



Single-test parallel assessment of multiple genetic disorders

We advocate a new paradigm for genetic diagnosis based on using customized array panels, each of which groups multiple genes and mutations associated with clinical profiles that are common to particular syndromic diseases. This parallel approach, based on a single-test multigene multiplexing strategy, compared with traditional sequential testing by gene-by-gene genetic analysis, drastically reduces the time and cost of diagnosis while maintaining accuracy and reliability. Faster diagnosis enables early decision-making to facilitate better patient management and outcomes at reduced costs to the healthcare system.

KEYWORDS: Array CGC ■ diagnostics ■ multiplexing panels ■ parallel genetic testing ■ syndromic genetic disorders

Sequential strategy of genetic assessment

During the past decade, we have witnessed continuous evolution in the field of human molecular genetics based on the advent of new high-content DNA genotyping technologies, the discovery of millions of human SNPs, mutations that occur in more than 1% of the population, and access to data from genome-wide association studies. A report by Khoury and colleagues has underscored the existence of over 6000 genetic diseases and 1100 molecular genetic tests [1]. The actual decision by a physician to order any particular genetic test is determined by familial and personal patient history and other clinical features as well as the proven clinical utility of the chosen test [2].

Diseases with underlying genetic components can affect different organ systems, not only as a primary disease, but often as a syndromic entity that can include metabolic, neurological, musculoskeletal or developmental symptoms. A syndromic entity may be associated with a single mutation in one of many different genes or from a combination of multiple mutations. In addition, the clinical features associated with any specific disease are often variable from one patient to another, due to the expression and penetrance of each condition, which may be subtle or absent at the time of examination. Alternatively, clinical characteristics of different diseases often overlap and the observed symptoms can correspond to more than one pathology. In this case, molecular diagnosis entails testing a large number of genes. However, even though important advances

regarding identification of genes associated with diverse diseases are being made on a daily basis, current conventional molecular methods in the clinic have not aggressively pursued high-content approaches and analysis.

To date, guidelines for diagnosis have been defined by clinical evaluation and step-by-step sequential differential genetic analysis, in which a proposed hypothesis is either confirmed or excluded by molecular techniques that probe for mutations in a specific gene that can explain the pathology (FIGURE 1). For each patient, a succession of genes are examined, one at a time (i.e., study gene A, if negative go to gene B, if negative go to gene C, and so on), implying an extended timeline and increased cost of diagnosis resulting from multiple physician appointments and tests that are sequentially ordered. Even for polygenic diseases, the current approach is primarily based on using classical DNA sequencing technology. However, present-day genetic diagnosis by DNA sequencing is still an expensive and time-consuming process that may have negative consequences on the patient's well being. Such is the case when there is a delay in making an appropriate therapeutic decision as physicians wait for test results from DNA sequencing. Additionally, this is especially relevant in prenatal molecular diagnosis where the timeframe for making informed decisions by pregnant women is vital. Anxiety created by the uncertainty of obtaining a diagnosis in an individual or family, along with the conventional approach of sequential testing, are important contributors to stress for the patient and their social environment.

Purificação Tavares^{1,2},
Luís Dias^{1,2},
Aida Palmeiro^{1,2},
Paula Rendeiro^{1,2}
& Peter Tolias³

¹CGC Genetics, 211 Warren St. Newark, NJ 07103, USA

²Rua Sá da Bandeira no.706 -1, 4000-432 Porto, Portugal

³Institute of Genomic Medicine, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, MSB F661, Newark, NJ 07101, USA

[†]Author for correspondence:

Tel.: +35 122 338 9900

Fax: +35 122 208 8710

mptavares@cgcggenetics.com

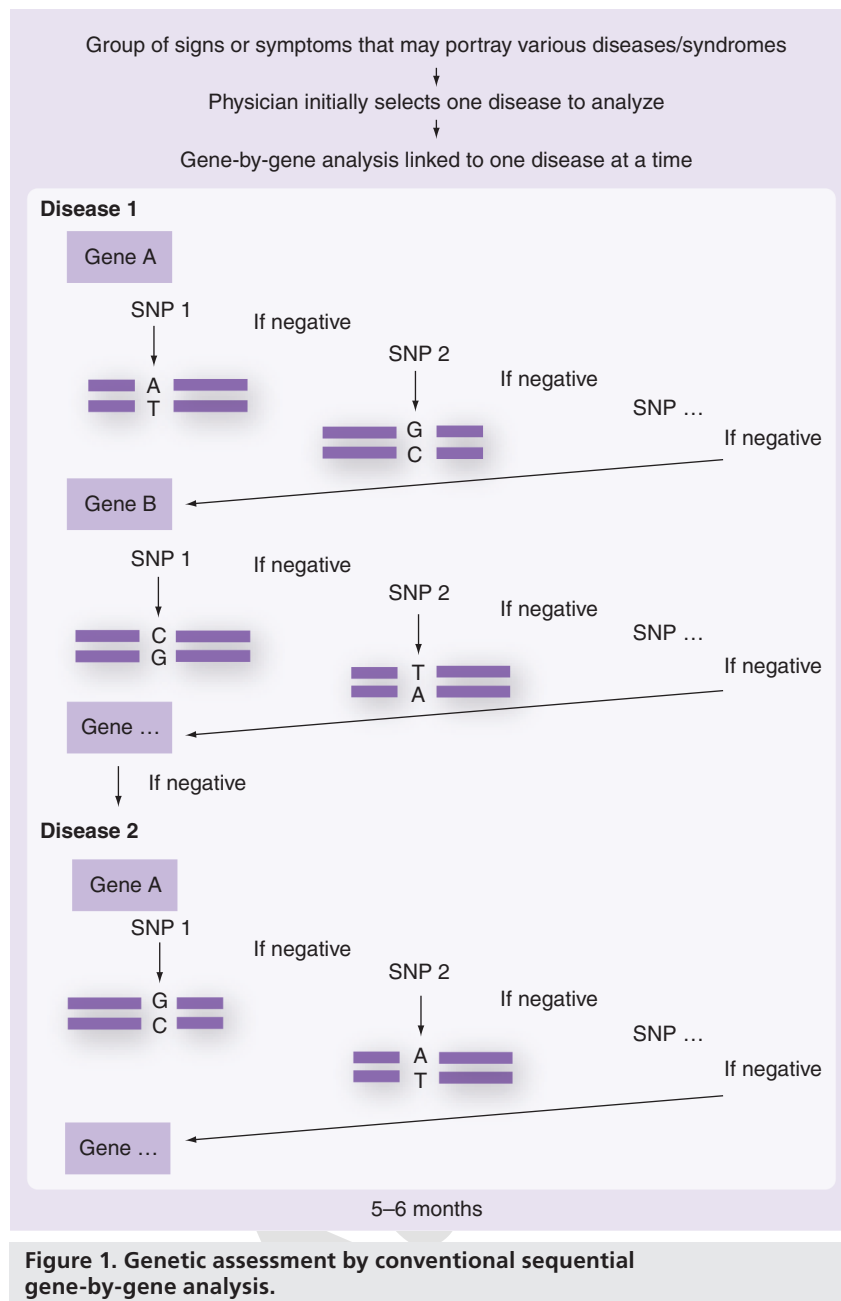


Figure 1. Genetic assessment by conventional sequential gene-by-gene analysis.

Parallel assessment of multiple genes

As an alternative to the focused sequential approach of genetic assessment on a gene-by-gene basis, a more efficient strategy is based on a parallel evaluation of important disease-associated genes and mutations incorporated on a customized multiplex panel (FIGURE 2). Particularly useful for syndromic diseases, this would cast a wider net when prescribing the first genetic test for a patient, by centering on assessment of the most common disease-associated mutations across a number of different genes rather than all possible deleterious mutations in a single gene. A positive result on such a test would reduce the time and cost of diagnosis, while maintaining accuracy

and reliability and enable early decision-making to facilitate better patient management and outcomes at reduced costs.

New clinical tests that enable parallel genetic assessment are available and we will focus on a number that we have developed (at CGC Genetics) known as Array CGC® (TABLES 1 & 2). These focused multiplex arrays assign different panels for analysis based on the observed clinical features of the disease or disorder (i.e., groups of symptoms), rather than just designing a test that probes for hundreds of thousands of unrelated SNPs. The accurate multiplex detection of genetic markers (up to 384) in a single test allows the organization of the panel in such a manner that mutations in genes associated with groups of diseases with overlapping symptoms or signs may be tested all at once. This approach drastically reduces the turnaround time and costs that usually characterize conventional genetic testing.

We have designed each of our Array CGC parallel genetic assessment tests on customized Illumina GoldenGate Genotyping (GGG) assays with detection on the VeraCode platform [101]. The GGG assay is a proven robust system for SNP genotyping. It was used to generate approximately 70% of the data from the Phase I International HapMap Project and is ideally suited for designing customized assay panels, allowing multiplex detection of as many as 384 SNPs or mutations within a single well of a standard microplate. This system also takes advantage of the fact that the VeraCode Beads are suspended in solution, which is favorable for assay kinetics and handling procedures, reducing hybridization time from 16 h (as in the Sentrix® Array Matrix protocols) to 3 h and decreasing the total assay time from 3 to 2 days.

The flexibility of the GGG bead-based system has reduced constraints that have limited the efficient application of microarray technology to genetic diagnosis. For each panel, different sets of oligo pool all (OPA; solution containing all of the allele-specific primers) are applied, avoiding nonspecific annealing that may occur when using allele-specific primers for mutations in close proximity on genomic DNA, where the minimum spacing normally recommended is approximately 60 bp [102]. Moreover, using different OPAs has facilitated the design of panels that analyze various SNPs in the same or in different genes or different mutations associated to the same disease. The optimization of each test panel is subjected to rigorous validation where DNA from control donors and patients with different mutations previously identified by DNA

sequencing are retested in order to confirm the accuracy of the method, using sensitivity, specificity and reproducibility as guidelines to determine the analytic validity.

The genes and mutations interrogated in all Array CGC panels were selected from reference sources such as the National Center for Biotechnology Information (NCBI), the Human Gene Mutation Database (HGMD) and the Online Mendelian Inheritance in Man (OMIM). Criteria for selecting genes and mutations for the different panels were based upon:

- Technical requirements (only point mutations were included);
- All the mutations directly causing the disease;
- Frequently occurring;
- Those described in more than one clinical case (where private and familial mutations were excluded).

Mutations with an ethnic distribution were also included, but to be used only within their respective groups.

Unlike other test methods such as DNA sequencing, which can produce data such as new unknown mutations that are difficult to interpret, CGC array test panels only include previously identified disease-causing or pathogenic point mutations. This clear and established

genotype/phenotype correlation allows a precise diagnosis of the medical condition. In cases where other sequence variants (insertions/deletions) are also described as frequent and important for the diagnosis, complementary tests with other techniques can be carried out, bearing in mind the fast and cost-effective purpose of this approach. Currently, whole-genome sequencing is not a time- and cost-effective approach for clinical diagnostics, particularly in prenatal situations [3].

The ability to analyze a group of genes associated with a specific clinical profile (e.g., a syndrome or a group of clinical features common to several diseases), represents a valuable asset that assists the physician in providing results with diagnostic value in a single step [4]. To achieve this end, a rigorous selection process that involved the categorization of clinical phenotypic groupings was accomplished by surveying a board of pediatricians and clinical geneticists who reviewed clinical phenotypes, determined shared signs and symptoms (e.g., mental retardation, short stature, hearing loss, craniosynostosis and bone dysplasias) and created groups of classified diseases organized by signs and symptoms. TABLE 1 presents the initial grouping of diseases/syndromes and signs that were suggested by the key opinion leaders who participated and the corresponding multiplex

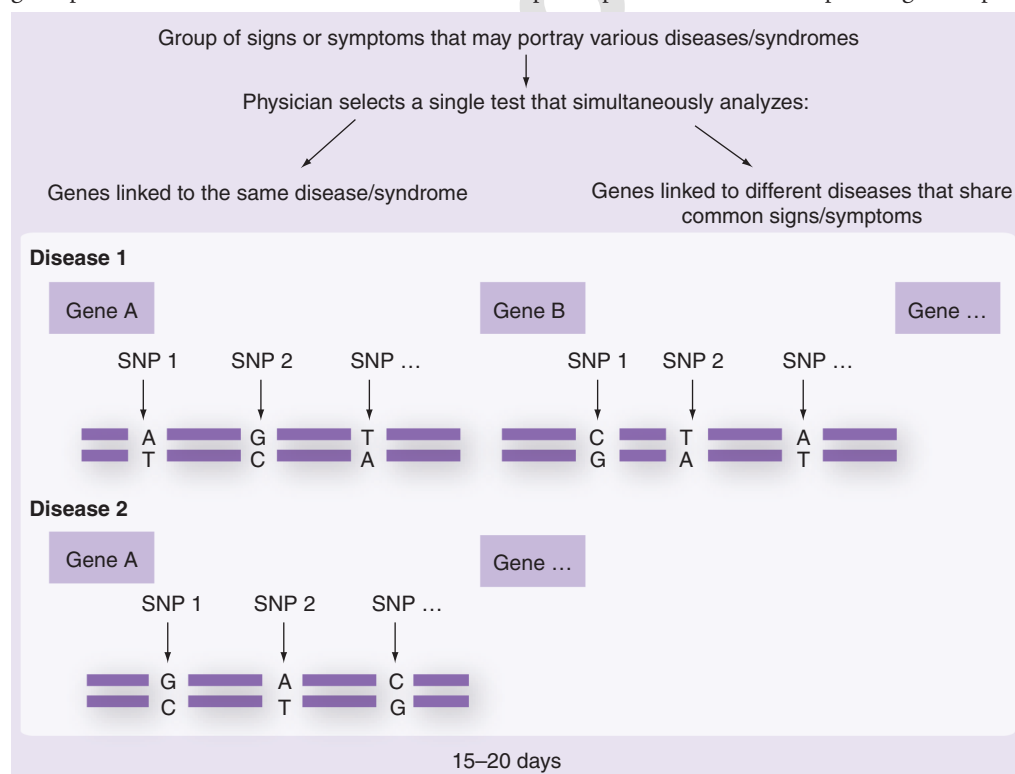


Figure 2. Genetic assessment by single-step parallel multiplexing.

Table 1. Genetic diseases by common signs and symptoms and list of available Array CGC panels.

Genetic diseases	Molecular diagnosis (Array CGC)	Mutations	Genes
Muenke, Pfeiffer, Apert, Crouzon and other syndromes associated with premature closure of calvarial skull bones, developmental delay, mental retardation	Craniosynostosis	52	<i>FGFR1, FGFR2, FGFR3</i> and <i>RAB23</i>
Skeletal disorders, bone dysplasias (achondroplasia, tanatoforic dysplasia, osteogenesis imperfecta and others)	Skeletal dysplasia	50	<i>FGFR3, COL2A1, SLC26A2, CRTAP, LEPRE1</i> and <i>SOX9</i>
Neuromotor disabilities, acidosis, ketosis, multisystemic manifestations	Metabolic disorders	93	<i>ACADM, ARSA, ATP7B, BTD, CLN2, CLN5, CLN8, CPT2, FAH, G6PC, GAA, GALC, GALT, GBA, HADHA, HEXA, HGD, MAN2B1, NPC1, NPC2, PEX1, PEX26, PPT1, PYGM, SLC37A4</i> and <i>TPP1</i>
Obesity, progressive pigmentary retinopathy, polycystic kidneys, congenital cardiopathy, progressive vision loss, development delay	Bardet–Biedl syndrome	130	<i>ARL6, BBS1, BBS2, BBS4, BBS5, BBS7, BBS9, BBS10, BBS12, MKKS, MKS1, TRIM32</i> and <i>TTC8</i>
Congenital deafness, vestibular disturbance	Nonsyndromic congenital hearing loss	136	<i>ACTG1, CDH23, COCH, CRYM, DNFA5, DIAPH1, GJA1, GJB2, GJB3, GJB6, KCNQ4, MYH14, MYO1A</i> and <i>MYO7A, OTOA, OTOF, POU3F4, SLC26A4, SLC26A5, TECTA, TMC1</i> and <i>WFS1</i>
Congenital deafness, development delay	Syndromic congenital hearing loss	176	<i>CDH23, EYA1, GJB2, KCNE1, KCNQ1, MYO7A, PAX3, PCDH15, SIX1, SIX5, SLC26A4, USH1C, USH1G</i> and <i>WFS1</i>
Thrombosis, venous thromboembolism, obstetrical complications, pregnancy loss	Thrombophilia and warfarin pharmacogenetics	15	<i>APOE, EPCR</i> , factor V Leiden, factor II, <i>MTHFR, PAI-1, ACE</i> , β -fibrinogen, factor XIII, <i>CYP2C9</i> and <i>VKORC1</i>
Noonan syndrome phenotype, heart disease, hypertrophic cardiomyopathy, postnatal short stature, psychomotor development delay, short or webbed neck, peculiar face	Noonan syndrome and other genetically related syndromes	80	<i>BRAF, KRAS, MAP2K, MAP2K1, PTPN11, RAF1</i> and <i>SOS1</i>

panel that was subsequently developed. For example, if a newborn presented postnatal short stature, heart defects and development delay, a physician would consult TABLE 1 and request the corresponding test that incorporated panels of genes and mutations that would probe all of these symptoms (e.g., Noonan syndrome phenotype in TABLE 1: postnatal short stature, broad or webbed neck, heart defects, including cardiomyopathy, psychomotor developmental delay and macrocephaly). The actual test that would be performed would include a panel that probes 80 different mutations in seven genes associated with several different diseases in a single 3-day test. If sequential methods of clinical analysis were applied to this example, it would include DNA sequencing of seven genes, covering approximately 100 exons, which would require at least the same number of sequencing reactions and a turnaround time of up to 1 year.

Conclusion & future perspective

The use of modified multiplexing panels for parallel detection of pathogenic SNPs associated with genetic diseases represents a major

opportunity for reducing the overall healthcare cost and turnaround time in the clinical diagnosis of human genetic disorders. This approach is particularly useful in prenatal diagnosis, as it drastically reduces the time needed to complete a thorough genetic evaluation and hence creates a window of opportunity for making informed decisions by pregnant women.

A recent review on the genetic diagnosis of nonsyndromic hearing impairment underscored the need to optimize technologies to allow the simultaneous analysis of deafness-related genes of such a heterogeneous pathology [5]. Tucker and colleagues have also commented on the potential use of massive parallel sequencing and its application in medical genetics [6]. However, at this time, parallel DNA sequencing would be very expensive and would generate enormous quantities of genetic data per individual, the vast majority of which would provide unknown clinical significance.

The advantage of using parallel multiplex genotyping in a highly organized manner to maximize clinical utility is not only useful for geneticists, but also for physicians of diverse

specialties. To this end, we have developed and implemented a series of different Array CGC panels (TABLE 2) that can be used individually or in groups depending on the initial clinical evaluation of the patient. The customization of the multiplex panels and their organization by signs and symptoms overcomes the consequences of long and expensive conventional testing. This allows physicians to obtain genetic information useful for clinical diagnosis by requesting a single test, in a fast and cost-effective way.

Financial & competing interests disclosure

Purificação Tavares, Luís Dias, Aida Palmeiro and Paula Rendeiro are employed by CGC Genetics, the company that has developed the Array CGC version of parallel genetic multiplexing discussed in this report. Peter Tolias discloses that he serves as a consultant for CGC Genetics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Sequential strategy of genetic assessment

- Diseases with genetic components can manifest as syndromic entities that can include metabolic, neurological, musculoskeletal or developmental symptoms.
- Diagnosis is defined by clinical evaluation and sequential molecular genetic analysis probing for mutations in each specific gene that may explain the pathology.
- Gene-by-gene analysis results in extended timelines and increased cost of diagnosis due to multiple physician appointments and tests that are ordered sequentially.

Parallel assessment of multiple genes

- Provides an alternative to the focused sequential approach of genetic assessment.
- Based on a parallel simultaneous evaluation of the most common disease-associated mutations across a number of different genes incorporated on customized multiplex panels such as the Array CGC line.
- Particularity useful for syndromic diseases by casting a wider net in a single genetic test that probes for the most common disease-associated mutations across many genes rather than all possible deleterious mutations in a single gene.
- A positive result reduces the time and cost of diagnosis enabling early decision-making to facilitate better patient management and outcomes at reduced costs.

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sequencing as well as other methods for identifying genetic variation.

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methods as well as other issues arising from vast amounts of information obtained through these new techniques.

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