Before discussing specific areas of drug discovery and development that we anticipate will be profoundly impacted by Generation II (or NextGen) sequencing, we first review current knowledge of nucleic acid variation, its functional correlates, and the current technical capabilities of Generation II sequencers. Deoxyribonucleic acid (DNA) molecules are composed of four nucleotide bases (the purines Adenine and Guanine, and the pyrimidines Thymine and methylated and non-methylated Cytosine). 3’-5’ phosphodiester bonds join the three billion bases of the human genome in 23 pairs of linear polynucleotide chromosomes (Chrs). Native DNA molecules are redundant, being formed through specific base pairing between purines and pyrimidines and resulting in two antiparallel, complementary strands. The two distinct copies of each Chr present in each diploid human cell form the basis for inheritance of traits and diseases. Thus, diploid human genomes contain six billion base pairs. Inherited variation between individuals has two principal components – approximately four million single nucleotide variants (single nucleotide substitutions [SNPs] and deletion insertion polymorphisms [DIPs], in a 10:1 ratio, comprising 1% of the genome) and approximately 10,000 structural variants (DNA stretches that are inserted, deleted, inverted or translocated [copy number variations, CNVs], comprising up to 10% of the genome]). Cells in an individual also develop genome variation, primarily somatic mutations (which are the primary driver in neoplasia), viral genome insertions and cytosine methylation (epigenetics). The human genome references which have been established comprise approximately 90% of the genome which can be cloned. Catalogues of somatic and meiotic genome variation remain very incomplete and understanding of the functional consequences of genetic variants is in its infancy. One of the first applications of Generation II sequencing has been maturation of reference genome sequences and variation catalogues, with compilation variant frequencies by sequencing of many human genomes (resequencing). These efforts are of critical importance for drug discovery and development since they form the reference sets against which disease- or drug-related changes are measured.
Approximately 25,000 protein-coding genes are interspersed throughout the genome as discontinuous sets of exons. Expression of genes occurs through transcription – the assembly of continuous, single stranded, messenger ribonucleic acid (mRNA) copies of exons, which are translated into proteins. Protein diversity largely results from multiple, alternative ways in which exons are assembled into mRNAs (alternative splicing)\(^\text{120}\). While exonic DNA, which accounts for 1-2\% of the genome, is the code of life, it is transcription that is the master controller of conversion of that code into cellular, organ and organismal activity. The emerging, primary function of the remaining 98\% of the genome is to control gene transcription precisely and ensure faithful copying of genomes during cell replication. It has recently been discovered that most of the genome is transcribed\(^\text{6-9}\). The non-exonic component of the genome accomplishes control primarily by transcription into a plethora of regulatory RNA types (including smRNA, ncRNA, tRNA, rRNA and antisense transcription) or by containing structural genomic DNA features that influence transcription and replication (both control elements and DNA methylation). The set of RNA molecules in a cell (transcriptome) is highly complex, dynamic and unique to that cell and its state at that moment. The activity of a gene in a particular cell and state can be inferred by measuring and integrating the abundance of its transcripts and regulatory RNAs. Since genes usually act in networks and pathways, integration of measurements of the abundance of sets of transcripts and regulatory RNAs is more biologically useful. Given the enormous complexity and dynamism of transcription, this is typically informative only when comparisons are performed on groups of samples that differ in only a few parameters. Thus experimental designs are a critical determinant of the translation of transcriptional measurements into biological knowledge. Another early application of Generation II sequencing is measurements of transcriptome components (digital transcript expression, DTE) with greater comprehensiveness, flexibility, sensitivity and precision than possible with array hybridisation by counting millions of random sequence tags\(^\text{121}\). DTE is emerging as an important area for drug discovery and development by identification transcriptional changes that were not apparent by array hybridisation, identification of novel transcript isoforms and identification of novel mechanisms of transcriptional control (such as small RNA), which may be amenable to intervention\(^\text{120,121}\).

Finally, given the current state of genome tools and knowledge, an immense current need is improved understanding of the functional consequences of genome and transcriptome features and variation (annotation). The third promise of Generation II sequencing is high throughput functional annotation by combining NextGen sequencing applications, such as genome resequencing and DTE, or methylome sequencing and DTE. For example, genome-wide association of SNP genotypes and mRNA sequencing enables identification cis- and trans-acting nucleotide variants that affect gene expression or splicing (eSNPs)\(^\text{10-20}\) (Kingsmore et al, unpublished). Identification of eSNPs is anticipated to be an important approach for translation of genome-wide association signals into knowledge of disease gene perturbations. In toto, these applications of Generation II sequencing are anticipated to have an immense impact on molecular diagnostics, pharmacogenetics, drug discovery and development\(^\text{21-25}\).
**Generation II DNA sequencing**

**Technology**
Generation II DNA sequencing uses Sanger chemistry and electrophoretic product separation to decode the primary structure (linear order of bases) of isolated (cloned) DNA molecules. It involves cycled, primer extension by incorporation of deoxynucleotide triphosphates (dNTPs) complementary to thousands of cloned, identical single-stranded DNA fragments, with reaction termination by dideoxynucleotide triphosphates (ddNTPs) lacking the 3'-hydroxyl group necessary to bond the 5'-amino group of the next dNTP. Thermostable DNA polymerase lacking 5'→3' exonuclease activity allows cycled primer elongation without degradation of templates or oligonucleotide products. Reactions use optimised ratios of dNTPs and four ddNTPs, each with a different label to generate nested sets of copies of the template of varying length that are labelled according to the terminal nucleotide. Electrochemical separation based on the basis of fragment length, together with label identification, allows determination of base composition. Generation I DNA sequencing instruments generate 96,800bp, high quality sequences per run at a cost of $1 per kb. Highly automated, Sanger production sequencing generates up to 2mb of sequence per instrument per day. Cost and the need to isolate individual clonal DNA templates (which greatly limits tag counting applications) are the principal limitations of high throughput Generation I sequencing. Sanger sequencing remains, however, the technology of choice for targeted, low and medium throughput, long, high quality sequencing.

**Generation II DNA sequencing technologies**
Currently, four Generation II sequencing approaches dominate the market and are established in the scientific literature and additional platforms are continuing to be released (Table 1, Figure 1). They include three sequencing-by-synthesis (SBS) platforms (pyrosequencing from Roche Applied Science XLR, the Danaher-Motion Polonator and the Illumina GA IIx) and the Applied Biosystems SOLiD 3.0 instrument, featuring sequencing-by-ligation (reviewed in 29-31). Generation II sequencing technologies have several features in common: First, all have similar DNA or cDNA template preparation procedures that obviate cloning vectors, propagation in a bacterial host, and clone isolation. Instead, DNA or cDNA templates are randomly fragmented, ligated to application-specific (and proprietary) adapters, and clonally amplified on a solid phase. This results in gigabase-per-day scalability and vastly improved ease-of-use. It also results in production of random (shotgun), rather than directed, sequences. Second, all sequencing reagents flow over a fixed array of template fragments, permitting digital image capture of fluorescent or chemiluminescent signals. This obviates the need for electrophoresis, another limitation of traditional sequencing. Third, they generate somewhat shorter, often paired reads with higher error rates than traditional sequencing. This increases the difficulty of accurate read alignment, assembly and variant detection (see below).

**Pyrosequencing using emulsion PCR**
Pyrosequencing was the first Generation II sequencing technology to become commercially available and, therefore, boasts the largest number of peer-reviewed application manuscripts (22-59) (Figure 1A). Templates are fragmented, adapters ligated and clonal amplification is performed by capture of single fragments on beads containing primers complementary to adapters and emulsion PCR. Following emulsion breaking, beads are deposited into microwells in a 1:1 bead:well stoichiometry. dNTPs are sequentially flowed over the wells, one at a time, and pyrophosphate, released when a nucleotide is incorporated by primer extension into the strand complementary to the template, is converted to ATP by sulfurylase, resulting in luciferase-generated chemiluminescence. Unincorporated nucleotides are degraded by apyrase and the dNTP flow sequence is repeated. Chemiluminescence intensities are algorithmically translated into basecalls. Sequential, identical bases produce an incremental signal increase. Advantages of pyrosequencing are relatively longer read lengths (500bp), availability of long paired reads (>2kb span) and applications reported in more than 300 peer-reviewed publications. Long paired reads are generated by circularisation of long DNA fragments with a biotin-labelled segment separating template ends. Following circle fragmentation and biotin-containing fragment capture, emulsion PCR is performed. The Roche XLR instrument currently generates 500mnb per run; however, pyrosequencing has a higher cost per gigabase than some other Generation II approaches, which limits its use to smaller projects, de novo sequencing, metagenomics, identification of genomic structural variants and targeted resequencing (in combination with Nimblegen capture arrays). In contrast, the Polonator (introduced in 2008) is a substantially lower cost platform, generating much shorter reads at low cost and using generic reagents, flexible chemistry and open-source informatics (72,82-84).
Sequencing-by-synthesis using bridge amplification (Illumina GA IIx)

The second commercially successful NextGen technology is marketed by Illumina, Inc and employed unique methods of solid-phase polony amplification ('bridge' PCR, described below) and fluorescently-labelled reversible chain terminators8,9,60-79 (Figure 1B). Templates are fragmented, adapters ligated and bridge PCR is performed to amplify polonies (polymerase colonies) anchored directly on a flowcell containing primers complementary to both adapters. Sequencing of resultant polonies is carried out in the flowcell using a primer complement oligonucleotide and four dNTPS with cleavable fluorescent labels and reversible terminators. Following the first cycle of SBS, images are captured, the blocked 3'-terminus and fluorescent tag are removed, and further cycles are performed. Paired reads are generated by flowcell denaturation following SBS using the first primer, followed by SBS using a primer complementary to the adapter at the other end of the polony fragment. The Illumina GA IIx instrument generates 420gb per paired run, dependent upon read length (36-106bp). Advantages of Illumina's SBS are lower cost per gigabase, large number of sequence tags (80 million per flowcell) for counting purposes, ease-of-use and practical applications established in more than 200 peer-reviewed publications. Principal applications of Illumina SBS relevant to drug discovery include genome resequencing and DTE.

Sequencing-by-ligation (Applied Biosystems’ SOLiD)

Originally described by Syndney Brenner85, sequencing-by-ligation (SBL) has been successfully commercialised by Applied Biosystems. The SOLiD 2.0 system (Figure 1C) employs nearly identical sample preparation as the Roche FLX. Following emulsion breaking, however, beads are attached to a flowcell similar to the method described for the Illumina GA IIx. SBL is performed by template-directed ligation of eight-base primers, labelled in one position and comprised of all possible sequence compositions, to the sequencing primer. Following image acquisition, the ligated primer is cleaved internally, exposing a 5’ phosphate, and four further rounds of ligation are performed. The sequencing primer and ligation product are then removed and a second, offset sequencing primer is annealed. This process is repeated, generating 35-50bp paired or singleton reads, respectively. The SOLiD instrument generates ~20gb per pair of slides. Advantages of SBL are low cost per gigabase, large number of sequence tags (120 million per slide) for counting applications, and very high accuracy (since each base is interrogated twice). Current limitations are the need to align to a reference database for basecalling (preventing use in de novo sequencing), and short read lengths. Emerging applications of SBL are genome resequencing and metagenomics, targeted resequencing.

Table I: Comparison of Gen I (AB 3730xl) and current Gen II sequencing technologies

<table>
<thead>
<tr>
<th></th>
<th>SANGER</th>
<th>AB SOLID 3.0</th>
<th>ILLUMINA GA IIx</th>
<th>ROCHE XLR</th>
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</thead>
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<tr>
<td>Max read length</td>
<td>1200</td>
<td>2 x 50</td>
<td>2 x 125</td>
<td>600</td>
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<td>Max reads per slide/plate</td>
<td>96</td>
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<td>100 million</td>
<td>1 million</td>
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<td>Max bases per slide/plate</td>
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<td>20 billion</td>
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<td>1 billion</td>
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<td>Run time for max bases</td>
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<td>Cost per GB</td>
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<td>99.9%</td>
<td>99%</td>
<td>99.5%</td>
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<tr>
<td>Ease of Use</td>
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<td>++</td>
<td>+++</td>
<td>++</td>
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<td>++</td>
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</tr>
<tr>
<td>Notable Applications</td>
<td>Targeted &amp; de novo sequencing</td>
<td>Resequencing, mRNA-seq</td>
<td>Resequencing, mRNA-Seq, methylation, ChipSeq</td>
<td>De novo sequencing, metagenomics, targeted resequencing</td>
</tr>
</tbody>
</table>
Emerging generation III DNA sequencing technologies

Helicos Biosciences’ HeliScope instrument and Pacific Biosciences Single Molecule Real Time (SMRT) technologies use a similar approach to the Illumina GA IIx but represent the first single molecule sequencing technologies, avoiding amplification-induced artifacts. By means of a melt-and-resquence method, Helicos eliminates an amplification step but requires a more expensive detection system, and is capable of sequencing multiple flowcells/slides simultaneously. The cost of the HeliScope and rapid advances in competitor platforms have deterred most customers to date. SMRT technology promises read-lengths that vastly exceed traditional Sanger sequencing, but is not yet commercially available.

Computational and bioinformatic implications of Generation II DNA sequencing

In Generation II resequencing and DTE applications, the sequence of short, random, clonal DNA fragments is decoded and either aligned to a reference database and/or overlapping fragment sequences are assembled into contiguous stretches (contigs). This is performed millions of times for each sample, in order to provide comprehensive coverage of all DNA species in that sample. Alignment and assembly are complicated by several technical considerations: Firstly, Generation II sequencing is relatively error prone (raw sequence accuracy of 98-99.5%), and, therefore, a consensus of redundant sequences is determined if nucleotide variants are to be distinguished from basecalling errors. By means of a melt-and-resquence method, Helicos eliminates an amplification step but requires a more expensive detection system, and is capable of sequencing multiple flowcells/slides simultaneously. The cost of the HeliScope and rapid advances in competitor platforms have deterred most customers to date. SMRT technology promises read-lengths that vastly exceed traditional Sanger sequencing, but is not yet commercially available.

Currently, DNA sequencing is used in four principal applications: 1) De novo sequencing to create near complete, first reference sets of sequences that render a species tractable to genomic investigation; 2) Resequencing in which genes, genome segments (eg quantitative trait locus intervals, chromatin immunoprecipitates), exomes (all exons), transcriptomes, genomes, methylomes (methylated cytosines) are sequenced in many samples, either to establish catalogs of variation or to undertake phenotyping associations or correlations. A typical Generation II sequencing instrument generates several terabytes of raw data per run and a typical Generation II sequencing project accrues tens to hundreds of gigabases of sequence. To put this in perspective, this corresponds to the entire DNA sequence in all public databases maintained by the National Center for Biotechnology Information (NCBI) as of April 2006. While the output of Generation II sequencing instruments is approximately quadrupling each year, similar gains in efficiency of data storage, computation and analysis have not occurred. Consequently, computational, software engineering and bioinformatic expertise and resources are rate limiting at present.

Current applications of second generation sequencing technologies

Currently, DNA sequencing is used in four principal applications: 1) De novo sequencing to create near complete, first reference sets of sequences that render a species tractable to genomic investigation; 2) Resequencing in which genes, genome segments (eg quantitative trait locus intervals, chromatin immunoprecipitates), exomes (all exons), transcriptomes, genomes, methylomes (methylated cytosines) are sequenced in many samples, either to establish catalogs of variation or to undertake phenotypes associations or correlations. A typical Generation II sequencing instrument generates several terabytes of raw data per run and a typical Generation II sequencing project accrues tens to hundreds of gigabases of sequence. To put this in perspective, this corresponds to the entire DNA sequence in all public databases maintained by the National Center for Biotechnology Information (NCBI) as of April 2006. While the output of Generation II sequencing instruments is approximately quadrupling each year, similar gains in efficiency of data storage, computation and analysis have not occurred. Consequently, computational, software engineering and bioinformatic expertise and resources are rate limiting at present.
marker – trait association studies; 3) RNA profiling (DTE), in which specific RNA species (small or large, coding or non-coding, sense or antisense) in sets of samples are converted to complementary DNA (cDNA) and sequenced in order to determine the composition and/or abundance of transcripts within samples (by tag counting) for marker – trait correlations; and 4) Metagenomics, in which DNA from samples containing more than one species are sequenced in order to determine the composition and/or abundance of species within samples (by tag counting) for environmental correlations. Of these applications, resequencing and DTE are anticipated to have the most profound and imminent impact on drug discovery and development and are the focus of the remaining discussion.

Resequencing
Resequencing refers to sequencing specific transcripts, genes, genomic regions or genomes from a number of specimens, usually in order to evaluate association between genotype (or haplotype) and a phenotype or trait. As with other systems biology experiments, it is divided into ‘Discovery’ and ‘Validation’ phases. In the Discovery phase a relatively small number of specimens are sequenced comprehensively and candidate associations are identified. In the validation phase, a few candidate associations are sequenced in many specimens. This design confers power for evaluation of statistical significance in the validation phase, provided attention is given to population structure. Hitherto, discovery studies were limited by cost and throughput to sequencing of small numbers of candidate genes or to genotyping of larger numbers of genes. A weakness of a targeted discovery phase is that experimentation is limited to the prevailing molecular knowledge (hypothesis-testing), rather than being systematic, and hypothesis-informing. During the past two years, genome-wide genotyping arrays have shown the immense power of systematic, hypothesis-informing approaches for identifying genetic associations in common, complex disorders (genome-wide association studies, GWAS, reviewed in 97). Resequencing applications of Generation II technologies are anticipated to further our understanding of the molecular basis of common diseases and pharmacophore response in at least three ways:

First, Generation II resequencing is being used to improve reference catalogs of genes, transcripts, nucleotide variants and structural variants. The ENCODE project, for example, is an NIH-sponsored initiative to catalog all human transcribed elements. The ENCODE project and similar efforts have indicated that alternative splicing may result in as many as 500,000 mRNA species in mammals. Of these, only one-tenth are currently annotated (present in RefSeq transcript or AceView databases), and 10,658 of the latter are putative or provisional genes that have been identified informatically from the human genome sequence (‘LOC’, ‘FLJ’ and ‘C_orf’ genes). These projects have also identified new classes of transcriptionally active regions (TARS) of the human genome that are poorly described or understood remain undefined. Second, Generation II mRNA or rRNA-depleted RNA resequencing enables comprehensive identification of splice isoforms and TARS. Incomplete or inaccurate reference catalogues can lead to erroneous conclusions that impact drug discovery and development. For example, numerous studies have sought associations between behavioural phenotypes and tryptophan hydroxylase (TPH), which is important in serotonin metabolism. It is now known that the gene (TPH1) examined in many of these studies is not expressed in serotonergic neurons. Examples of large Generation II resequencing efforts to improve reference catalogs of nucleotide variants and structural variants are the 1000 Genomes Project and the Personal Genome Project.
in drug discovery to translate signals identified in GWAS into drug targets. Array-based GWAS are predicated on identification of associations between complex traits and ~1 million random, common (minor allele frequency >5%) SNP genotypes or imputed haplotypes (by comparison with genome maps of SNP-tagged haplotype blocks, 108) in thousands of cases and controls (reviewed in 97). During the past two years, the effectiveness of GWAS for identification of replicable, common, susceptibility variants in complex diseases has been unequivocally established97. However, many GWAS signals are located in genomic regions lacking annotated genes or with multiple genes. Even GWAS signals located adjacent to a single gene do not usually indicate the causal variant or functional consequence of the variant. Generation II resequencing is being used, in combination with target enrichment strategies, for GWAS interval resequencing and for high throughput functional annotation of nucleotide variants. For example, genome-wide association of SNP genotypes and mRNA sequencing enables identification of cis- and trans-acting nucleotide variants that affect gene expression or splicing (eSNPs)10-20 (Kingsmore et al, unpublished). Identification of eSNPs is anticipated to be a principal approach for translation of genome-wide association signals into knowledge of disease gene perturbations. This approach, termed Genome-Tissue Expression Analysis (GTEx), is likely to be the subject of a large NIH effort in the near future.

Third, Generation II resequencing is being used in drug discovery to directly identify variants associated with common traits by non-hypothesised-directed sequencing of many human genomes (resequencing). For example, Generation II mRNA resequencing has shown utility for identification of somatic mutations in cancers38,54,55,59,104. One recent project sought to identify novel, putatively damaging, somatic mutations expressed in malignant pleural mesothelioma (MPM) surgical specimens using Generation II resequencing104. MPM is a prototypic environmentally-induced cancer (due to asbestos) and does not exhibit mutations in established oncogenes. 1.7gb of cDNA sequence was generated from six surgical specimens (four microdissected MPMs, one lung adenocarcinoma and one normal pleura) using Roche pyrosequencing. A web-based software system was used for sequence management, pipelining, visualisation, and statistical analysis (Figure 2). Singleton pyrosequencing reads aligned to ~16,000 genes and identified ~1,300 eSNPs in each sample, of which ~1,000 were known, inherited, common variants. 69 novel, putatively damaging mutations were identified, of which 15 nsSNPs were found in tumour but not normal cosogenic tissue, reflecting somatic mutations, RNA editing, LOH due to chromosomal deletions and epigenetic silencing, including Chr X inactivation. Three of seven somatic nsSNP mutations were identified in additional MPM tumours. In response to proofs-of-concept, such as this, the International Cancer Genome Consortium proposes to generate genome sequences from 500 patients with each of 50 types of cancer in the next decade109. For inherited diseases and traits, array-based GWAS alone are insufficient to fully elucidate their genetic basis. Specifically, array based GWAS have limited effectiveness at recombination hotspots (~20% of the genome) or recent or ‘private’ mutations (which are anticipated to be important in the ‘common disease – rare alleles’ hypothesis110). For example, recent mutations and genetic heterogeneity have been suggested to be important in causality of schizophrenia111, prompting our group to launch the Schizophrenia Genome Project112. This rationale is also driving the EUVADIS project, which proposes to sequence the human genomes of 1,000 European citizens with 10 common disorders113. Since 20-fold coverage of the haploid human genome (60gb) still costs ~$100,000 with Generation II sequencing technology, a major current focus of resequencing is on mRNA or comprehensive exon (exome) selection, which comprise approximately 2% of the genome but conceptually enables delineation of the vast majority of biologically relevant nucleotide variation in human cohorts32,64,71. Exome sequencing is being pursued in several disorders as part of the NIH medical resequencing programme and by the Psychiatric Genomics Center at Cold Spring Harbor Laboratory134,135.

The pharmaceutical industry is becoming increasingly involved in these type of projects, particularly in therapeutic areas of greatest interest to individual organisations. Ultimately, Generation II (and emerging Generation III) sequencing is anticipated to play an important role in identification of novel drug targets, establishing genetic profiles related to drug response, confirming pharmacogenetic associations and accelerating the development of new drugs and companion diagnostic tests.

RNA profiling
RNA profiling is a well established and ubiquitous tool for target discovery, target validation and biomarker development. Commonly profiled RNA types are polyadenylated messenger RNA and small RNA molecules (smRNAs). The latter are 20-30 nucleotide molecules that block translation or induce
degradation of target mRNAs. smRNAs include microRNAs (miRNAs) and trans-acting small interfering RNAs (tasiRNAs) that regulate mRNA stability and translation, and small interfering (si) RNAs that cause post-transcriptional gene silencing and are important in cytosine DNA methylation. Gene silencing is important to proper cellular development and proliferation. As an epigenetic modification, RNAi may be reversible and is a promising therapeutic target. The goal of expression profiling experiments is typically to understand the dynamics of transcript network and pathway abundance between states or temporal events. Usually, this involves the identification of sets of transcripts whose expression differs as an external parameter is varied (time, treatment, dose, genotype, etc). The technology specifications for comprehensive gene expression profiling experiments are well established:

- Ability to measure most transcripts simultaneously (requiring a six log10 dynamic range).
- Small mass of RNA sample input (<1µg total RNA).
- High throughput (hundreds of samples).
- Precision (coefficients of variation of <10%).
- Moderate price.

The most prevalent technology for transcript profiling is array hybridisation. It has limitations of closed architecture (features are constrained by available exon sequences at time of array design), ratiometric measurement (rather than absolute mass measurement), limited sensitivity, limited dynamic range and relatively high imprecision. It is becoming clear that the ~25,000 mammalian107 genes encode more than an order of magnitude greater number of transcripts via alternative initiation, splicing and termination6-9,10,82,85,98-103. Thus Generation II sequence-based RNA profiling provides absolute measurement of gene abundance with a sensitivity that is determined by the number of reads generated per sample. An economically practical level of sensitivity is a single channel of Illumina SBS or AB SBL (ie 10 million reads), Generation II sequencing is approximately 2.5 x log10 more sensitive than array hybridisation120, detecting up to one RNA molecule per 30 cells (Hayashizake et al, personal communication). A variety of Generation II RNA profiling approaches have been described, including random mRNA sequencing, rRNA-depleted total RNA sequencing, smRNA profiling, 3’ end tagging, sense- and antisense-5’ cap tagging, paired read profiling8,9,33-35,37,39-41,45,46,48-50,52,53,56,58. These approaches allow profiling of specific transcript subsets or testing of specific hypotheses. Other potential benefits of Generation II sequencing for RNA profiling include: Sequence verification for each measurement, allowing discrimination of paralogs with high sequence similarity; Detection of all transcripts and isoforms, known and novel; utility in any species, whether sequenced or not; low run-to-run imprecision (~3.5%); absence of interference from abundant transcripts (eg globin); linear dose-response characteristics; decreased dependence on RNA integrity and, extensibility to concomitant detection of nucleotide and structural variation104,120,121. The only drawback to Generation II-based RNA profiling is approximately a three-fold increase in cost. As this cost differential decreases, it is anticipated that Generation II sequencing will largely replace array hybridisation as the technology of choice for RNA profiling. RNA profiling is the first Generation II sequencing application which is a mainstream component of pharmaceutical discovery and development and most pharmaceutical companies are evaluating and implementing Generation II sequencing technologies for this application at present. Excitingly, pilot studies are identifying many biologically relevant gene expression differences that are undetectable by array hybridisation. Proofs-of-concept in drug discovery applications are in preparation for publication.

Conclusions
Several Generation II sequencing technologies have become available within the last three years, marketed currently by Roche Applied Science, Illumina, Applied Biosystems Danaher Motion, and Helicos. Generation II technologies are largely distinguishable from Generation I sequencing by enabling a variety of functional genomics applications and by up to 20,000-fold greater cost-effectiveness. Established applications of Generation II sequencing are de novo genome and transcript sequencing, resequencing of genes, genomic segments, exons (exome), transcripts (transcriptome) and genomes, digital expression profiling of several classes of transcripts (including mRNA, 3’ mRNA end-tags and smRNA) and methylome and chromatin immunoprecipitation sequencing. Currently, Generation II sequencing technologies are having a profound impact on basic and translational research, creating improved understanding of human genome variation and transcriptome complexity, improving catalogs of genes, splice isoforms and functional nucleotide variants and identifying
new molecular mechanisms underpinning disease development and drug response. The pharmaceutical industry is evaluating the utility of Generation II sequencing for digital transcript expression and resequencing related to target discovery and validation and biomarker development, particularly related to oncology and biotherapeutic development.

The advent of molecular cloning and Generation I sequencing created the biotechnology and biopharma industries. Generation II sequencing technologies are becoming established in several applications and enabling mRNA, exome and genome resequencing projects of unparalleled scale. Generation II sequencing is anticipated, in combination with GWAS, to lead to a new level of understanding of molecular mechanisms, molecular staging and subsetting of common diseases and drug responses. The pharmaceutical industry is starting to evaluate the potential impact of these technologies on drug discovery, drug development and personalised medicine. Within the next two years, Generation II sequencing technologies are anticipated to replace array hybridisation as the gold standard technology for RNA profiling. In the next five years, the fruits of these efforts will be novel drug targets for common diseases, identification of molecular biomarkers that inform clinical trials of investigational new drugs and discovery of companion molecular diagnostic tests that allow altered reimbursement of targeted therapies to patient subsets.

In the longer term, Generation III sequencing technologies are anticipated to decrease the cost of genome sequencing to $1,000. Generation III sequencing technologies will allow genome sequencing of 5% of the US population. Personal genome sequences will establish personalised medicine in which medical care will routinely include genome-based inference. In tandem, molecular diagnostic testing is anticipated to continue to be the largest growth segment of the diagnostics industry, Generation II and Generation III sequencing technologies are thus anticipated to become key enabling technologies for drug approvals that reflect segmented efficacy for groups of patients with labels referring to the appropriate companion diagnostic. Such targeted therapies are anticipated to reduce attrition of new, innovative medicines and improve reimbursement.

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