Flexible universal tag genotyping on a bead-based platform

By Doug Carey, Dr. Roman Zastawny and Dr. Richard Janezczko

The scramble for a platform

Putting the human genome to work is an obvious and almost self-evident intention of the Human Genome Project but in practice this is not always easy to realise. In the last few years, manufacturers of human genetic diagnostics have struggled to find the ideal platform to meet the potential growth of the clinical marketplace. In the absence of commercial assays, many clinical laboratories have found it necessary to develop “home brews” for genetic diagnostics or perform expensive DNA sequencing.Neither whole genome screening for point mutations nor whole genome sequencing is currently practical. For this reason, a number of platforms have been developed specifically to screen for point mutations, single nucleotide polymorphisms (SNPs), duplications and/or deletions.

While national organisations such as the National Screening Committee in the UK [1] or the French Association for the Screening and Prevention of Infant Handicaps (AFDPHE) in France [2] or the American College of Medical Genetics (ACMG) [3] in the United States began to suggest and recommend genetic testing as a standard of care in areas including, among others, cystic fibrosis (CF), diagnostic companies scrambled to meet the demands. Early systems [4] such as the Elucigene enzymatic platform (Tepnel Diagnostics, Abingdon, UK) which uses ARMS (Amplification Refractory Mutation System) technology or Immogenet’s (Gent, Belgium) Line Probe Assay (LiPA) were adequate but not ideally designed to cope with the increasing number of tests required. ARMS technology utilises limited multiplex primers followed by gel visualisation which does not lend itself to higher throughput needs nor provide automated analysis. LiPA too provided an early solution to genetic analysis. The technology is based on direct hybridisation on pads embedded on a nitrocellulose strip. Positive reactions are visualised using a NBT/BCIP chromagen. However, while this technology is semi-automated for processing, it is prone to visual subjectivity and not well suited for automated data analysis. Still other technologies [4] attempted to solve this growing need for an ideal platform. This quest included Abbott/Celera’s (Abbott Park, USA and Alameda, USA) OLA (Oligo Ligation Assay) technology on the Applera/Applied Biosystems (ABI) platform. While providing more automation, the platform is relatively expensive to purchase. Here, limited multiplexing is possible with the use of capillary electrophoresis where mutants and wild type alleles are discriminated by the addition of different sized probes through oligo ligation. Third Wave’s (Madison, USA) novel Invader technology utilising the cleavase enzyme, takes advantage of lower technology instrumentation. A PCR free technology, it works by creating a cleavage structure with binding of two probes. The cleavase enzyme then cuts a flap with one of the probes where a fluorescent probe would bind, and then cleaves the fluorescent probe resulting in a release of fluorescence. While having the advantage of being a homogenous assay, the drawback with this technology is that it is unable to analyse multiple mutations in a single tube (multiplexing). In Third Wave’s CF screening assay for instance, this lack of multiplexing leads to complex screening algorithms and therefore has not been conducive to the increasing numbers of tests required.

What the clinical market requires is a robust technology which is multiplexed, requires relatively inexpensive hardware, and retains flexibility to adapt to the ever changing recommendations of national and international genetic organisations. Tm Bioscience (Toronto, Canada) takes advantage of the development of a set of universal tags/anti-tags which are designed to be resistant to non-specific hybridisation with native DNA and among each other, a common source of error in biochips. These 24mer tag sequences and their complements (anti-tags) were combined with Allele Specific Primer Extension (ASPE) chemistry and the Lumixen xMAP (Austin, USA) bead based system for the development of a number of assays. This technology forms the base of the Tag-It platform.

The Tag-It platform

Sample DNA (whole blood, blood spot, etc.) which may be extracted by a variety of methods, undergoes a single tube multiplex PCR reaction to amplify regions of the genome that are of interest. Multiple copies of the target DNA containing the mutations being queried are generated. The amplified DNA is then subjected to ASPE. During this process, a thermostable DNA polymerase is added to the DNA along with two tagged allele-specific primers which overlap the mutation or SNP on the amplified target DNA. Each 5’ tagged primer represents either a wild type or mutant allele. A perfectly matched primer will hybridise to the target DNA and be extended incorporating biotin. Non-complementary primers are not extended or labelled due to the 3’ mismatched base. The biotin incorporated extension products with a 5’ tag are then incubated with a set of spectrally addressable (5.6 micron) polystyrene beads or microspheres. Each spectrally distinct bead set has a chemically coupled tag complement or anti-tag. During hybridisation, all extended and unextended tagged product binds with high specificity to its corresponding anti-tag bead. Conjugation of a fluorescent reporter molecule (streptavidin-phycoerthrin) with the biotin extension product allows identification of present alleles [Figures 1, 2, 3].

Analysis of extension product/bead complex is carried out using a Lumixen xMAP platform. The xMAP platform is a bench top flow cytometer which is able to discriminate up to 100 spectrally distinct beads (therefore can analyse up to 100 alleles or 50 bi-allelic mutations and includes information on both wild type and mutant alleles in the same tube). Here samples are analysed in a 96 well plate. Each sample is

Figure 1. Illustration of the proprietary tag system showing a polystyrene bead with attached anti-tag bound to a complementary tag with attached DNA probe sequence.

Figure 2. Five steps of the Tag-It system. Multiplex PCR amplifies target region, multiplex ASPE increases discrimination of target sequence, universal array sorting sorts alleles, xMAP detection detects presence of alleles, and data analysis performs automated allele calling based on allelic ratios.
aspirated into the xMAP instrument where each bead is analysed individually by two lasers. A red laser identifies the bead and therefore identifies the allele being queried. A green laser identifies the presence of the reporter molecule which indicates an extended product and thus the presence of the allele. Typically, 100 beads from each population are read to give a statistically significant sampling. Subsequently, automated calling software analyses the signal comparing extended product vs the non-extended background. An allelic ratio - mutant signal/(mutant signal + wild type signal) - is then calculated to determine the genotype of the sample. Sample results therefore yield genotypes of wild type, heterozygous, or mutant for each SNP queried [Figure 4].

The platform in practice

The Tag-It platform has been applied to a number of disease states including thrombophilia [5] and drug metabolism. A further example of this application is in the area of cystic fibrosis. Here, the assay analyses 40 CFTR mutations and another 4 polymorphisms in a single tube. The multiplexing capability of the assay improves productivity in the laboratory by consolidating the testing of each mutation into one tube. Another good example of the multiplexing advantages lies in the platform’s Ashkenazi Jewish Panel. Here 8 different disease states (31 mutations) that are prevalent in persons of Ashkenazi Jewish descent are tested for simultaneously in a single tube. Analysis for Tay-Sachs, Canavan, Familial Dysautonomia, Gaucher, Bloom Syndrome, Fanconi Anaemia GrpC, Niemann-Pick, and Mucolipidosis Type IV are all combined together in a single test which would otherwise typically be run as 8 different tests in a laboratory. The savings of both time and resources can be directly attributed to the flexibility and design of the platform.

While other diagnostic companies such as Ambion (Austin, USA) are now taking advantage of the Luminex platform, the combination of a Tag based system and ASPE chemistry lend some benefits over other Luminex-based formats. ASPE improves SNP discrimination based on the high sequence specificity of the enzyme over an oligo hybridisation which is determined by sequence context and reaction conditions. Because the 24mer Tag/Anti-tag system was designed to operate within the same reaction conditions, robustness is improved and very little assay optimisation is required should the content of the assay require changes (which is common in today’s genetic world). Furthermore, Tm’s tag technology is available in an open platform format in the form of a universal bead set FlexMAP (Luminex, Austin, USA). This allows development of “home brew” assays using the same technology as Tag-It for the addition of auxiliary tests or mutations to augment current assays.

References


The authors

Doug Carey,
Roman Zastawny, PhD,
Richard Janeczko, PhD
Tm Bioscience Corporation
439 University Ave. Suite 900
Toronto, Ontario M5G 1Y8, Canada
Email: dcarey@tmbioscience.com