

A targeted gene capture next-generation sequencing panel for genetic screening of newborns

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Abstract

Objective: The application of next-generation sequencing (NGS) will greatly promote the screening and diagnosis of genetic diseases. This study aimed to implement and validate a targeted NGS panel for genetic screening of over fifty types of genetic disorders in newborns.

Methods: A targeted gene panel consisting of 104 known genes related to genetic diseases with a target size of 347.8 kb was designed. Genes were selected through reference to databases including HGMD, OMIM, GeneReviews®, and Genetic Home Reference, and the latest peer-reviewed publications associated with the genetics of hereditary diseases.

Results: The average coverage for all targeted exons was 596X, and the mean targeted region coverage of 1X, 10X, 20X and 50X reads for each sample were 99.8%, 99.2%, 98.8%, and 95.3%, respectively. The panel showed 100% consistency in detecting 8 pathogenic insertion/deletion (indels) variants ranging from 1 to 16 bp in size and 20 pathogenic single-nucleotide variations (SNVs) across 32 samples previously confirmed by Sanger sequencing.

Conclusions: A dried blood spot (DBS)-based targeted NGS panel for efficient genetic screening of a wide variety of genetic diseases in newborns was developed and evaluated. Furthermore, our panel will contribute to providing accurate diagnosis for genetic disorders and will be helpful for gene therapy for specific diseases.

Keywords: Genetic screening, Next-generation sequencing, Dried blood spot, Inherited metabolic disorders, Gene therapy. (JPMA 70: 1789; 2020) DOI: <http://doi.org/10.5455/JPMA.28637>

Introduction

It is estimated that 7.9 million babies worldwide are born annually with birth defects of genetic or partially genetic origin.¹ Hereditary diseases, especially congenital diseases present at birth, are genetic problems caused by one or more abnormalities in the genome. These diseases include hearing loss, primary immunological deficiencies, traditional inborn errors of metabolism (IEM), which are classified as disorders of amino acid metabolism, carbohydrate metabolism, organic acid metabolism, or lysosomal storage diseases, and other various genetic conditions.²

Inherited metabolic disorders, also referred to as IEM, are mostly rare diseases with a prevalence estimated to range between 1/50,000-1/150,000. Nevertheless, IEM may have an overall prevalence of approximately 1:1000 among live births.³ Most IEM-causing mutations are small DNA changes that affect one or more nucleotides.⁴ Mutations generally affect specific proteins or enzymes, the abnormal function of which results in changes in a particular metabolic pathway.⁵ Enzyme methods have

traditionally been considered the gold standard diagnostic method for IEM. Nevertheless, these methods are highly diverse, cumbersome, time consuming, and have low output and rigorous sample requirements.⁶⁻⁸ As an alternative, genetic detection has been used to diagnose suspected IEM disease in an effort to explore genotypic pathogenicity.⁹⁻¹³ Hearing loss is one of the most common sensory disorders, which severely affects the quality of daily life. It has various etiologies, and it is estimated that over two-thirds of cases of congenital deafness are associated with genetic factors.¹⁴⁻¹⁶

Over the past decade, NGS technologies have significantly affected various fields of molecular research, mainly because they reduce costs and raise the throughput of DNA sequencing.¹⁷ Indeed, NGS technologies have been used in research, clinical trials and diagnoses of hereditary diseases.^{18,19} In recent years, targeted NGS has been validated in population-based carrier screening and patient-based testing of hereditary Mendelian diseases, allowing for simultaneous testing of multiple genes at a relatively low cost as well as an understanding of disease etiology.^{20,21}

In the present study, a targeted NGS panel for genetic screening of genetic diseases in newborns was established. The platform can simultaneously achieve mutation screening of genes related to fifty types of

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genetic diseases in newborns. As many babies affected by inherited disorders are not diagnosed until severe and irreversible symptoms occur later in life, detection of a disease through genetic screening at an early stage can enable appropriate and timely medical intervention before more serious and sometimes irreversible health issues arise. This study aimed to implement and validate a targeted NGS panel for genetic screening of over fifty types of genetic disorders in newborns.

Materials and Methods

A total of 25 healthy subjects and 32 patients from Children's Hospital of Dongguan in Southern China were included in this study between January 2017 and June 2018. Among them twenty-two patients with hereditary hearing loss, two with phenylketonuria, two with tuberous sclerosis, one with methylmalonic acidemia, one with X-linked agammaglobinemia, one with

Wilson's disease, one with G6PD deficiency, one with mucopolysaccharidosis type II and one with severe myoclonic epilepsy in infancy. All of the patients were identified through newborn screening, and whole blood samples were collected on specialized filter paper. Sensitivity and specificity were determined using Cohen's Kappa coefficient by comparing with results obtained by the standard testing of Sanger sequencing.²²

This study was approved by the Ethics Committee of Children's Hospital of Dongguan, and the protocol was conducted in accordance with the Declaration of Helsinki. For each proband and their family members, informed written consent was obtained to participate in this study.

Target enrichment panel design: Target enrichment followed by NGS was used to develop clinical genetic test panels for 104 genes (347.8 kb target region) related to over 50 different genetic diseases, mainly IEM (mainly

Table-1: Disorders and corresponding genes contained in the panel.

Disorders	Genes	Disorders	Genes
G6PD deficiency	G6PD	Fabry disease	GLA
Propionic acidemia, PA	PCCA, PCCB	Wilson disease, WD	ATP7B
X-linked agammaglobinemia, XLA	BTX	Citrullinemia type I	ASS1
Tuberous Sclerosis, TSC	TSC1, TSC2	Phenylketonuria	PAH
Methylmalonic acidemia, MMA	MUT	Citrin deficiency	SLC25A13
N-acetylglutamate synthetase; NAGS	NAGS	Galactosaemia	GALT
β -ketothiolase deficiency	ACAT1	Nijmegen breakage syndrome, NBS	APEX1, MRE11A
Carbamoyl phosphate synthetase deficiency	CPS1	Argininosuccinic aciduria, ASA	ASL
Short-chain acyl coenzyme A dehydrogenase deficiency	ACADS	Multiple carboxylase deficiency	BTBD
Ataxia-telangiectasia	ATM	Arginase deficiency	ARG1
Ornithine transcarbamylase deficiency, OTC	OTC	Glycogen storage disease type II, GSD II	GAA
Carnitine palmitoyltransferase I deficiency, CPT I	CPT1A	Hereditary fructose intolerance, HFI	ALDOB
Carnitine palmitoyltransferase II deficiency, CPT II	CPT2	Isovaleric acidemia, IVA	IVD
Mucopolysaccharidosis type I, MPS I	IDUA	Krabbe disease	GALC
Mucopolysaccharidosis type II, MPS II	IDS	Pendred syndrome	SLC26A4
Cartilage-hair hypoplasia, CHH	RMRP	Severe myoclonic epilepsy in infancy, SMEI	SCN1A, SCN9A
Cystic fibrosis, CF	CFTR, FCGR2A, TGFB1, ZFPM2	Nonsyndromic hearing loss	GJB2, GJB3
Primary carnitine deficiency, PCD	SCD, SLC22A5, TSHR	Trifunctional protein deficiency, TFPD	HADHA, HADHB
Medium chain acyl-CoA dehydrogenase deficiency, MCAD	ACADM	3-methylcrotonyl-CoA carboxylase deficiency, MCCC	MCCC1, MCCC2
Very long-chain acyl-CoA dehydrogenase deficiency	ACADL, ACADVL	Tyrosinemia type I, HT1	FAH
Neiman-Pick disease, NPD	SMPD1, NPC1, NPC2	Cystathionine β synthase deficiency hyperhomocysteinemia	CBS
Glutaric acidemia II, GA-II	ETFA, ETFB, ETFDH	Glycogen storage disease type Ia, GSD Ia	G6PC
Tetrahydrobiopterin deficiency, BH4D	PTS, GCH1, QDPR, PCBD1	Glycogen storage disease type Ib, GSD Ib	G6PC, SLC37A4
Haemophagocytic lymphohistiocytosis, HLH	STXBP2, FHL1, PRB3 PRF1, FHL3, UNC13D REEP2, STX11, FHL5	MSUD	BCKDHA, BCKDHB, DBT
Methylmalonic acidemia	ACSF3, MMAA, MMAB, MTRR, MTR, MMACHC, ABCD4, MMADHC, LMBRD1, HCFC1, ALDH6A1, MCEE, MOCS1, MOCS2, SUCLA2, SUCLG1	Severe combined immunodeficiency disease, SCID	ADA, IL2RG, IL4, IL7R, RAG1, RAG2, DCLRE1C, PTPRC, NHEJ1

categorized into amino acid, organic acid, fatty acid and lysosomal storage disorders), severe combined immune deficiency and hereditary deafness. The hereditary disease genes were selected from HGMD (<http://www.hgmd.org>), RefSeq (<https://www.ncbi.nlm.nih.gov/refseq/>), OMIM (<https://www.omim.org/>), Genetic Home Reference (<https://ghr.nlm.nih.gov/>). All of the 1355 regions of these 104 genes were covered by coding exons, and the 20 bases of flanking and intronic regions of each exon were prepared based on solution-based custom capture. A list of all 104 genes included in this assay is provided in Table-1. Two samples were run in duplicate to evaluate reproducibility.

DNA extraction: In this study, dried blood spots (DBSs) were punched from routine newborn filter cards. DNA was extracted from DBSs according to the protocol of a MagPure Tissue and Blood DNA LQ Kit (Magen Technologies, Guangzhou, China). DNA quantity and quality assessments were performed using an ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA) and agarose gel electrophoresis according to a standard protocol. The amounts of isolated DNA fluctuated between 400 and 500 ng. The yield of pure DNA at the 260/280 ratio should be between 1.8-1.9.

Targeted genome capture and next-generation sequencing: Overall, 200 ng of extracted DNA was fragmented (NEBNext®), and T4 DNA polymerase, Large (Klenow) fragment and T4 PNK were used to convert the overhanging sequences resulting from fragmentation into blunt ends. Adapters were attached to the DNA fragments, and the fragments were purified and selectively enriched. PCR amplification was performed, during which an index tag was introduced to the adapter, and a library quality test was performed. The final library was sequenced using a proton platform (BioelectronSeq 4000).

Bioinformatics: The sequencing data were analyzed using an inhouse bioinformatic analysis pipeline. Alignment of sequence reads to the human reference genome (hg19) was performed using TMAP (Torrent Mapping Alignment Program). Variants were called using TVC (Torrent Variant Caller). ANNOVAR software was used to functionally annotate genetic variants, providing a list of variants with information of chromosome, start and end position, reference nucleotide and observed nucleotides. Further genome annotation data were obtained from the Genome Aggregation Data, Exome Aggregation Consortium, 1000 Genomes Project, and dbSNP databases.

Results

The average targeted exon coverage was 596X when sequence reads were aligned to the human reference genome. The mean targeted region coverages of 1X, 10X, 20X and 50X reads for each sample were 99.8%, 99.2%, 98.8%, and 95.3%, respectively (Figure-1).

To test and verify this panel as a genetic testing tool, 25 negative samples and 32 positive DNA samples previously confirmed by Sanger sequencing were used to evaluate performance. Samples from patients having different mutation states (homozygous, heterozygous and

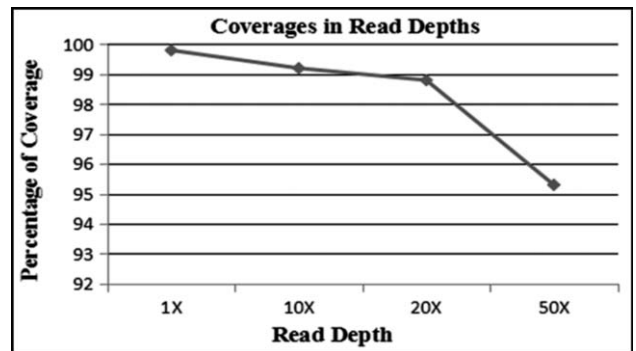


Figure-1: Mean coverage percentages for the 1X, 10X, 20X and 50X read depths for the samples in this study.

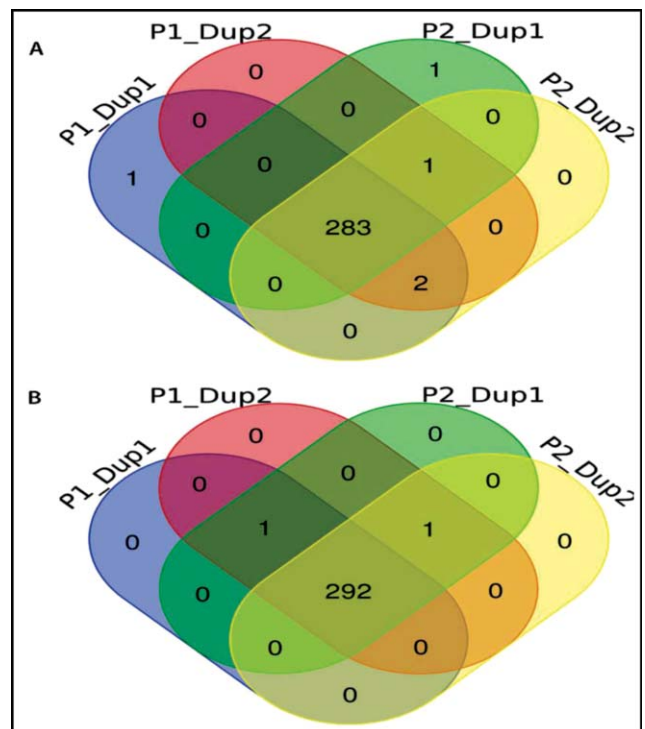


Figure-2: Mutation detection in duplicate reproducibility testing.

Table-2: Identified causative variants in 32 samples.

Sample ID	Gene	Transcript	Nucleotide change	Protein Change
1	PAH	NM_000277.2	c.[728G>A];[728G>A]	p.[Arg243Gln];[Arg243Gln]
2	PAH	NM_000277.2	c.[728G>A];[721C>T]	p.[Arg243Gln];[Arg241Cys]
3	MMACHC	NM_015506.2	c.[609G>A]	p.[Trp203Ter]
4	BTB	NM_000061.2	c.[872dupC]	p.[Ala291fs]
5	G6PD	NM_000402.4	c.[1466G>T]	p.[Arg489Leu]
6	ATP7B	NM_000053.3	c.[c.2333G>T]; [2605G>A]	p.[Arg778Leu];[Gly869Arg]
7	IDS	NM_000202.8	c.[1400C>T]	p.[Pro467Leu]
8	SCN1A	NM_006920.5	c.[680T>G]	p.[Ile227Ser]
9	TSC1	NM_000368.4	c.[1904_1905del]	p.[Thr635Argfs]
10	TSC2	NM_000548.4	c.[226-2A>G]	Aberrant splicing
11	GJB2	NM_004004.5	c.[235delC];[235delC]	p.[Leu79Cysfs];[Leu79Cysfs]
12	GJB2	NM_004004.5	c.[235delC];[257C>G]	p.[Leu79Cysfs];[Thr86Arg]
13	GJB2	NM_004004.5	c.[299-300delAT];[c.512insAACG]	p.[His100Argfs];[Trp172ThrfsTer39]
14	GJB2	NM_004004.5	c.[235delC];[c.299-300delAT]	p.[Leu79Cysfs];[His100Argfs]
15	GJB2	NM_004004.5	c.[235delC];[c.176_191del16]	p.[Leu79Cysfs];[Gly59Alafs]
16	GJB2	NM_004004.5	c.[235delC];[299-300delAT]	p.[Leu79Cysfs];[His100Argfs]
17	GJB2	NM_004004.5	c.[176_191del16];[511_512insAAC]	p.[Gly59Alafs];[Ala171Glu]
18	GJB2	NM_004004.5	c.[35_36insG];[235delC]	p.[Val13Cysfs];[Leu79Cysfs]
19	GJB2	NM_004004.5	c.[235delC];[235delC]	p.[Leu79Cysfs];[Leu79Cysfs]
20	GJB2	NM_004004.5	c.176_191del16;GJB2:c.299-300delAT	p.[Gly59Alafs];[His100Argfs]
21	GJB2	NM_004004.5	c.235delC;GJB2:c.299-300delAT	p.[Leu79Cysfs];[His100Argfs]
22	GJB2	NM_004004.5	c.235delC;GJB2:c.427C>T	p.[Leu79Cysfs];[Arg143Trp]
23	SLC26A4	NM_000441.1	[IVS7-2A>G];[IVS7-2A>G]	Aberrant splicing; Aberrant splicing
24	SLC26A4	NM_000441.1	c.[2168A>G];[IVS7-2A>G]	p.[His723Arg]; Aberrant splicing
25	SLC26A4	NM_000441.1	c.[697G>C];[1160C>T]	p.[Val233Leu];[Ala387Val]
26	SLC26A4	NM_000441.1	c.[1174A>T];[IVS7-2A>G]	p.[Asn392Tyr]; Aberrant splicing
27	SLC26A4	NM_000441.1	c.[1174A>T];[IVS7-2A>G]	p.[Asn392Tyr]; Aberrant splicing
28	SLC26A4	NM_000441.1	c.[1226G>A];[IVS7-2A>G]	p.[Arg409His]; Aberrant splicing
29	SLC26A4	NM_000441.1	c.[1229C>T];[c.349delC]	p.[Thr410Met];[Leu117SerfsTer9]
30	SLC26A4	NM_000441.1	c.[1105A>G];[IVS7-2A>G]	p.[Lys369Glu]; Aberrant splicing
31	SLC26A4	NM_000441.1	[IVS7-2A>G];[IVS15+5G>A]	Aberrant splicing ;Aberrant splicing
32	SLC26A4	NM_000441.1	[IVS7-2A>G];[IVS14+1G>A]	Aberrant splicing; Aberrant splicing

compound heterozygous) and types (missense, nonsense, frameshift) for genes PAH, MMACHC, BTB, GJB2, SLC26A4, TSC1, TSC2, ATP7B, G6PD, IDS and SCN1A were used for this study. In the limited thirty-two positive samples with 28 mutations, the panel showed 100% analytical sensitivity in detecting 8 pathogenic insertion/deletion (indels) mutations ranging from 1 to 16 bps in size and the 20 pathogenic SNVs (Table-2). The assay did not identify any unexpected variants, confirming that there were no unreported variants in the positive validation cohort. Furthermore, no mutation was identified by this assay in the negative samples.

For the two blinded duplicates used for reproducibility test, each sample was detected twice by two technologists, and four tests were performed for each sample. A total of 286 mutations were detected in sample A; of these, 283 mutations were detected in 4 replicates (consistency rate was 98.95%) (Figure-2A). A total of 294

mutations were detected in sample B; of these, 292 mutations were detected in 4 replicates (consistency rate was 99.32%) (Figure-2B). The inconsistency was possibly due to (1) GC-rich regions, (2) poly-regions, (3) mismapping of INDELs, and (4) undetected sites at lower depths.

Discussion

An NGS panel-based assay was designed and validated. This assay is DBS adaptable and rapid and able to diagnose a variety of genetic diseases such as IEM and other genetic diseases, which are usually not evident at birth but become clinically evident during late childhood or adolescence.

Although several laboratories offer NGS for genetic diseases, considering the DNA amount needed, most of them use whole blood samples for DNA extraction. The opportunity for genotyping on DBS-extracted DNA has

opened up a new avenue for newborn screening as well as the study of the genetics underlying many complex diseases.^{23,24} We performed a preliminary study of thirty-two cases of hereditary diseases and twenty-five negative samples, and our experience with NGS using DNA isolated from DBSs demonstrated that the DNA extracted can be used reliably. The platform we designed in this study proved to be reliable and robust.

The main constraint of comprehensive genetic testing for many hereditary diseases is that multiple genes are often involved in the etiology and pathogenesis of a single disease.²⁵ Sanger sequencing is generally used to assess genetic diseases, but sequencing multiple genes is expensive, and the test volume is small. The ability to sequence numerous genes and multiple patients simultaneously renders NGS suitable for solving the limitations of traditional sequencing technology.^{26,27} NGS has been rapidly developed in the genetics field and has provided a large number of accurate and immediate sequence information; thus, it is a valuable tool for evaluating clinical diseases caused by multiple gene mutations. Coupled with advances in data processing and analysis, technology is becoming a standard tool in research and clinical genetics.^{28,29} Moreover, such sequencing technology has great potential for disease diagnostics and newborn screening.³⁰ The sensitivity of this technology is rapidly increasing, and the standardized off-the-shelf specifications allow the use of nanograms of high-quality input DNA for whole-genome sequencing (WGS).³¹

Detecting the genetic defect underlying a disease is useful for genetic counseling and helpful for applying suitable treatment. Such genetic data also provide the basis for genetic counseling in family planning and prenatal diagnostics; such data may also help families prepare for the onset and progression of diseases and prevent unnecessary diagnostic procedures.³² Compared with traditional screening, genetic testing with the same DBS sample will greatly reduce false positive results and prevent unnecessary stress on families due to recalls. As a form of validation testing, NGS has proven to be a useful tool for explaining abnormal metabolite concentrations in DBSs and distinguishing affected patients from individuals with only a heterozygous variant.

Conclusions

In conclusion, our study indicated that reliable NGS could be performed on archived neonatal DBSs using only partially accessible material. This further increases the application of neonatal DBSs in future genetics studies, diagnostics and screening projects.

Disclaimer: None to declare.

Conflicts of Interest: No potential conflict of interest was reported by the authors.

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