupled with different detection platforms, such as capillary electrophoresis (21), plate readers (12), DNA arrays (22), and others.

Depending on the genotyping assay and the detection technology, two or more steps can be combined. For example, homogeneous methods for allelic discrimination (23, 24) allow combining amplification, discrimination, and detection in one step.

For the detection of mutations with a known sequence, molecular diagnostic laboratories need specific, reliable, and affordable high-throughput methods that can be easily automated. One of the strategies that can certainly reduce costs and increase throughput is multiplex analysis. Multiplexing allows for the simultaneous detection of several different mutations in a single reaction vessel. Examining multiple mutations in the same reaction tube reduces time, labor, and cost compared with single-reaction-based detection methods.

One of the approaches that allow massive parallel mutation detection and analysis is the use of DNA microarrays. Allele-specific oligonucleotides (ASOs) are printed on glass slides (25) or glass plates (26) by dedicated instrumentation, synthesized in situ on a silicon wafer (27), or driven by an electric field toward a specific address on the solid support (28). The address (hybridization target) of a single mutation depends on the position of the ASO on the array. After immobilization on the array, the ASOs are ready for hybridization. The DNA fragments to be queried for mutations are first amplified in single or multiplex PCR using fluorescently labeled primers. Subsequently, the labeled PCR products are hybridized to the ASO immobilized on the array by use of either a hybridization chamber or a fluidic station. After hybridization is complete, the array is subjected to several washings and then scanned with a fluorescence microarray scanner. Signal is collected as light emitted from the fluorescent groups incorporated into the PCR products, which after hybridization are bound to the immobilized ASO. Perfect matches generate stronger signals than do those duplexes containing single-base-pair mismatches. Genotypes are assigned based on fluorescence intensities and their ratios.

Gel-based capillary electrophoresis and mass spectrometry, which can separate a mixture of allele-specific products generated with technologies such as oligonucleotide ligation or primer extension, have also been used as multiplex detection systems (29, 30).

Another means of multiplex detection is the use of fluorescently labeled microspheres that are individually identified by a flow cytometer based on their unique fluorescence labels (31–36). This has been achieved by coating the microspheres with various concentrations of two fluorescent dyes, thus generating sets of microbeads, each with a unique spectral signature determined by the ratio of the included fluorophores. Unique synthetic oligonucleotide sequences are covalently attached to microspheres containing various dye combinations; thus, each fluorescent microbead becomes the address for a single mutation. The array of microbeads coupled with specific identifying DNA sequences allows for multiplex mutation detection. Recognition of the microbead–oligonucleotide pairs by their unique signatures leads to the identification of allele-specific products that are captured by hybridization to the tag sequences attached to the microbeads. The identification of these products is mediated by the use of a third fluorescent dye, which has emission maxima different from those of the fluorophores used for “painting” the microbeads.

A variety of allele-specific technologies have been used for genotyping by the microbead approach. Armstrong et
al. (31) attached ASO sequences to microspheres to perform allelic discrimination by ASO hybridization. Four ASOs were synthesized for each mutation, such that each oligonucleotide had a different base at the polymorphic site (32-plex assay). Fluorescently labeled multiplex PCR products were captured by hybridization in an allelic-specific manner to oligonucleotides attached to microbeads characterized by their unique fluorescent signatures. Similar to DNA microarrays, perfect matches generated stronger signals than those duplexes containing single-base-pair mismatches. Fluorescence intensities and their ratios were used to perform genotyping. Other publications demonstrated that the oligonucleotide ligation assay (36) and single base chain extension (32) could be used for genotyping DNA samples with the microbead approach. A limitation of the previously mentioned technologies is the fact that the allelic-specific reactions are performed in two reaction vessels. To overcome this disadvantage, Ye et al. (34) and Taylor et al. (33) used allele-specific primer extension (ASPEx). This method allows genotyping reactions to be performed in a single tube. The assay consists of four steps: PCR or multiplex PCR, ASPEx, microbead decoding, and detection. The first step involves amplification of the target DNA sequence containing the mutation. The amplification step is followed by a biochemical reaction that involves the use of two allele-specific primers. Each primer for the ASPEx reactions includes two different sequences, each one having a different role. The 5’ portion of the primer is complementary to the tag oligonucleotide sequence attached to a microbead, and the 3’ sequence is locus specific with its 3’ nucleotide complementary to the polymorphic nucleotide. DNA polymerases extend primers with a mismatched 3’ nucleotide with much lower efficiency than perfect matches. When the allele-specific primers are extended in the presence of biotin-dCTP, the resulting extended products are labeled with the biotinylated nucleotide. Subsequently, the allele-specific products formed in the previous step are hybridized to the tag sequence attached to the microbeads, incubated with streptavidin–phycoerythrin, and then injected into the flow cytometer. Finally, fluorescent signals are detected and genotypes are assigned according to the fluorescent intensities and their ratios.

In this issue of Clinical Chemistry, the power of multiplexing using the microbeads approach is demonstrated by two groups of researchers (37, 38). In one report, Johnson et al. (37) describe the development and validation of the Multicode® PLx system for the detection of mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) gene. I will focus on the technology described in this report and not on the clinical importance of identifying cystic fibrosis mutations.

The authors demonstrated that the Multicode PLx system for mutation detection provides several advantages compared with previously published genotyping methods based on the use of microbeads and flow cytometry. They used isoguanosine (iG) and 5-Me-isocytosine (iC), two nonnatural bases that under specific conditions form stable base pairs and do not hybridize to natural bases (39). These nonnatural bases were used to generate the tag sequences attached to the microbeads. The tags, which usually are 10 bases long, contain a mixture of natural and nonnatural bases. These tag sequences hybridize to their complements, which are part of the allele-specific primers used for ASPEx reactions. Because the cross-reactivity of iG and iC with natural bases is minimal, allele-specific products can be captured at room temperature (liquid decoding) with high specificity. This is a great advantage over protocols that require long incubations at high temperatures to achieve good decoding specificity. The nonnatural bases are also involved in labeling ASPEx products. Primers containing an iC at the 5’ end are used for target amplification. PCR products with the iC at the 5’ end are used as templates for ASPEx reactions. During this step, the extended products incorporate a biotin-labeled 2’-deoxy-isoG triphosphate (diGTP) at their 3’ ends. After hybridizing to the tag sequences, these biotin molecules are subsequently incubated with streptavidin–phycoerythrin and detected by a flow cytometer. The novel labeling system using biotin-diGTP is advantageous compared with ASPEx protocols that require biotin-dCTP. Protocols that use dCTP require a PCR clean-up step with shrimp alkaline phosphatase to inactivate remaining nucleotides (dCTP) that would interfere with the performance of biotin-dCTP.

The authors evaluated the specificity of the Multicode PLx system with a retrospective study that involved the analysis of 225 DNA samples prepared from whole blood and a prospective study that included more than 400 newborn DNA samples prepared from Guthrie cards (37). In both studies, >99% of the samples included were correctly genotyped. The method developed by Johnson et al. (37) is fast, and all of the reaction steps are performed in a single vessel. The processing of 24 DNA samples for the analysis of 27 cystic fibrosis mutations and 4 reflex polymorphisms by a manual approach took ~3 h. The analysis of the same number of samples by an automated approach produced similar reaction times. Although not yet validated, an improved version of the test was able to process 96 DNA samples in a 96-well plate in 3 h.

In a second report in this issue of the journal, Bortolin et al. (38) report the development of the Tag It™ platform for mutation detection; this system is also based on the use of fluorescently labeled microspheres. An interesting application of the Tag It platform was developed for the detection of six mutations believed to be associated with thrombotic risk.

The authors addressed the specificity of both the hybridization-decoding step and the allele-specific assay used for genotyping. The sorting capabilities of the array were successfully validated by use of a mixture of 100 microbeads, each one having attached a different anti-tag sequence. The specificity of ASPEx genotyping method was evaluated with a method-comparison study in which DNA from 132 individuals was genotyped by both the Tag It platform and DNA sequencing. Genotyping results
showed a 100% correlation between both methods. Although this method showed great specificity, its turnaround time is not as short as that reported by Johnson et al. (37). Because the Tag IT method uses tag-unmodified oligonucleotides, the hybridization-decoding step is done at 37 °C for 1 h. In addition, the protocol requires a 45-min treatment with shrimp alkaline phosphatase and exonuclease I for preparation of the PCR products for the ASPE reaction (38).

Where do these platforms have potential within the molecular diagnostic laboratory? Because they have the potential to accommodate the simultaneous detection of up to 50 biallelic single-nucleotide polymorphisms, they are well suited for the detection of heterogeneous genetic disorders caused by numerous mutations, such as α- and β-thalassemia and cystic fibrosis. They can also be useful for studying highly polymorphic systems such as the human major histocompatibility system (HLA); for testing of risk panels (e.g., for thrombosis, cancer, or infectious diseases); and for newborn screening panels.

References


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