Next generation sequencing in clinical diagnostics

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Introduction to next generation sequencing

Next generation sequencing (NGS) is a moniker used to represent multiple high-throughput or massively parallel nucleic acid sequencing technologies that have emerged since the mid 2000s. These technologies collectively constitute some of the greatest advances made in biomedical sciences in the past three decades (Meldrum et al., 2011). Utilizing NGS approaches, it has become possible for a single laboratory to sequence an entire human genome in a matter of days to a couple of weeks on a single instrument for a cost that is rapidly approaching $1000 per genome (Natrajan and Reis-Filho, 2011). When compared with the first published genome sequence assembly that was completed in 2003 using farms of Sanger sequencers over a period of 13 years involving more than 3000 scientists in 6 countries at a cost of nearly $3Bn, the astounding progress made, in less than a decade, is even more impressive (Diamandis, 2009). Not since the introduction of the polymerase chain reaction (PCR) in the mid 1980s has a platform technology emerged with such potential to transform medical diagnostics.

The completion of the human genome project was supposed to herald a paradigm shift in the way medicine was practiced, with a change from the traditional reactive therapy approach to a personalized approach based on the patient's genetic makeup. While the information explosion resulting from the genome projects has precipitated great strides in the basic sciences, much to the disappointment of many, the impact on general clinical medicine has been relatively modest (Hall, 2010; Manolio, 2010). Now, however, with the availability of NGS technologies, we finally have the tools to translate the knowledge gleaned from these efforts into individual patient care. In this perspective we provide a brief overview of NGS approaches and discuss some of the advantages, challenges, and progress made in integrating NGS into routine clinical practice.

The NGS platforms and their chemistries

In contrast to traditional Sanger sequencing, where typically a few sequential or parallel sequencing reactions of relatively long read lengths (700 – 1000 bp) generate a modest amount of data, the shared basis of most NGS technologies is the simultaneous execution of millions of sequencing reactions of relatively short read length (30 – 500 bp) in parallel, and generation of gigabases (Gb) of sequence data per run. The common steps in most NGS approaches consist of (i) fragmentation of the DNA to be sequenced, (ii) addition of adaptor molecules to the ends of the fragments for priming of amplification and sequencing reactions (iii) clonal amplification of the fragments (e.g. emulsion PCR or bridge PCR), (iv) sequencing of the fragmented pieces (e.g. sequencing by synthesis or sequencing by ligation), (v) acquisition of raw data (e.g. image capture or ion detection) and (vi) conversion/deconvolution of the raw data into nucleotide base calls. A detailed discussion of the technologies behind each of these platforms and their relative merits and demerits is beyond the scope of this article. However, in order to give context to the discussion ahead, we provide a brief description of the principles behind some of the major NGS technology platforms. For additional information, readers are directed to several excellent reviews that describe these platforms in considerable detail (Glenn, 2011; Mardis, 2008; Metzker, 2010; Shendure and Ji, 2008; Thompson and Milos, 2011; Voelkerding et al., 2009).

The Roche/454 pyrosequencing (GS FLX series and GS Junior), Life Technologies' semiconductor sequencing (Ion Torrent Personal Genome Machine and Ion Proton), and Illumina/Solexa (Genome Analyzer IIx, HiSeq and MiSeq) platforms generate sequence data during synthesis of a complementary strand of DNA The Roche pyrosequencing platform, which was the first NGS product to be released, and the Life Technologies' semiconductor sequencing platform both make use of emulsion PCR (emPCR) to clonally amplify small fragments of DNA captured on magnetic beads within droplet microreactors (Glenn, 2011; Metzker, 2010; Shendure and Ji, 2008; Voelkerding et al., 2009). Roche/454 sequencers use the principle of pyrosequencing, wherein natural nucleotides of each type are added sequentially to the millions of sequencing reactions physically separated on picotitre plates by means of micro-wells that hold only a single bead each (Glenn, 2011; Metzker, 2010; Shendure and Ji, 2008; Voelkerding et al., 2009). Within each reaction, when a nucleotide is incorporated into the growing strand by a polymerase, a cascade of reactions involving sulfurylase and luciferase results in the generation of a burst of light. A CCD camera captures this light, the intensity of generated light being proportional to the number of nucleotides incorporated. A correlation between the type of nucleotide provided in a cycle and the intensity of light yields the sequence on that particular fragment. Life Technologies' semiconductor sequencing platform is based on a similar principle. In place of the pyrosequencing reaction cascade that leads to release of a burst of light on the Roche platform, the semiconductor-sequencing platform exploits the change in pH resulting from H+ ion released following incorporation of a nucleotide which is detected by a microfabricated pH meter (Glenn, 2011; Metzker, 2010; Shendure and Ji, 2008; Voelkerding et al., 2009). The Illumina platform, on the other hand, works on a slightly different principle. Fragmented DNA pieces are captured in a flow cell on a solid surface and amplified in local clusters through a process known as bridge amplification (Glenn, 2011; Metzker, 2010; Shendure and Ji, 2008; Voelkerding et al., 2009). Sequencing of each cluster is carried out using reversibly terminated fluorescently labeled nucleotides, which are added to the sequencing reaction iteratively. In every cycle, the color of the nucleotide incorporated into the growing strand is optically captured resulting in the identification of the base, after which the terminator chemistry is cleaved. This cycle repeats, resulting in generation of sequence data. For all the platforms, millions of short overlapping reads are put together like a giant jigsaw puzzle to yield the final sequence.

Life technologies' SOLiD (sequencing by oligo ligation detection) system also makes use of emPCR for clonal amplification of the fragment library. Sequencing is carried out through multiple cycles of hybridization and ligation (Glenn, 2011; Metzker, 2010; Shendure and Ji, 2008; Voelkerding et al., 2009). Instead of the addition of a single nucleotide per sequencing reaction cycle, it makes use of octamers that add two nucleotides in a reaction catalyzed by DNA ligase. The octamer is structured such that the first two nucleotides are for interrogating the sequence, the next 3 nucleotides are degenerate and the last 3 nucleotides are universal nucleotides such as inosine. The octamers carry 4 different fluorescent labels at the 5' end, each corresponding to 4 possible dinucleotide combinations. Following ligation, fluorescence is captured by a CCD camera, and the fluorescent label removed by cleaving the octamer. Multiple ligation cycles are conducted to generate the sequence. In this system, each nucleotide position ends up being sequenced twice, thereby increasing the fidelity of the data.

Single molecule sequencing (third generation platforms): The platforms described above are often referred to as second generation sequencing technologies. Recently, a new generation of sequencing platforms, capable of long-read single-molecule sequencing, have debuted. Many of these platforms eschew the clonal amplification steps (Thompson and Milos, 2011). The earliest of these was HeliScope from Helicos BioSciences based on 'true single molecule DNA sequencing' technology, which makes use of cyclic addition of reversibly terminated fluorescently labeled nucleotides and extremely sensitive optics. Pacific Biosciences' PacBio/SMRT (single molecule real-time) platform is another single-molecule sequencing technology that works on the basis of optics that limit fluorescence and detection to individual DNA molecules, and their cognate-coupled polymerases during synthesis using a zero-mode wave-guide (ZMW) (Thompson and Milos, 2011). Oxford Nanopore recently revealed their new sub $1000 nanopore-based sequencing platform (Eisenstein, 2012). The company announced two products GridION – for genome scale sequencing, and MinION – a disposable device capable of sequencing gigabases, both in under an hour. In nanopore-based platforms, sequence data is generated when nucleotides pass through individual nanopores embedded in a synthetic membrane platform (Eisenstein, 2012). In the Oxford Nanopore system, an exonuclease tethered to one side of the nanopore cleaves the DNA molecule being sequenced and directs the cleaved nucleotides one-by-one into the nanopore (Sanderson, 2008). A cyclodextrin plug inserted into the other end of the nanopore allows each different nucleotide (including 5-methylcytosine which is involved in epigenetic regulation) passing through the nanopore to generate a characteristic signal thus generating sequence data in real time. Life Technologies/VisiGen's Starlight (Quantum Dot FRET) represents another promising single-molecule sequencing platform on the horizon. The system makes use of fluorescently labeled nucleotides and polymerase molecules conjugated to quantum dots (fluorescent semiconductor nano-crystalline-particles) (Thompson and Milos, 2011).

NGS in diagnostics research

NGS technologies have had a profound impact on understanding of genetics and genome biology (Meldrum et al., 2011). The high throughput and low sequencing costs provided by NGS systems have greatly accelerated research studies involving de novo genome sequencing, complete genome resequencing, whole exome sequencing, targeted genomic resequencing, transcriptome profiling, metagenomic analysis, methylome profiling, and chromatin immunoprecipitation sequencing (ChIP-Seq) (Shendure and Ji, 2008). After fundamentally altering the way genetic research was conducted, the next frontier for application of NGS technologies was diagnostics. A flurry of studies have appeared in the last couple of years that showcase the use of NGS technologies in identifying the genetic variation underlying some particularly challenging cases including several rare conditions (Ng et al., 2010). These studies have lead to the discovery of more than 50 new disease genes (Calvo et al., 2012). NGS based diagnostic studies have been used to study conditions representing much of the spectrum of potential medical disorders including metabolic, endocrine and other rare disorders (Bonnefond et al., 2010; Choi et al., 2009; Elliott et al., 2012; Haack et al., 2012; Leidenroth et al., 2012; Lim et al., 2011; Lupski et al., 2010; Ng et al., 2010; Ng et al., 2009), oncology (Duncavage et al., 2012; Hou et al., 2012; Ley et al., 2010; Thompson et al., 2012; Walsh et al., 2010; Xu et al., 2012), cardiology (Dames et al., 2010; Meder et al., 2011), prenatal and preconception testing (Chen et al., 2011; Chiu et al., 2011; Ehrich et al., 2011; Hahn et al., 2011; Lo, 2011; Lo et al., 2010; van den Oever et al., 2012), infectious diseases (Chen et al., 2010; Mellmann et al., 2011; Vogel et al., 2012), gastrointestinal disorders (Worthey et al., 2011) and HLA typing for transplantation (Erlich et al., 2011; Holcomb et al., 2011). In one of the earliest demonstrations of the use of NGS for diagnostics, Choi et al combined the Roche/NimbleGene array based whole exome capture technique with the Illumina NGS (Genome Analyzer) platform to make an unanticipated diagnosis of congenital chloride diarrhea in a suspected case of Bartter syndrome (a renal salt-wasting condition) by the detection of a homozygous missense mutation in the SLC26A3 gene (Choi et al., 2009). Around the same time, Sarah Ng and colleagues also made use of exome capture and Illumina based NGS to genetic variants implicated in Freeman-Sheldon Syndrome (a multiple congenital contracture syndrome) (Ng et al., 2009). Lupski and colleagues identified compound, heterozygous causative alleles of the SH3TC2 gene in the primary author's personal genome and in that of his family, sufferers of Charcot-Marie-Tooth neuropathy, an inherited demyelinating disease of the peripheral nervous system, by whole genome sequencing using Life Technologies's SOLiD platform (Lupski et al., 2010). Thompson et al demonstrated the use of single-molecule sequencing using the Helicos platform to detect mutations in the BRCA1 gene (Thompson et al., 2012). Recently Elliot and colleagues at Ambry Genetics demonstrated that the Ion Torrent Personal Genome Machine could be used to reliably identify all but one of the 23 ACMG/ACOG (American College of Medical Genomics / American College of Obstetrics and Gynecology) recommended mutations in the cystic fibrosis transmembrane regulator (CFTR) gene illustrating the suitability of the platform for this type of testing, albeit with caution (Elliott et al., 2012).

Next generation sequencing platforms are a mature set of technologies that are ready to be implemented into routine clinical practice. Continuing evolution of NGS platforms will further bring down costs, simplify the process flow, and improve the quality of data. these technologies are poised to have as profound an influence on routine patient care as they have had on basic research.

Potential and challenges for the use of NGS in routine clinical diagnostics

There are a number of characteristics of NGS systems that make them a perfect tool to be used in clinical diagnostics of genetic disorders. Sequence generation using NGS technologies is simple, cost effective - nearly four orders of magnitude cheaper as compared with Sanger sequencing (Bamshad et al., 2011), less labor intensive, and generates large amount of data in a relatively short amount of time, thereby making a large number of genes amenable to analysis within the typical constraints of medical diagnostics. This is particularly important in the case of several Mendelian disorders, many of which are caused by multiple genes. Routine genetic diagnostics almost always involve the interrogation of just one or a few genes due to limitations and costs of current molecular platforms, a problem which can be alleviated through NGS (Baudhuin et al., 2012).

The aim of clinical diagnostics is to provide clinicians with reliable and actionable information from individual patients in reasonable amounts of time and at acceptable costs. Since NGS technologies are significantly different from the traditional Sanger based sequencing techniques that clinicians have developed some amount of familiarity with, it will take a

certain amount of time and effort before they gain confidence and comfort with these platforms (Haspel et al., 2010; ten Bosch and Grody, 2008). The technical feasibility of using NGS for diagnostics has already been demonstrated via examples described above, however, several other issues must be addressed before NGS platforms can be incorporated into routine clinical diagnostics.

Analytical issues and selection of an appropriate NGS platform: Several characteristics of diagnostic platforms such as accuracy, precision, analytical sensitivity and specificity, and reportable and reference ranges must be understood before they can be incorporated into routine clinical use. Some of the technical factors that must be taken into consideration during the selection of an NGS platform for diagnostics use are read length, platform bias, accuracy, and total sequence output. Individual read-lengths vary for the various platforms – ranging from ~400bp on the Roche platform down to ~35-75bp on the SOLiD platform - and must be considered while selecting a platform for a clinical application. De novo sequencing applications, sequencing of regions with repeats or pseudogenes, and cases where the cis/trans linkage of polymorphisms need to be resolved are better suited on instruments with longer read lengths while other applications can yield good results even with the short read platforms. Similarly platforms may suffer from various sequencing biases. The Ilumina platform tends to under-represent AT rich regions, whereas pyrosequencing, semiconductor sequencing and some of the single molecule sequencing platforms are prone to homopolymer sequencing errors (strings of one type of nucleotide) leading to insertion/deletion (indel) errors (Baudhuin et al., 2012; Dames et al., 2010; Raca et al., 2010). Furthermore, inherent bias of the pre-analytical amplification step must also be considered. The accuracy of these platforms also varies compared with Sanger based sequencing and amongst themselves. In their comparison of the three bench-top NGS platforms - Roche 454 GS Junior, Illumina MiSeq, and Life Technologies' Ion Torrent PGM – Loman et al showed that the Illumina MiSeq has the highest throughput and fidelity, followed by the Roche GS Junior and the Ion Torrent PGM. However even with it's lower performance, the Ion Torrent PGM was more than sufficient to provide biologically relevant information on the enterohaemorrhagic E. coli O104:H4 which caused an outbreak of food poisoning in Germany in 2011 (Loman et al., 2012). Lastly, the large amount of data produced by NGS systems works both, for and against their routine use in diagnostics. When sequencing the whole genome of large animals and humans, the high throughput of NGS systems is useful, however, for diagnostics, since only a small region of the genome or a handful of genes are relevant to the disease, this might be overkill. In such cases, sequencing of whole genomes is wasteful and could lead to ambiguity in identification of underlying causes.

Sample preparation and library generation: Whole genome sequencing (WGS) is rarely needed for diagnostic purposes. Whole exome sequencing (WES) which captures only the coding region of DNA is a far more efficient way of sequencing for disease causing mutations because these regions account for only ~1% of the genome while containing ~85% of the known mutations (Ku et al., 2012a; Ku et al., 2012b; Ware et al., 2012). Targeted sequencing or resequencing is another approach to sequence only few genes or a genomic area of interest and may be assisted with target enrichment technologies from Roche/NimbleGen, RainDance Technologies, Agilent, Fluidigm and Febit (Hoppman-Chaney et al., 2010; Meldrum et al., 2011; Voelkerding et al., 2010; Voelkerding et al., 2009). Apart from the overall approach to sample preparation, there are several platform specific differences. In their analysis of E. coli E. O104:H4 described above, Loman and colleagues also highlighted the relative ease of sample preparation (automated template amplification) on the Illumina MiSeq platform as compared with the Ion Torrent and 454 GS Junior instruments.

Bioinformatics and data storage: The clinical utility of NGS is hindered more by our limited ability to interpret the data than with our ability to generate it in the first place (ten Bosch and Grody, 2008). As more sequence data is generated, more variants will be uncovered whose physiological significance will need to be determined. Due to higher error rate, short read length and greater depth, analysis of NGS data is very computationally intensive and complicated. Moreover, most of the analysis of NGS data is not yet standardized with multiple tools available and these tools open source, which may or may not be well supported. Most of the NGS systems can generate GBs of data per run. Storing, sharing and analyzing the data need high-end hardware, which can add to the capital and information technology (IT) support cost.

Ethics: There are several ethical and legal issues associated with the data generated from NGS-based diagnostics (Hastings et al., 2012; Ku et al., 2012a). Due to the scale of sequencing, unintended or incidental information of physiological relevance might be generated. Should this be revealed to the patient? What if this data has potential to affect relatives or unborn children? Should it be revealed to those potentially affected? How should this data be stored and who can it be shared with? Can it be used for research purposes? Prior to NGS analyses informed consent must be taken from patients and return and management of data must be codified. Frameworks must be set so that discrimination based on genetic information does not occur in areas such as access to health insurance and employment.

Education: It is hard enough for seasoned molecular biologists to keep pace with the developments of NGS, for clinicians, many of who don't have training in these areas, it is even harder (Haspel et al., 2010; ten Bosch and Grody, 2008). There needs to be improved physician training, patient education and improved communication between genetic counselors, research and clinical scientists, informaticians and clinicians.

Economics: Even though the per base sequencing costs of NGS platforms are significantly lower than Sanger based platforms, the cost of the instrumentation is still extremely high for most labs that perform routine molecular genetic diagnostics. Coupled with the fear that the rapid progress in technology development will quickly make current platforms obsolete, labs are reluctant

to invest in these platforms. Another issue is the cost of the test to the end user. While the NGS based tests are often cheaper than their cognate Sanger based equivalents, the cost for the cheapest tests is still on the order of $1000 and until insurance reimbursement systems are modified to handle NGS tests, their adoption into routine clinical settings is likely to be hampered.

Other issues: Finally few other critical issues, including guidelines for the reporting of results (providing meaningful data), regulatory compliance, and QA/QC frameworks need to be established.

Recent progress in the adoption of NGS into clinical practice

Instrumentation and protocols: Some of the newer NGS systems, such as Illumina's MiSeq, Roche's GS Junior and the Ion Torrent PGM, launched over the last year, address the excessive throughput issue while maintaining the benefits of the larger systems. These are all bench top sequencers with throughput in the range of 50 Mb to 1 Gb with sequencing protocols that can be run in a single day and lower cost than the larger NGS instruments. Additionally, these systems allow easier multiplexing, which means that multiple samples can be sequenced in a single run by adding a unique barcode sequence to each of the samples. This further reduces the cost of sequencing and allows smaller sequencing reactions to be run.

Emerging guidelines: For their part, several regulatory authorities and professional bodies have initiated efforts to ease the transition of NGS technologies into routine clinical use. The US-FDA have partnered with other federal organizations (NCBI, NHGRI and NIST) to create a regulatory path for new emerging technologies while the CDC and AMP have created working groups to address the issues and create guidelines. In their policy statement of March 27, 2012 the American College of Genetics and Genomics (ACMG) have stated that NGS technologies may be indicated in molecular testing of disorders with high degree of genetic heterogeneity and disorders where a specific genetic test is not available or current tests have been unable to arrive at a diagnosis. The policy statement also provides guidelines for pre-testing considerations (patient consent and education), clinical testing (lab with a board certified director), result reporting, and data management.

Commercial deployment: Finally, in the surest validation of NGS as a viable clinical diagnostic platform, several reputed laboratories around the world have begun to commercially offer genetic tests that utilize NGS platforms in place of standard Sanger sequencing (Baudhuin et al., 2012; Natrajan and Reis-Filho, 2011). Launched assays include tests for cardiomyopathies (GeneDx, Centrogene GmbH, Oxford Medical Genetics Laboratories, Harvard Laboratory for Molecular Medicine), mitochondrial disorders (GeneDx, Baylor Medical Genetics Laboratory, Medomics), X-linked intellectual disabilities (Ambry Genetics, Emory University), congenital muscular dystrophy and congenital disorders of glycosylation (Emory University), epilepsy, amyotrophic lateral sclerosis and Parkinson's disease (CeGaT, Germany), cystic fibrosis, sick cell anemia, Tay-Sachs disease, long QT syndrome and periodic fever syndromes (GeneDx), hereditary breast cancer, ovarian cancer and Li-Fraumeni syndrome (University of Leeds Institute of Molecular Medicine) and prenatal genetic tests (Good Start Genetics). Ambry Genetics, CeGaT, GeneDx, NCGR (National Center for Genome Resources, Santa Fe, NM) and others continue to rapidly increase the number of individual and panel-based NGS-genetic tests offered. Even though the turn-around times for these tests currently range from 4-16 weeks, they are sure to reduce once the sample numbers rise and the testing infrastructure scales.

Conclusions: Next generation sequencing platforms are a mature set of technologies that are ready to be implemented into routine clinical practice. Continuing evolution of NGS platforms will further bring down costs, simplify the process flow, and improve the quality of data. Once issues such as regulatory approval of instruments and reagent kits, standardized protocols, clear guidelines, improved software, increased automation and integration, physician education and close collaboration between teams of scientists, clinicians, genetic counselors, bioinformaticians, statisticians and platform vendor have been satisfactorily addressed, these technologies are poised to have as profound an influence on routine patient care as they have had on basic research.

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