EXPERT REVIEWS

Progress and prospects of next-generation sequencing testing for inherited retinal dystrophy

John (Pei-Wen) Chiang*[1], Tina Lamey[2], Terri McLaren[2], Jennifer A Thompson[2], Hannah Montgomery[2] and John De Roach*[2]

[1]*Casey Eye Institute Molecular Diagnostic Laboratory, Oregon Health Science University, Portland, OR, USA*
[2]*Australian Inherited Retinal Disease Register and DNA Bank, Department of Medical Technology and Physics, Sir Charles Gairdner Hospital, Western Australia 6009, Australia*
Authors for Correspondence:
chiangj@ohsu.edu;
john.deroach@health.wa.gov.au

Next-generation sequencing, also known as massively paralleled sequencing, offers an unprecedented opportunity to study disease mechanisms of inherited retinal dystrophies: a dramatic change from a few years ago. The specific involvement of the retina and the manageable number of genes to sequence make inherited retinal dystrophies an attractive model to study genotype–phenotype correlations. Costs are reducing rapidly and the current overall mutation detection rate of approximately 60% offers real potential for personalized medicine and treatments. This report addresses the challenges ahead, which include: better understanding of the mutation mechanisms of syndromic genes in apparent non-syndromic patients; finding mutations in patients who have tested negative or inconclusive; better variant calling, especially for intronic and synonymous variants; more precise genotype–phenotype correlations and making genetic testing more broadly accessible.

The diagnostic challenges of inherited retinal dystrophies

Inherited retinal dystrophies (IRDs), characterized by retinal involvement as the main clinical presentation, comprise many overlapping conditions, such as retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), and Stargardt disease. The disease incidence for RP is 1/3500–4000 people in the USA and Europe; for Stargardt disease, it is 1/8000–10,000 individuals and for LCA it is 2–3/100,000 newborns [1].

In most cases, retina is the only affected tissue (non-syndromic conditions). However, additional tissues/organs can be involved (syndromic conditions) [2], and it can be difficult to assign a precise clinical diagnosis of non-syndromic versus syndromic conditions. For some syndromic conditions, such as juvenile

Batten disease, retinal dystrophy is the earliest clinical sign, and variable phenotypes are not uncommon. In addition, the presence of some clinical signs can be co-incidental, such as the presence of both RP and hearing loss in patients not affected with Usher syndrome, which may reflect the relatively high incidence of these diseases'. Even for non-syndromic IRDs, assigning a definitive clinical diagnosis can be difficult. Clinical presentations can be overlapping and/or progressive, and it may take several decades for the full disease spectrum to manifest. Moreover, intrafamilial variability is not uncommon, to the extent that family members with the same causative mutation(s) can show different phenotypes. Mutations in particular genes can be causative for both non-syndromic and syndromic conditions. For instance, *CEP290* mutations have been identified in patients with LCA (non-syndromic) [3],

Bardet-Biedl (BBS) [4], Joubert [5], Meckel [6], and Senior-Løken [7] syndromes, while *USH2A* mutations can manifest as either Usher syndrome type II or non-syndromic RP [8].

The large number of genes identified in IRDs (locus heterogeneity; >200 genes) [9] has complicated molecular diagnosis, and thus, the clinical utility of target sequencing only the more common RP genes is limited. In fact, the overall mutation detection rate for many IRD-associated genes is very low, except for conditions such as Stargardt disease and achromatopsia where disease-specific panels are successfully utilized due to the limited number of genes involved. Furthermore, even though certain genes implicated in IRDs are associated with specific inheritance modes (autosomal recessive, autosomal dominant, or X-linked), the difficulty in reliably establishing an inheritance mode for certain patients makes targeted gene sequencing less effective in these instances. Allelic heterogeneity also adds complexity. Although over 4000 mutations have been characterized in ~200 IRD-associated genes [10], in general, their prevalence is low.

The existence of multigenic inheritance patterns and genetic modifiers further obscure molecular diagnoses, with evidence for phenotypic variability due to complex genetic architectures emerging for IRDs. In the case of Usher syndrome, *PDZD7* is implicated as a retinal disease modifier, and a contributor to digenic disease in combination with mutations in *USH2A* and *GPR98* [11] and also *DFNB31* [12]. The interaction of the BBSome components (complex of 7 BBS proteins) with CEP290 in *CEP290*-related ciliopathies amply illustrates the complexity of multigenic traits, and partly explains both inter/intrafamilial phenotypic variability and phenotypic overlap among distinct syndromes [13].

Locus and allelic heterogeneities therefore limit the effectiveness of targeted strategies, as many pathogenic variants go undetected. Taken together with multigenic inheritance patterns and genetic modifiers, sequencing all known genes implicated in IRDs simultaneously seems the best current approach for the definitive molecular diagnosis of IRDs, and next-generation sequencing (NGS) has revolutionized this field. This review will compare NGS with other molecular diagnostic strategies for IRDs with a particular focus on the challenges ahead.

Sanger sequencing versus NGS

Before the arrival of NGS, when Sanger sequencing was routinely performed, common approaches to molecular diagnosis included: targeted sequencing of selected genes; screening known mutations in a large number of IRD-associated genes by array; and screening mutations by PCR and single strand conformation polymorphism. Targeted NGS of IRD-associated genes was established in 2012 [14–17], and in just 3 years NGS has become the preferred testing method for IRD patients.

While the unparalleled specificity of PCR and the 'to-see-is-to-believe' method of Sanger sequencing have long been the benchmark for molecular diagnosis of IRDs, the arrival of NGS has dramatically changed this field [14]. The Illumina NGS sequencing platform is becoming the *de facto* method for laboratories working on rare genetic diseases, with target enrichment one of the most critical steps. The introduction of hybridization-based enrichment methods, such as Agilent Sure-Select reagents, has markedly simplified enrichment procedures [15]. Hybridization baits from any number of genes can now be ordered from several vendors. However, serious issues have been created by this advancement.

The gold standard for target enrichment in the era of Sanger sequencing has been PCR-based, and it offers unmatched target specificity. However, three major factors limit its role in this NGS 'gold rush':

- Primer design has mostly been a slow, labor-intensive process. Any attempt at automation requires synthesizing redundant primers to improve coverage. The rule of thumb for automation, especially with respect to complicated biological systems, is that it can never be perfect. This is true for PCR primer design and for the designing of baits for the capture method. Making redundant primers when targeting large numbers of genes can significantly increase costs. Even with extra primers, 100% coverage is still impossible.
- Allele dropout is a concern, and is mostly a random event. The presence of SNPs in the primer regions may increase the chance of allele dropout.
- Manual PCR set-up is laborious and error-prone, and primer maintenance and storage requires additional effort. When large numbers of amplicons are sequenced, the task becomes increasingly labor intensive and less manageable.

The arrival of a liquid-phase, hybridization-based method [18,19], and other modified methods, have eliminated the preceding first and third factors. The single tube method can streamline enrichment, and is especially desirable for clinical laboratories. However, users should be aware of its limitations; non-specific enrichment and enrichment of homologues and pseudogenes cannot be avoided, and the on-target sequencing rate is lower than the more specific PCR-based method. Therefore, more sequencing coverage is required to identify mutations. In essence, the unparalleled sequencing power of NGS can sequence indiscriminately. Both non-specific and false-positive variants are then filtered out during data analysis. This approach of indiscriminate sequencing followed by variant filtering is at the core of hybridization-based enrichment methods. However, to increase coverage of target regions to, for example, 95%, the sequence depth is usually lowered to, approximately, 20×. Based on our experience, when the sequence depth is lowered, additional false-positive variants appear. To eliminate these, a more stringent filtering threshold is implemented that increases false-negative rates. We believe this is one of the most common causes of false negativity. However, there is currently no consensus as to what the best sequence depth rate should be. To add complexity, Illumina offers several different NGS sequencers (MiSeq, NextSeq, and HiSeq). The balance of cost/run time with test specificity/sensitivity is a matter of judgment for each laboratory.

Whole exome sequencing

About 5 years ago, WES was heralded as the 'panel killer' and promoted as the one-stop testing platform for rare genetic diseases. This expectation seemingly stemmed from widespread frustrations associated with testing rare genetic diseases, given that clinical diagnoses are not infrequently incorrect [20–22]. For a patient with differential diagnoses, running entire panels for multiple diseases can be cost-prohibitive. Therefore, the arrival of a simple, comprehensive testing step (WES) was particularly attractive. The uneven performance of WES, however, has limited its clinical utility, and it was clearly an intermediary before whole genome sequencing (WGS). From a cost perspective, WES was favorable, but this is diminishing as WGS is becoming cheaper. At this time, limiting analysis to less than whole exomes, such as medically relevant exomes, is popular for some clinical testing. In one published study, IRD-limited exome analysis outperformed WES [23]. The current consensus about WES is that it can be an alternative approach after exhausting the more specific panel testing. The rapidly declining cost for WGS may see it replace WES as an alternative approach in the foreseeable future.

Panel sequencing still makes sense today but whole genome sequencing is coming

Is the US$1000 genome a reality [24]? The first public record of US$1000 genomes was in December 2001 [25]; when it will finally become a reality in clinical practice is purely speculative at this time. Following the history of WES in the clinical market, it is safe to say that clinical sequencing is at least several years behind research sequencing. It is difficult to argue that WGS is a viable or sustainable business model currently for the clinical market, even with the introduction of HiSeq X 10 from Illumina. The true cost from a stand-alone and sustainable, commercial molecular diagnostic laboratory is much higher than the advertised cost of sequencing a whole genome today. Furthermore, it is unclear whether most 'junk DNA', which constitutes >90% of our genome, has major biological functions [26]. For conditions such as IRDs, clinical presentations are relatively specific and the total number of genes involved is currently approximately 230 [9]. Sequencing all known genes (at least the coding regions) implicated in IRDs simultaneously will certainly reach the US$ 1000 milestone in the near future. Ultimately, sequencing the whole genome and extracting specific data for analysis will make more economic sense for IRDs.

Smartpanel – a high throughput & fast enrichment method based on simplex PCR

Currently, PCR still offers unmatched specificity for target enrichment. However, due to the large number of IRD-associated genes, sequencing all known genes simultaneously will require thousands of PCRs per patient, and is thus unsuitable in the clinical setting. Multiplex PCR can potentially reduce the workload: however, designing a multiplex PCR platform on this scale is difficult to achieve as PCRs compete less favorably in a multiplex environment. Recently, a lab-on-a-chip (SmartChip) platform was developed to run up to 5184 simplex PCRs [27]. The PCR primer set is printed in each individual well, and duplicated to avoid allele dropout. SmartChips are manufactured and packed individually and stored at room temperature, simplifying PCR set-up to the addition of reagents onto the chips, with no requirement for primer storage or maintenance. We have developed several disease-specific SmartPanels based on the SmartChip platform. For example, our RD Smart-Panel (version 7) covers 233 genes with 4658 simplex PCRs duplicated on two SmartChips. On average, six low coverage regions (<100× sequencing depth) occur, which are compensated for by manual PCR and Sanger sequencing. Our Stargardt/macular dystrophy panel covers 10 genes with 551 simplex PCRs duplicated on one SmartChip. Entire coding regions from all 10 genes and the reported deep intronic mutations in *ABCA4* are 100% covered (>100× sequencing depth). This new approach offers unprecedented advantages over traditional PCR set-up, multiplex PCR set-up, and capture/hybridization-based methods. A simple, specific, comprehensive, reproducible, and reliable platform is important to improve mutation detection rates. Therefore, SmartPanels are a fast, reliable, and less expensive first-line screening tool.

There are three major limitations for SmartPanel analysis: insertion/deletion mutations cannot be reliably detected by PCR enrichment unless deletions are homozygous; mutations outside coding/target regions are not sequenced unless specific primer sets are designed to amplify mutation-specific regions (e.g., *ABCA4* deep intronic mutations); and the extremely repetitive region in *RPGR* ORF15 cannot be sequenced by specific PCR amplification of <500 bp amplicons. Array CGH analysis for the detection of insertion/deletion mutations and specific methods to sequence *RPGR* ORF15 are therefore still required for comprehensive testing.

The clinical utility of molecular diagnoses for IRD patients is becoming clearer

Until recently, the clinical utility of molecular diagnoses was limited owing to factors including: the absence of impending therapeutic options; the time and cost of testing methods; and the use of hypothesis-driven approaches, for which 'success' was impeded by the limited number of known IRD-associated genes, the variable confidence in diagnoses and/or inheritance patterns and the difficulty in interpreting variant pathogenicity.

IRDs are particularly amenable to gene therapy, and today many clinical trials are in progress, for which knowledge of the causative gene is required. This is also true for family planning options, such as pre-implantation genetic diagnosis. Moreover, identifying the disease mechanism in early stage disease, and in syndromic cases where IRD is the first clinical sign, can reduce medical costs and patient anxiety by avoiding unnecessary testing, resulting in better patient management. The molecular diagnosis of IRD patients therefore represents an excellent model for cost savings, and will undoubtedly become increasingly popular.

Table 1. Comparison of molecular diagnostic strategies for inherited retinal dystrophies.

Approach	Method		Advantages	Disadvantages	Detects	
					Novel variants	*Novel genes*
Hypothesis driven	SSCP		Low cost	Small DNA fragments only; optimal fragment size 150–200bp. Requires confirmation by Sanger. Sensitivity (mutation detection rate) less than Sanger sequencing, at ~80–90%	✓	✗
	Microarray		Low cost. Detects mutations across multiple genes	Only known mutations can be identified	✗	✗
	Sanger sequencing		Well-established method for clinical testing	High cost; cannot be scaled up. Large/ heterozygous insertion/deletion mutations cannot be detected (but smaller homozygous insertion/ deletion mutations can be detected). Mutations outside the sequenced regions cannot be detected	✓	✗
	Targeted NGS	Disease-specific panel	Low cost; massively paralleled sequencing of selected genes simultaneously	Less cost-effective when clinical diagnosis is not certain. Typically longer turnaround time. Detection of insertion/deletion mutations can be a challenge. Mutations outside the sequenced regions cannot be detected. Repetitive regions may not be covered well	✓	✗
Non-hypothesis driven	Panel NGS	Standard RD panel	Broader target region than disease-specific panels. More specific than WES; offering higher and typically better coverage of the genes of interest	Detection of insertion/deletion mutations can be a challenge. Mutations outside the targeted regions cannot be detected. Repetitive regions may not be covered well. Coverage varies	✓	✗
		Smart RD panel	Unmatched specificity by simplex PCR; a simple and fast method of target enrichment	Insertion/deletion mutations cannot be detected. Mutations outside the sequenced regions cannot be detected. Repetitive regions may not be covered well	✓	✗
	Whole exome NGS	WES	An acceptable choice when clinical diagnosis is uncertain. Lower cost than ordering multiple panels	Coverage is uneven. Incidental findings can create ethical dilemmas. Detection of insertion/ deletion mutations can be a challenge. Mutations outside the captured regions cannot be detected. Repetitive regions may not be covered well	✓	✓
	Whole genome NGS	WGS	No target enrichment bias	Repetitive regions may not be covered well. Data interpretation is a major bottleneck. Years away from truly affordable clinical testing. Incidental findings can create ethical dilemmas; should the entire data be returned back to patients? Data delivery and storage issues	✓	✓

NGS: Next-generation sequencing; SSCP: Single strand conformation polymorphism; WES: Whole exome sequencing; WGS: Whole genome sequencing.

In the past few years, the generation of fast, powerful, and cost-effective gene discovery and mutation detection methods, in addition to the development of variant pathogenicity assessment tools, has markedly increased the clinical utility of molecular diagnoses. With >230 IRD-associated genes identified [9], the non-hypothesis-driven approach of testing all known IRD-associated genes by targeted-capture NGS is now the method of choice, often revealing unsuspected disease mechanisms [16].

Correspondingly, the simultaneous sequencing of a large number of genes has resulted in increased detection of variants of unknown significance, which require interpretation for clinical purposes. The development of databases such as ClinVar [28] and WES variant allele frequency by ExAc Browser [29] have gradually improved variant interpretation. Similarly, programs such as SIFT [30], PolyPhen-2 [31], and NNSPLICE [32] are now widely used to predict the influence of a variant on protein localization, structure, and/or function. However, *in silico* predictions are not always consistent with functional studies and, despite recent advances, pathogenicity assessment remains challenging, particularly for hypomorphic, synonymous and non-coding variants. Ultimately, better tools are required, as well as improved knowledge of gene regions and, in the case of WES and WGS, genes of unknown significance.

Despite the potential benefits, several ethical issues remain. The generation of new guidelines and legislation has not kept pace with the rapid advances in mutation detection discovery. For example, there are currently no restrictions on testing asymptomatic minors for later onset conditions. In this context, incidental findings are also of considerable concern, particularly for WES and WGS, and increasingly for specific panel testing with the progressive addition of syndromic genes.

At the laboratory level, while there are arguments against strict regulation in some contexts (such as for rare genetic conditions where not enough controls exist to validate tests), a general consensus and additional guidelines are required. The recent release of reference DNA by National Institute of Standards & Technology (NIST) is a good starting point, enabling clinical laboratories to sequence the same reference DNA for comparison. However, as each clinical testing laboratory still sets its own criteria, including for coverage, cutoff threshold, variant filtering, and variant calling, more work is clearly required to maximize the potential of molecular diagnoses for greater clinical utility.

Expert commentary

Overall, the progress in molecular diagnosis of IRDs is unprecedented, with the arrival of NGS opening a new frontier. WES, WGS, and specific panel sequencing each have their own pros and cons (TABLE 1), and there is still no single platform offering the ideal solution today, especially when cost is factored in. Insurance coverage is not available in many cases, mainly because the clinical utility is yet to be proven [33]. Even with this limitation, an exciting trend is emerging. The non-hypothesis-driven approach has already generated some exciting results. *CLN3*, the gene implicated in juvenile Batten disease, is

also mutated in some patients with non-syndromic IRD [34]. Similarly, *BBS1*, implicated in Bardet–Biedl syndrome, is also mutated in some patients with non-syndromic IRD [35]. It is probably true that most of the common IRD-associated genes are already identified, and some patients may in fact have mutations in recognized syndromic genes, which have never been analyzed in non-syndromic patients. Alternatively, mutations can also be present outside typically sequenced regions, such as the deep intronic *ABCA4* mutations [36,37], or cannot be identified through a PCR-based approach, such as insertion/deletion mutations. Therefore, sequencing broader and deeper will likely improve mutation detection rates. Moreover, unconventional mutation mechanisms relating to genetic modifiers, and multigenic and multiallelic inheritance will need to be more systematically studied. Nonetheless, even with the current mutation detection rate of between 50 and 60% [23,38,39], this is already a very significant improvement from only a few years ago.

Five-year view

The field of molecular diagnosis, in general, is fluid and highly competitive. Any attempt to predict the status 5 years from now will most likely be somewhat off the mark. It is likely that in 5 years, if not sooner, WGS will be where WES stands today. In our opinion, WES served as an intermediary between the unleashing of sequencing power and the full implementation of WGS, and we predict it will most likely lose its appeal as a diagnostic platform. PCR-based enrichment may still have a role to play, but the extraction of target genes will likely happen after sequencing the whole genome (selective analysis of a set of genes), unless there is a significant pricing advantage for PCR enrichment. US$ 1000 IRD genomes will arrive, even if not through WGS.

Technological advancements will most likely be the catalyst for lowering costs and improving accessibility of testing. Large-scale genotype–phenotype correlation studies will be required to unravel complex genetic mechanisms for some IRD patients. The concept of a single-gene disease will be challenged, and many genetic modifiers will be identified. As a result, the value of molecular diagnosis as a first-line diagnostic tool may be truly appreciated, especially in less developed countries with limited retina specialists. Even in developed countries, the many benefits of molecular diagnosis are likely to elevate it to a standard medical diagnostic procedure for IRDs.

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Key issues

- Inherited retinal dystrophies (IRDs), complicated genetic conditions with specific clinical presentations and continually enlarging number of genes identified, are coming to the center stage as a model to showcase the potential of personalized medicine.
- The molecular diagnosis of IRDs has come a long way, especially with the rapid progress experienced after the arrival of next-generation sequencing, which is becoming a key tool for the clinical diagnosis of IRDs.
- The non-hypothesis-driven approach, by sequencing all known genes implicated in IRDs simultaneously, offers the best opportunity for genotype–phenotype correlation studies.
- The finding of multiple mutations in different IRD-associated genes is not uncommon, which challenges the simple concept of single-gene diseases in IRDs.
- Multigenic and multiallelic inheritance patterns and genetic modifiers will become the new frontiers in the study of disease mechanisms of IRDs.
- Deep intronic mutations and mutations outside the typically sequenced regions may account for some of the missing, second mutations in autosomal recessive conditions.
- Most of the common IRD-associated genes have likely already been identified and syndromic genes, which have never been sequenced before in non-syndromic patients, may harbor many of the missing mutations.
- A true US$1000 genome is still many years away but the US$1000 IRD genome (at least the coding regions of all the known IRD-associated genes) is within reach.
- The value of molecular diagnosis as a first-line diagnostic tool for IRDs has significant potential in less-developed countries, where clinical services may be limited.
- Even in developed countries, the benefits of more timely and definitive answers will make molecular diagnosis a standard medical diagnostic tool for IRDs.

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