3billion's state-of-the-art molecular diagnostic test for rare Mendelian diseases

3billion

Your one answer

One step closer to a molecular diagnosis

Table of Contents

Introduction

Exome sequencing and genome sequencing are now being routinely used to diagnose suspected rare genetic (Mendelian) disorders by rapidly identifying the disease-causing-variants in an unbiased way. Identifying the molecular diagnosis for patients with rare genetic disorders is extremely important as it not only provides the patients with personalized clinical care and management plan but also opens genetic counseling opportunities for their family members.

Nevertheless, a substantial number of patients with suspected rare genetic diseases remain undiagnosed. A few of the reasons are: 1) limited access to genomic tests because of a relatively high cost and challenges with insurance coverage, 2) limited knowledge of gene-disease association, and 3) technical limitations with sequencing data analysis and variant interpretation. However, with increasing amounts of sequencing data being generated every day from a number of laboratories, and significant efforts to further advance analytical and interpretation skills, some of these challenges are getting resolved.

3billion has joined this global effort since October 2016, with the vision of providing an affordable test to patients with suspected rare genetic disorders and maximizing the variant interpretation skills and speed to ensure every patient who walks into 3billion's system can promptly get a clear molecular diagnosis.

3billion's Genomic Tests

3billion’s genomic test menu includes 3B-EXOME for exome sequencing test, 3B-GENOME for genome sequencing test and 3B-VARIANT for searching variants reported from 3B-EXOME or 3B-GENOME tests in related family members. 3B-EXOME and 3B-GENOME are based on next-generation sequencing (NGS) technology while 3B-VARIANT uses a traditional Sanger sequencing method. Both 3B-EXOME and 3B-GENOME are comprised of four main parts:

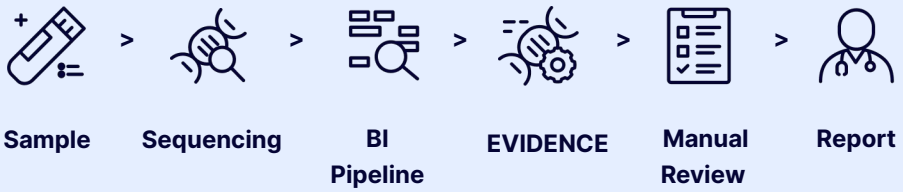


Figure 1. Schematic diagram of 3billion’s genomic test workflow

1. High-quality sequencing:

Sequencing library preparation and sequencing are performed using clinically validated Standard Operating Procedures (SOPs). 3billion’s laboratory (3billion Co LTD Molecular Diagnostics Laboratory) is accredited by CAP (College of American Pathologists) and CLIA (Clinical Laboratory Improvement Amendments). See below for more details.

2. Sequencing data analysis:

Once the sequencing data is generated, 3billion’s bioinformatics workflow is run on each sample, also following the clinically validated SOPs. See below for more details.

3. Variant annotation and prioritization by EVIDENCE following the ACMG/AMP guidelines:

EVIDENCE is 3billion's state-of-the-art, highly automated and cost-effective analytical system developed in-house. Through its annotation, classification, and phenotype matching process only a handful of variants are left for the expert to interpret. See below for more details.

4. Variant interpretation in the context of the patient's symptoms and reporting of disease-causing variants:

Once EVIDENCE prioritizes the top candidate variants/genes, 3billion's highly-trained clinical/medical geneticists manually curate each variant to identify the disease-causing variant for reporting. See below for more.

3B-EXOME

3billion performs exome capture with IDT xGen Exome Research Panel v2.0 (Integrated DNA Technologies, Coralville, Iowa, USA) and sequencing on NovaSeq 6000 (Illumina, San Diego, CA, USA). The IDT panel was selected after a thorough evaluation of the coverage statistics in comparison with other commercially available capture kits. Currently, the minimum depth-of-coverage (DOC) per exome is 100 x with a minimum 98% of the targeted region covered at 20x DOC.

Once the sequencing is complete, the base call (BCL) sequence files generated by NovaSeq 6000 are converted and demultiplexed to FASTQ files using bcl2fastq v2.20.0.422 [1]. Sequence reads in the FASTQ files are aligned to the human reference genome (GRCh37/hg19 from NCBI, February 2009) using BWA-mem 0.7.17 [2] to generate BAM files. BAM files are processed following the GATK best practices (GATK v.3.8) [3] for single nucleotide variants (SNV) and small insertions/deletions (indel) variant calling to generate VCF files [4, 5]. Conifer [6] and 3bCNV are used for copy number variant (CNV) calling based on depth-of-coverage (DOC) data. Due to the lack of sequencing data between exons, the resolution of CNV calls is minimum 3 consecutive exons and for most of the CNVs, exact breakpoints are not identifiable. AutoMap v1.2 [7] is used for Region of Homozygosity (ROH) detection from the VCF file (Figure 2). Various quality control metrics such as Q30, mapping rate, PCR duplication rate, capture efficiency, total number of variants, heterozygous/homozygous (het/homo), and transition/transversion (ts/tv) ratios are used to ensure the sequencing data is within an acceptable range for a clinical test.

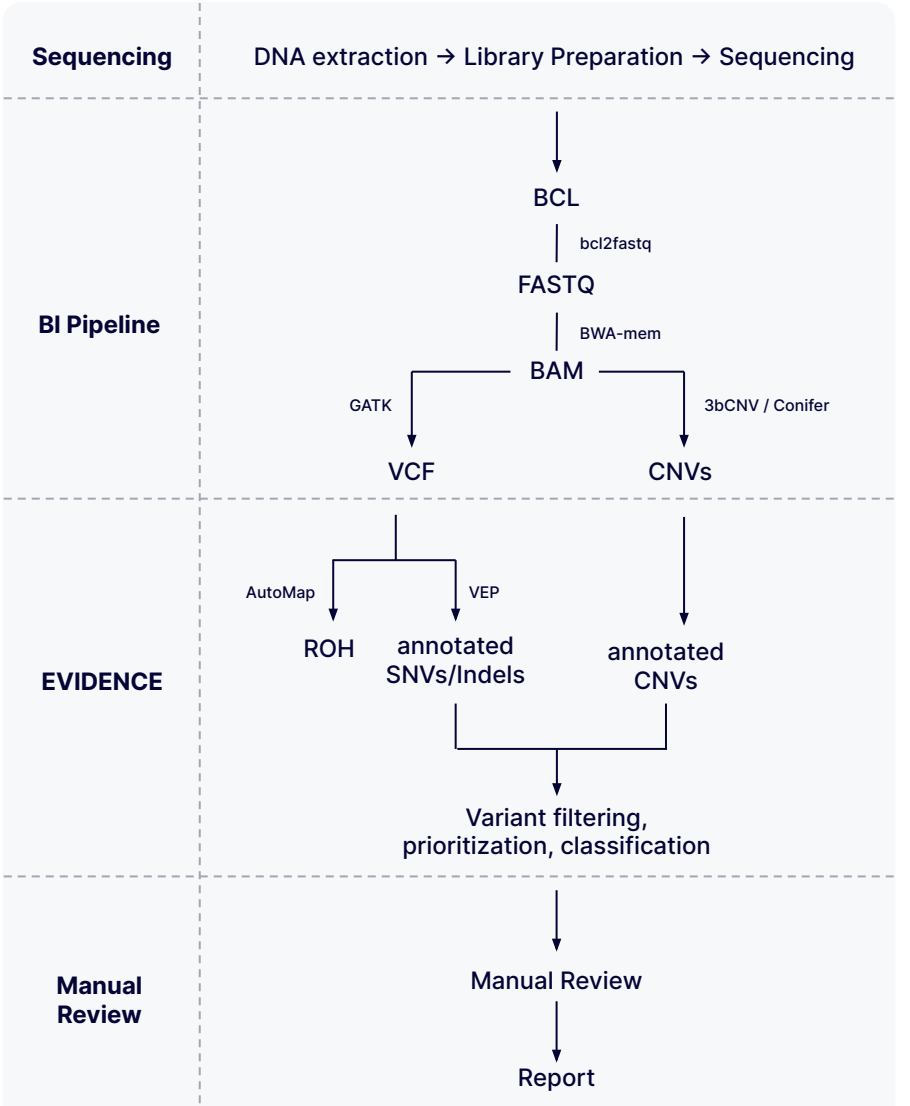


Figure 2. Schematics of 3B-EXOME analysis workflow

3B-GENOME

Genome sequencing libraries are generated using TruSeq DNA PCR-Free Low Throughput Library Prep Kit (Illumina, San Diego, CA, USA) and sequencing is performed on NovaSeq 6000 (Illumina, San Diego, CA, USA). Currently, the minimum depth-of-coverage (DOC) of autosomes per genome is 30x with a minimum 95% of the autosomes covered at 20x DOC. Once sequencing is complete, the base call (BCL) sequence files generated by NovaSeq 6000 are converted and demultiplexed to FASTQ files using bcl2fastq v2.20.0.422 [1]. Sequence reads in the FASTQ files are aligned to the human reference genome (GRCh38.p14 from NCBI, February 2022) and revised Cambridge Reference Sequence for mitochondrial genome (GeneBank accession number: NC_012920) using BWA-mem 2.2.1 [2] to generate BAM files. BAM files are processed following the GATK best practices (GATK v.4.2.0.0) [3] for SNV (single nucleotide variants) and small indels (insertions and deletions) variant calling to generate VCF files [4, 5]. Structural variants, including CNVs, inversions, translocations, repeat expansions and mobile element insertions, are also called from the BAM files using 3bCNV (in-house), MANTA v1.6.0 [8], ExpansionHunter [9] and MELT v2.2.2 [10]. AutoMap v1.2 [7] is used for Region of Homozygosity (ROH) detection from the VCF files (Figure 3). Various quality control metrics such as Q30, mapping rate, PCR duplication rate, total number of variants, het/homo and ts/tv ratios are used to ensure the sequencing data is within an acceptable range for a clinical test.

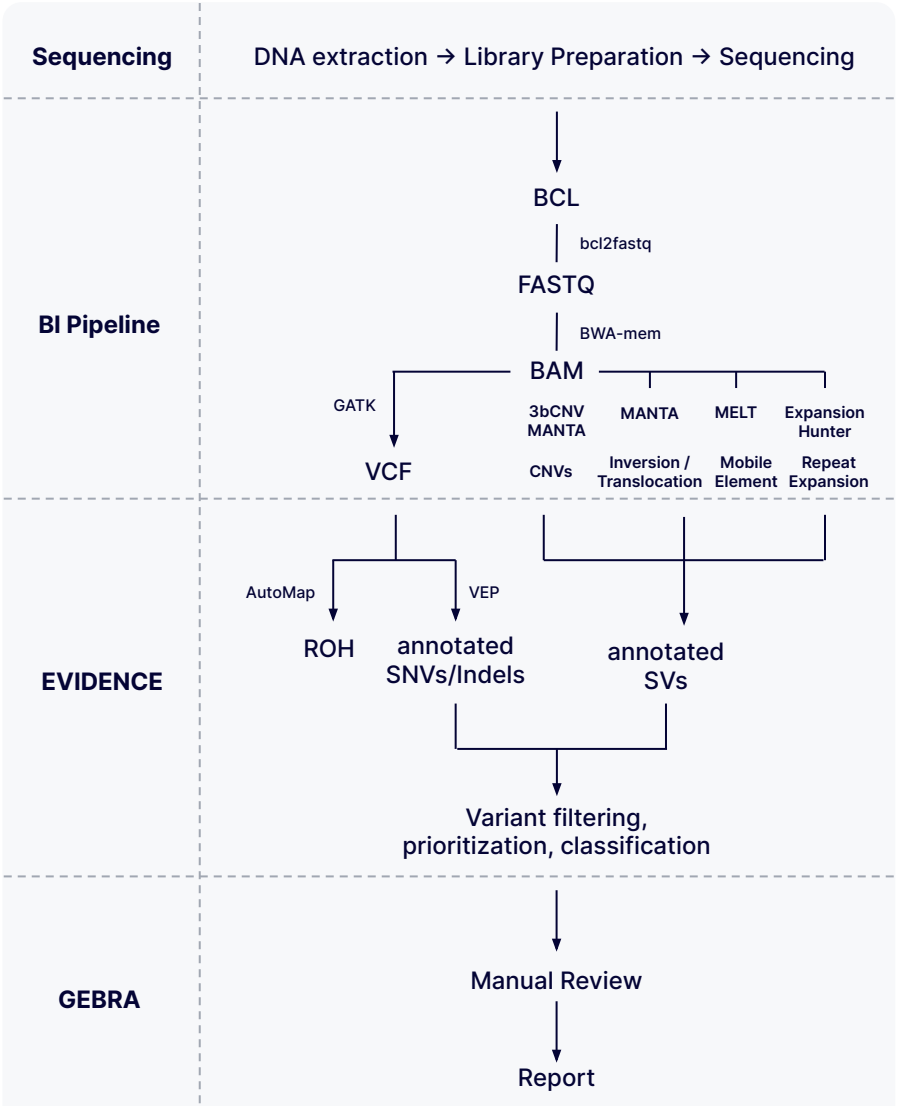


Figure 3. Schematics of 3B-GENOME analysis workflow

3B-VARIANT

3B-VARIANT, also called variant specific test (VST), uses Sanger sequencing for genotyping a specific variant position in family members. Once a proband is reported with a variant by 3B-EXOME or 3B-GENOME, the presence of the same variant in proband's parents or other family members can be tested with 3B-VARIANT. The test provides a cost-effective method for determining whether the proband's variant is inherited or not, which is often crucial for evaluating its pathogenicity. Extending the test to other family members can also enable genetic counseling, expanding to other family members by either confirming the diagnosis in other affected members or informing potential disease risk.

Genomic DNA is extracted from whole blood, buccal swab or dried blood spot (DBS) samples, using QIAamp blood (QIAGEN, GmbH, Germany), AccuBuccal DNA Prep kit (AccuGene, Incheon, Korea), and AccuFAST DBS Prep Kit (AccuGene, Incheon, Korea), respectively. PCR primers are designed using Primer3 (v. 0.4.0), [11, 12] and NCBI GenBank reference sequence. PCR amplification and Sanger sequencing are performed following the standard protocol using PCR Master Mix Kit (ThermoFisher Scientific, Waltham, MA, USA) and SeqStudio Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing results are manually analyzed using Sequence Scanner Version 1.0 (Applied Biosystems, Foster City, CA, USA).

Each case is then comprehensively reviewed by our clinical team of physicians, geneticists and informaticists.

Quality metrics for Sanger validation of identified variants

Even though next-generation sequencing (NGS) has settled down to be a robust technology for molecular diagnostic tests, because Sanger sequencing is oftentimes still considered as the gold standard in the field, variants identified by NGS have been subject to Sanger confirmation prior to being reported. This confirmation process results in delayed turnaround time and increased cost. Multiple groups, including 3billion, have investigated the needs of Sanger confirmation for NGS-based tests to uniformly report that Sanger confirmation is not necessary for variants with 'good' quality scores as long as sufficient validation and quality control measures are implemented [13, 14, 15]. 3billion has performed a thorough validation study (Figure 4) to determine a conservative threshold using the variant quality score generated by GATK and variant allele frequency (VAF) to define 'good' variants that do not require Sanger confirmation. This reduced the number of variants requiring Sanger confirmation by more than 90%.

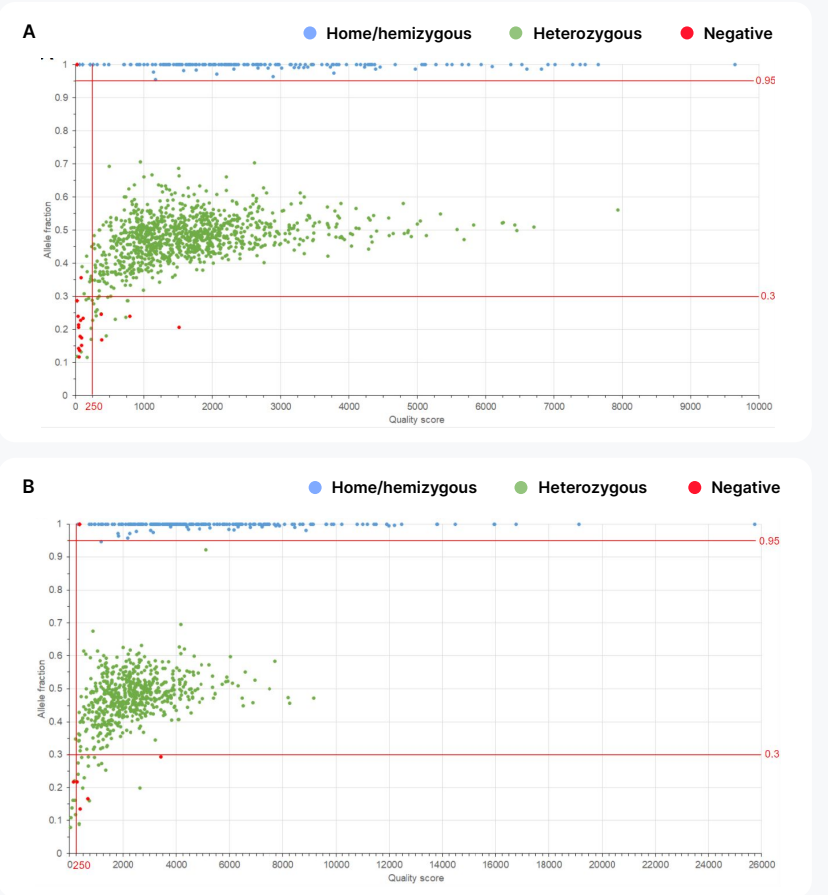


Figure 4. Variants are plotted by their quality score and VAF. A. Single nucleotide variants (SNV), B. Small insertion and deletion variants (INDEL). Blue dots are variants called homozygous or hemizygous by WES and Sanger sequencing, green dots are variants called heterozygous by WES and Sanger sequencing and red dots are variants called as homozygous/hemizygous or heterozygous by WES but not confirmed by Sanger sequencing. Variants with (quality score> 250) and (VAF> 0.3 (heterozygous) or >0.95 (homozygous)) and (read depth>=10) were determined to be defined as 'good' variants without the need of Sanger confirmation.

EVIDENCE:

Automatic variant prioritization system

EVIDENCE is an automated variant prioritization system that has been developed to facilitate genomic sequencing analysis.

EVIDENCE is composed of 3 key modules:
1. variant annotation module with daily updated database
2. customized variant classification module
3. phenotype similarity scoring module

1. Variant annotation module with daily updated database

Annotating each variant with public and private (in-house) data is the first step of variant analysis as this collective annotation data is used as supporting evidence for the variant classification. As new information on genes, variants, and disorders become available everyday, it is important to update and integrate various databases such as ClinVar, HGMD (Human Gene Mutation Database) professional, OMIM (Online Mendelian Inheritance in Man), ENSEMBL Genes, NCBI Genes, HGNC (HUGO Gene Nomenclature Committee) PubMed, in-house database, etc as often as possible. The more information on each variant we can access, the more accurate molecular diagnosis we can make. Various databases are available at the variant level, gene level, and disease level. Insufficient or outdated information for variant interpretation can lead to an incorrect molecular diagnosis with incorrect variant classification. To minimize this risk, 3billion checks for any updates on each database every single day. The newer version of the updated database is downloaded and internally validated before it is applied to the variant analysis. See below Table 1 for the database list currently used at 3billion.

Table 1. Database list

Category	Database	Source	Version
Sequence	GRCh37/19 GRCh38/hg38	https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/ https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.40	GRCh37.p13 GRCh38.p14
Population frequency	gnomAD (variant and SV)	https://gnomad.broadinstitute.org/downloads (GRCh37) https://gnomad.broadinstitute.org/downloads (GRCh38)	v2.1.1 v3.1.2
Gene	HGNC	https://ftp.ebi.ac.uk/pub/databases/genenames/new/tsv/hgnc_complete_set.txt	Daily up to date
	NCBI gene	https://ftp.ncbi.nih.gov/gene/DATA/GENE_INFO/Mammalia/Homo_sapiens.gene_info.gz	Daily up to date
Transcript	RefSeq	https://ftp.ncbi.nlm.nih.gov/refseq/H_sapiens/annotation/annotation_releases/105.20220307/GCF_000001405.25_GRCh37.p13/GCF_000001405.25_GRCh37.p13_genomic.gff.gz https://ftp.ncbi.nlm.nih.gov/refseq/H_sapiens/annotation/annotation_releases/110/GCF_000001405.40_GRCh38.p14/GCF_000001405.40_GRCh38.p14_genomic.gff.gz	GRCh37.p13 GRCh38.p14
	Ensembl	https://ftp.ensembl.org/pub/grch37/release-87/gtf/homo_sapiens/Homo_sapiens.GRCh37.87.gtf.gz https://ftp.ensembl.org/pub/release-109/gtf/homo_sapiens/Homo_sapiens.GRCh38.109.gtf.gz	GRCh37.87 GRCh38.109
	GTEx	https://www.gtexportal.org/home/datasets	V8
Disease	OMIM	https://www.omim.org/downloads	Daily up to date
	Orphanet	https://www.orpha.net/consor/cgi-bin/index.php	2022.12
	CGD	https://research.nhgri.nih.gov/CGD/download/txt/CGD.txt.gz	2022.10
	HPO	https://raw.githubusercontent.com/obophenotype/human-phenotype-ontology/master/hp.obo, http://purl.obolibrary.org/obo/hp/hpoa/phenotype.hpoa	2023.01
	In-house database		Daily up to date
Variant	ClinVar	https://ftp.ncbi.nlm.nih.gov/pub/clinvar/xml/weekly_release/ClinVarFullRelease_00-latest_weekly.xml.gz	Weekly up to date
	UniProt	https://www.uniprot.org/downloads	2022.12
	DGV	http://dgv.tcag.ca/dgv/docs/DGV.GS.March2016.50percent.GainLossSep.Final.hg19.gff3 http://dgv.tcag.ca/dgv/docs/DGV.GS.hg38.gff3	2016.05.13
	HGMD	https://www.hgmd.cf.ac.uk/	version 2022.4
	In-house database		Daily up to date
Domain	UniProt	https://www.uniprot.org/downloads	2022.12
Prediction tool	dbNSFP (REVEL, GERP++RS)	http://database.liulab.science/dbNSFP	v4.3a
	dbscSNV (ADA_score, RF_score)	http://www.liulab.science/dbscsnv.html	v1.1
	SpliceAI	https://github.com/Illumina/SpliceAI	v1.3.1
	3Cnet	In-house database	
	RepeatMasKer	https://www.repeatmasker.org/RepeatMasker/	4.1.4
	REVEL	https://sites.google.com/site/revelgenomics/	May 3, 2021
	GERP	https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1001025	
Scientific literature	PubMed and Google Scholar		

2. Customized variant classification module

The American College of Medical Genetics and Genomics (ACMG) and the American Molecular Pathology (AMP) have put together standards and guidelines for variant interpretation in 2015 initially [16]. These guidelines and any updates followed are commonly adopted by many diagnostic laboratories. However, it is also known that even when the same guidelines are used, a variant can be given different classifications by different laboratories due to condensed/vague descriptions of various rules in the guidelines [17, 18]. 3billion tried to scrutinize and customize each rule in the guidelines to make them more precise based on existing knowledge gathered from the public databases and the internal database. This effort was developed into the variant classification module of EVIDENCE.

Variants are classified as pathogenic (P), likely pathogenic (LP), variants of uncertain significance (VUS), likely benign (LB), or benign (B) based on the guidelines suggested by the American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG/AMP). The ACMG/AMP guidelines have provided a framework for assessing the pathogenicity of genetic variants by considering a wide range of evidence. Various information such as variant type, predicted consequence, variant frequency, segregation, in silico prediction, and in vitro functional effect are integrated to determine the pathogenicity of each variant.

Nevertheless, the interpretation of genetic variants may result in discrepancies, leading to divergences between distinct testing facilities and even within a given laboratory, resulting in inconsistent classifications of the variants. 3billion has customized the guideline embodying each criteria with more specific rules and strengths so that at least within 3billion, variants are classified more consistently across different interpreters or timepoints.

This is described in more detail in Seo et al., 2020 [19].

a) Single nucleotide variants and small insertions/deletions (SNVs, indels)

ACMG/AMP guidelines proposed 28 criteria that can be assessed when determining variant pathogenicity.

1) Pathogenic criteria

PVS1

Null variant (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where the loss of function (LOF) is a known mechanism of disease.

- PVS1 criteria have been modified with reference to two articles [20]
- Exception

  PVS1 could be claimed when the absence of gene expression or protein production is experimentally proven through methods such as RNA sequencing, RT-PCR for mRNA expression, etc.

- Start loss variant: an alternative start codon should not be present in a near downstream region as in-frame or in another transcript (alternate transcript). Our system monitors the presence of previously reported pathogenic variants upstream of the new potential start codon. Classification is upgraded or downgraded accordingly.

PS1

Same Amino acid change as the previously established pathogenic variant, regardless of the nucleotide change.

- Variant type: missense variants.
- Definition of the established pathogenic variant: variants with P/LP determined by the ACMG guidelines' criteria, referenced from the reputable variant database (Table 1). Furthermore, medical geneticists perform a manual review of all previously documented pathogenic variants in order to verify their consistent pathogenicity.

PS2

De novo (maternity and paternity confirmed) variant, with matching highly specific symptoms from the disease and with no previous family history of the disease.

- Variant type: all types
- PS2 can be claimed for a previously reported de novo variant, with matching, highly specific symptoms. The variants reported as de novo in literature or in the in-house database have been manually curated by medical geneticists. Strength can be increased for recurrent de novo variants.

PVS1 evaluation

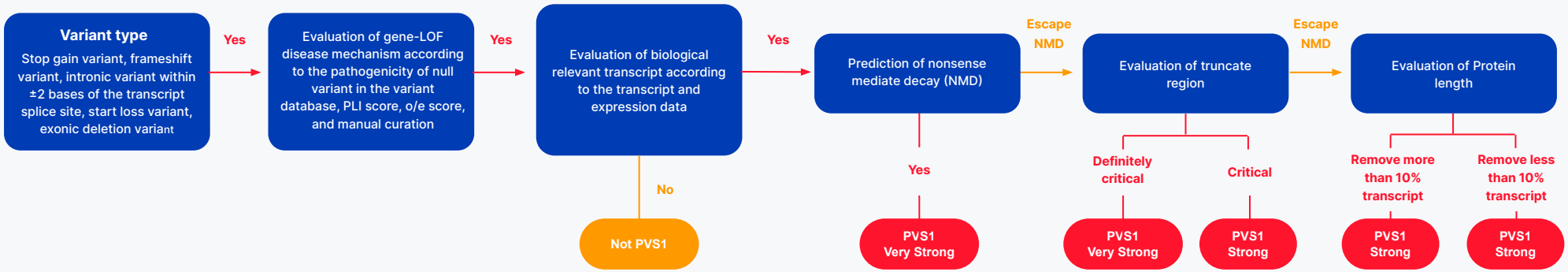


Figure 5. Schematic of PVS1 evaluation

PS3

Well-established in vitro or in vivo functional studies supporting a damaging effect on the gene or gene product.

- Variant type: all types
- PS3 can be applied if there is solid functional study data on the variant. Our Medical geneticists manually review the functional study data from external resources to determine if it was performed robustly.

PS4

Variant prevalence in the affected individuals is significantly higher than in the controls.

- Variant type: all types
- For exceedingly rare variants, a moderate level of evidence may be used: 1) insufficient case-control studies may be available to obtain statistical significance; 2) the variants for the identical phenotype are found in multiple unrelated patients, but not in the general population. The strength would be upgraded depending on the number of reports of variants in unrelated families [21].

PM1

Variant located in a mutational hot spot and/or a critical and well-established functional domain (e.g., the active site of an enzyme) without benign variation.

- Variant type: missense variants and in-frame variants
- Domain and variant databases are utilized to evaluate "well-established functional domains without benign variants". A mutational hot spot is determined by the distribution of pathogenic variants extracted from reputable databases.

PM2

Variant is absent from controls (or at extremely low frequency if recessive; see Table 6) in the Exome Sequencing Project, 1000 Genomes, or ExAC.

- Variant type: all variants
- The population frequency database evaluates the PM2, BA1, BS1, and BS2.
- The disease-specific allele frequency threshold (dMAF) is used to estimate the rarity of variants based on prevalence and penetrance [22]. If the prevalence of the disease is unknown, the prevalence is assumed to be 1/1,000,000.

Dominant disease

$$dMAF = \frac{Prevalence(d)}{2 * Penetrance(d)}$$

Recessive disease

$$dMAF = \sqrt{\frac{Prevalence(d)}{Prevalence(d)}}$$

PM3

Variant detected in trans with another Pathogenic variant for recessive disorders. Parental testing is required to determine a phase.

- Variant type: all types
- PM3 can be claimed for a previously reported variant in the trans phase with highly specific, matching symptoms. Phases of the variants from the literature and the in-house database are reviewed and updated manually by medical geneticists. The strength would be adjusted for recurrent occurrences.
- Markedly, variants found within 200 base pairs are assessed for phase status by each read, indicating that the interpretation of variants includes potential phase results.

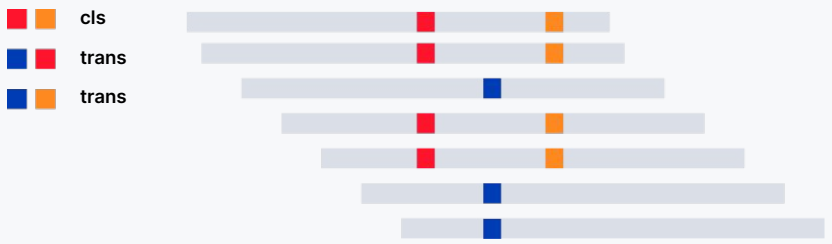


Figure 6. Phasing analysis based on reads

PM4

Changes in protein length due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.

- Variant type: in-frame deletion/insertions, stop loss variants
- The repeat region is determined by RepeatMasker.
- To avoid double-counting the same evidence, PM4 will not be claimed for variants already issued with PVS1.

PM5

Novel missense changes in amino acid residues where an alternative missense change has been previously reported to be pathogenic.

- Variant type: missense variants
- Definition of the established pathogenic variant: variants with P/LP determined by the ACMG guidelines' criteria, referenced from the reputable variant database (Table 1). In addition, medical geneticists review every previously reported pathogenic variant to confirm the established pathogenicity.

PM6

Assumed de novo, but without any confirmation of paternity and maternity.

- Variant type: all variants
- PM6 can be claimed for variants previously reported as assumed de novo variants if highly specific symptoms are matched. The assumed de novo variants in literature or in the in-house database will also be updated by medical geneticists.

PP1

Co-segregation of a causative gene and disease in multiple affected family members.

- Variant type: all types
- PP1 can be claimed for co-segregated variants with a previously reported disease in multiple affected family members. The updated variants would be manually curated by medical geneticists. The strength can be increased by the number of meiosis and affected relatives.

PP2

Missense variants in a gene where missense variants are observed as a common disease mechanism.

- Variant type: Missense variants

PP3

Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.).

- Variant type: missense variants, splice region variants outside +/-2-bp of a splicing junction, synonymous variants, and intron variants
- The functional effect of missense variants is predicted using programs such as REVEL [23], 3Cnet [24], and Splice AI [25].
- Splice region variants outside +/-2-bp of a splicing junction, synonymous variants, and intron variants are analyzed to predict the functional effect using Splice AI, ADA, and RF scores [26].

PP4

Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.

- Variant type: all types
- PP4 requires a similarity score >5 between the patient's phenotype and disease symptoms. Attention must be paid to applying this rule, as the symptoms provided may not be sufficient.

PP5

Variants reported as pathogenic in reputable sources, but the evidence might not be available for laboratories to perform an independent evaluation.

- Variant type: all types
- In 2018, ACMG/AMP made a recommendation to discontinue the use of PP5, due to the risk of possible double-counting [27]. However, external databases such as ClinVar are still actively used as important evidence for variant classification. To avoid the risk of missing such important evidence, 3billion applies the PP5/BP6 rules based on the level of evidence, after extensive review and evaluation of the variant by medical geneticists.

2) Benign criteria

BA1

Allele frequency is above 5% in the Exome Sequencing Project, 1000 Genomes, or ExAC.

- Variant type: all types
- Allele frequency is >0.05 in any general continental population dataset of at least 2,000 observed alleles. Non-continental populations (Jewish and Finnish groups) were excluded.
- A BA1 exception list has also been integrated [28].

BS1

Allele frequency is greater than expected for a disorder

- Variant type: all types
- Applied to variants with an allele frequency 10-fold or more in PM2 threshold.

BS2

Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age.

- Variant type: all types
- BS2 is applied depending on the inheritance pattern. Diseases with adult-onset and/or incomplete penetrance were excluded.

BS3

Well-established in vitro or in vivo functional studies showing no damaging effects on protein function or splicing.

- Variant type: all types
- Functional studies would be validated and proven by solid reproducibility in well-established clinical laboratory settings. Medical geneticists review the functional study data related to the variants.

BS4

Lack of segregation in affected family members.

- Variant type: all types
- BS4 can be claimed when disease variants are not segregated in the previously reported multiple affected family members. The updated variants are manually reviewed by medical geneticists.

BP1

Missense variant in a gene where premature termination variant is an expected mechanism of pathogenicity.

- Variant type: missense variants

BP2

Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern.

- Variant type: all types
- Variants located within 200 base pairs are evaluated for phase status read by read (Figure 6). BP2 can be accepted as a label when separate variants are confirmed to be located in the cis phase.

BP3

In-frame deletions/insertions in a repetitive region without known function.

- Variant type: in-frame deletion/insertion variants
- The repeat region is selected using the RepeatMasker.

BP4

No expected impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) measured by computational tools.

- Variant type: missense variant, splice region variant outside +/-2-bp of a splicing junction, synonymous variant, and intron variant
- The functional effect of missense variants is predicted by programs such as REVEL [23], 3Cnet [24], and Splice AI [25].
- Splice region variants outside +/-2-bp of a splicing junction, synonymous variants, and intron variants are analyzed to predict the functional effect using Splice AI, ADA, and RF scores [26].

BP5

Variants found with a disease that has an alternate molecular basis.

- Not applicable

BP6

Variants reported as benign in reputable sources, but the evidence might not be available for laboratories to perform an independent evaluation.

- Variant type: all types
- refer to comments on PP5

BP7

A synonymous (silent) variant predicted to have no impact on the splice consensus sequence or the creation of a new splicing site by splicing prediction algorithms, AND the nucleotide is not highly conserved.

- Variant type: synonymous variants
- Splice AI, ADA, RF score, and GERP++RS are used to predict the functional effects of synonymous variants.

(The criteria strength could be upgraded or downgraded via a manual review of our expert panel)

3) Rules for Combining Criteria to classify variants

Pathogenic	**1. Very Strong (PVS1) *AND*** a. ≥ 1 Strong (PS1 - PS4) *OR* b. ≥ 2 Moderate (PM1-PM6) *OR* c. 1 Moderate (PM1-PM6) and 1 Supporting (PP1-PP5) *OR* d. ≥ 2 Supporting (PP1-PP5) **≥ 2 Strong (PS1-PS4) *OR*** **1. Strong (PS1-PS4) *AND*** a. ≥ 3 Moderate (PM1-PM6) *OR* b. 2 Moderate (PM1-PM6) *AND* ≥ 2 Supporting (PP1-PP5) *OR* c. 1 Moderate (PM1-PM6) *AND* ≥ 4 Supporting (PP1-PP5)
Likely Pathogenic	**1. Very Strong (PVS1) AND 1 Moderate (PM1-PM6) *OR*** **1. Strong (PS1-PS4) *AND* 1-2 Moderate (PM1-PM6) *OR*** **1. Strong (PS1-PS4) *AND* ≥ 2 Supporting (PP1-PP5) *OR*** **≥ 3 Moderate (PM1-PM6) *OR*** **2 Moderate (PM1-PM6) *AND* ≥ 2 Supporting (PP1-PP5) *OR*** **1 Moderate (PM1-PM6) *AND* ≥ 4 Supporting (PP1-PP5)**
Benign	**1. Stand-Alone (BA1) *OR*** **≥ 2 Strong (BS1-BS4)**
Likely Benign	**1. Strong (BS1-BS4) and 1 Supporting (BP1-BP7) *OR*** **≥ 2 Supporting (BP1-BP7)**

** Variants should be classified as Uncertain Significance if other unmet or benign and pathogenic criteria are contradictory.

The 2015 ACMG/AMP guidelines marked all variants with conflicting evidence as VUSs. It would be reasonable if the level of evidence for pathogenicity and strength is comparable. However, the level of VUS can differ depending on the number and strength of criteria claimed to support pathogenicity. Notably, for SNV and small indel, a Bayesian framework is used to quantify the variant pathogenicity and make a final decision to determine accuracy by overcoming the limitations of the 2015 ACMG/AMP guidelines [29]. 3billion exploits the original guidelines along with the Bayesian scores and professional judgment for accuracy and validity in analyzing variants.

b) Copy number variants (CNVs)

ACMG/AMP guidelines proposed a semi-quantitative point-based scoring metric for CNV classification when determining variant pathogenicity. Separate scoring criteria have been developed for copy-number-loss and copy-number-gain and are interpreted using 5 different sections [30].

Section 1. Genomic content evaluation

Section 1 evaluates the genomic content in the affected CNV area. Based on reputable databases (Table 1), each CNV is checked if it contains any protein-coding regions, protomers, enhancers, or other regulatory regions. CNVs only containing non-coding/non-regulatory regions (UTR, intron, pseudogene) are more likely to be benign than pathogenic.

Section 2. Gene dosage evaluation

Section 2 evaluates individual genes that are inside the affected CNV region and determines whether the genes are known to be haploinsufficient or triplosensitive from reputable databases. Tools that predict haploinsufficiency or triplosensitivity are also used to support their pathogenicity. If the breakpoints are located inside the genes of interest and expected to result in loss of function is also vetted.

Section 3. Gene number evaluation

CNV is evaluated based on the number of genes within. CNVs that encompass a larger number of genes are expected to be more pathogenic than smaller ones.

Section 4. Evaluation of literature and public databases.

Section 4 compares a CNV to previously reported CNVs in the literature and reputable databases that overlap. Evidence such as the number of previously reported cases, reported segregation data, phenotype similarities alongside how unique they are, and, if possible, the prevalence of reported CNVs are all used to determine the pathogenicity.

Section 5. Evaluation of Patient Being Studied

In the final section, proband specific information is evaluated. Segregation information and specificity of patient phenotypes are used to determine the pathogenicity of a given CNV.

3. Phenotype similarity scoring module

Ultimately, the variant interpretation is carried on in the context of the patient's phenotype. EVIDENCE uses a 'symptom similarity scoring' module that scores how well the symptoms between the patient's phenotype and disease phenotype match. The symptom of each patient is converted to the corresponding standardized Human Phenotype Ontology (HPO) term, which in turn is used to compare to the HPO terms for each of the ~7,000 rare genetic disorders. The similarity between the patient's symptoms and the reported phenotypes of a certain disease is evaluated and presented as a similarity score ranging from 0 to 10. Empirical data suggests that a gene with 3billion's symptom similarity score >=6 has a significantly higher chance of being the diagnosis.

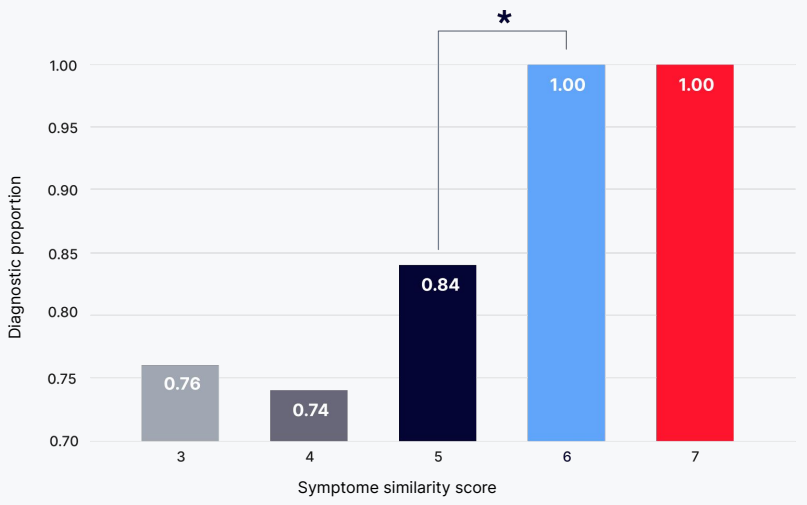


Figure 7. Probability of a gene being the diagnosis according to its symptom similarity score. There is a significant jump between the score of 5 and 6 (*P < 0.05).

3Cnet:

Pathogenicity prediction of human variants using multitask learning with evolutionary constraints

Missense variants are common, corresponding to 83% of nonsynonymous variants in the population, and many genetic disorders are caused by missense variants. According to dbNSFP, the possible number of missense variants within the human genome is 82,755,468. However, less than 100,000 missense variants are known to be pathogenic or benign with strong confidence, leaving the pathogenicity of most of the variants unknown. Therefore, various attempts have been made to develop artificial intelligence (AI)-based diagnostics using the rapidly increasing volume of genomic data.

3billion developed 3Cnet, which employs deep recurrent neural networks (RNNs) to predict pathogenicity based on the protein sequence around the variant [24]. This AI model can identify disease-causing variants of patients 2.2 times more sensitively. For the interpretation of variants, 3Cnet is only used to evaluate missense variants following the ACMG guideline. With its recent update to version 2, its capability of predicting the pathogenicity covers 99.99% of variants including start-loss, stop-gain, stop-loss, in-frame deletion, frameshift, in-frame insertion, delins, duplication, 5' extension, and 3' extension.

3Cnet makes use of 3 different genomic databases to train pathogenicity of variants effectively, and to avoid overfitting of the model network. 1) Clinical data which consists of pathogenic and benign variants from ClinVar database, 2) Common variants observed in the general human population from GnomAD database, 3) Conservation data, which refers to the simulated variants that we generated based on evolutionary conservation using UniRef database. The network architecture of 3Cnet is composed of two modules, feature extractor and pathogenicity classifier (Figure 8).

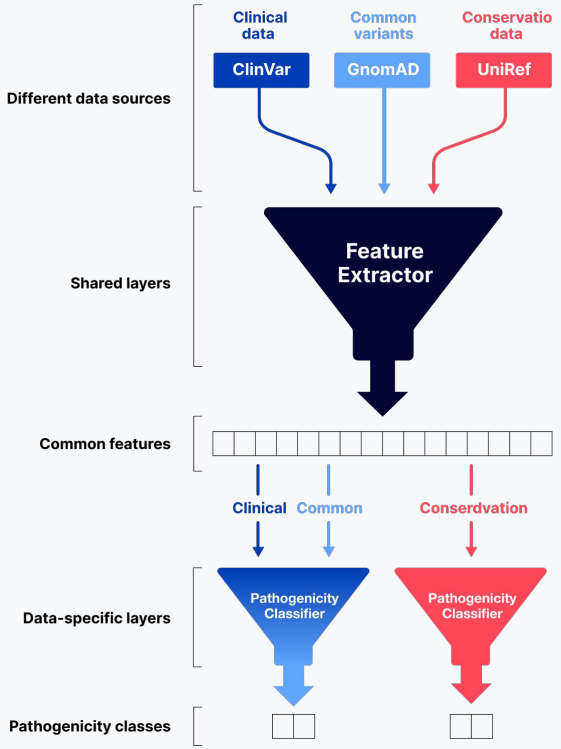


Figure 8. The deep learning network of 3Cnet. Multi-task learning using various genomic databases to avoid overfitting of the network to a small number of clinical data. conservation data reflects the evolutionary constraints given on genes.

3Cnet can classify pathogenic and benign variants the most accurately compared to other methods including REVEL, VEST4, SIFT, Polyphen2, PrimateAI, CADD, FATHMM, and DANN. Also, it can discover disease-causing variants in patient genomes with 2.2 times greater sensitivity than currently available tools, thereby improving diagnosis rates (Figure 9).

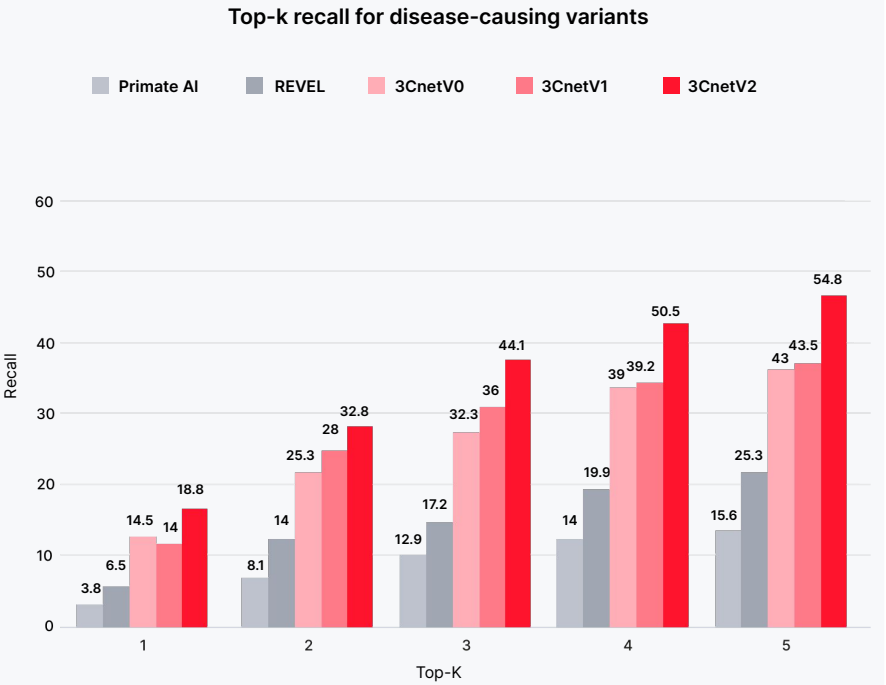


Figure 9. Prediction sensitivity of 3Cnet to discover disease-causing variants. The top-k recall rate implies the probability of determining the true disease-causing variant(s) among the top ranked variants using prediction scores.

3ASC:

Variant recommendation system

Although NGS-based genomic tests are routinely performed, analysis of its data and interpretation still requires much resources. Variant prioritization tools help the variant interpretation step and accelerate the process. Most variant prioritization tools are based on genotype-phenotype knowledge combined with the variant data. Currently, there is no single tool that outperforms and much improvements are warranted. 3billion also developed such a tool, called 3ASC [31]. 3ASC is a data driven machine learning model, which is composed of multiple weak learners. It leverages three features, patient's symptoms, ACMG Bayesian score, and variant calling quality to predict which variant is more likely to be disease-causing. The prediction performance was improved by learning variant interpretation patterns of clinical/medical geneticists. In a real world dataset including 3billion's patient data, 3ASC outperformed other variant prioritization models including LIRICAL and Exomiser (Figure 10). For 95.7% of the time, the disease-causing variants were within the top-5 variant list when 3ASC was used. Additionally, an ablation test was conducted to assess the contribution of artifactual variant risks. The approach leveraging the risk of artifactual variants showed significantly superior performance (Figure 11).

With its high-performance, 3ASC is now helping 3billion's clinical geneticists to interpret exome and genome results more efficiently.

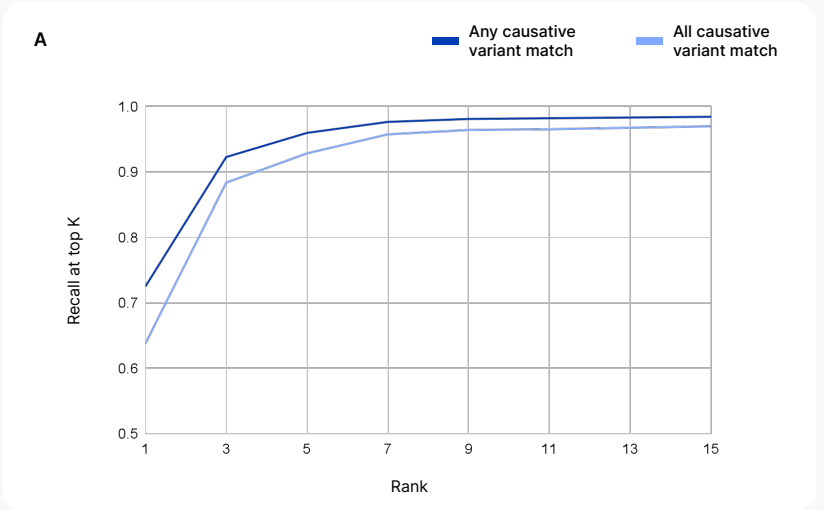


Figure 10. A. Model performance based on single match (any causative variant) and full match (all causative variants match) B. Comparison of recall of Exomiser, LIRICAL, and proposed model by gene-level match

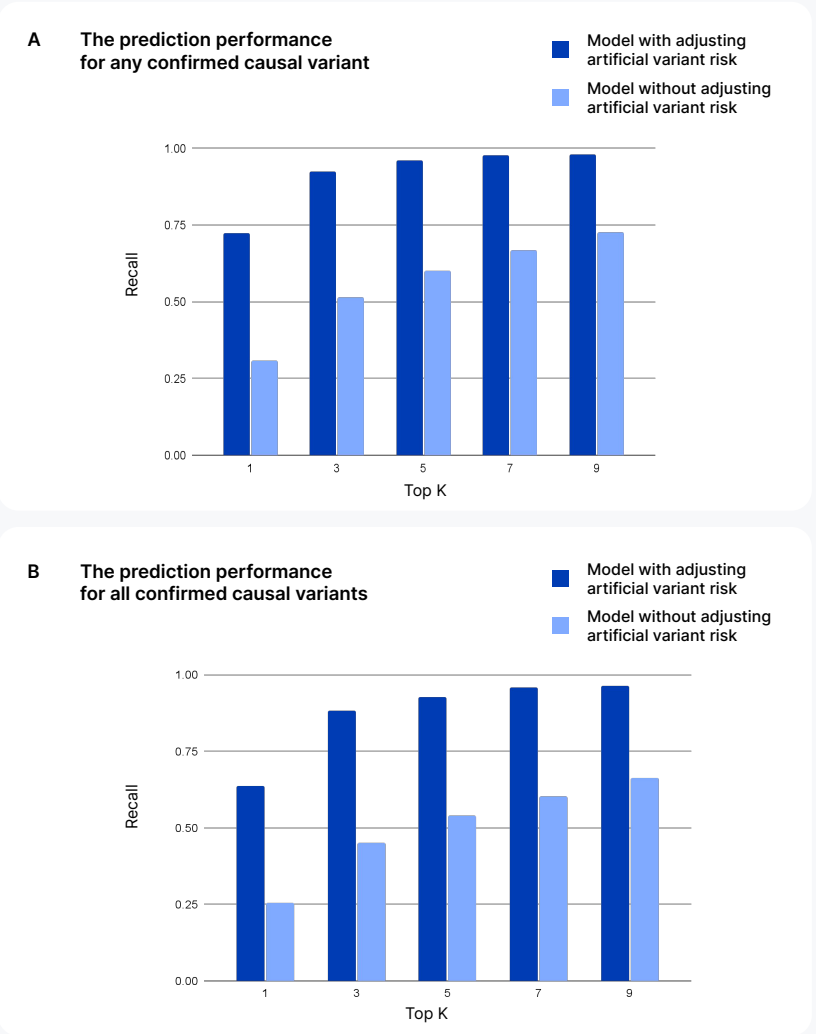


Figure 11. For the prediction of any confirmed causal variant, Figure 11-A showed that the model with adjusting the artificial variant outperformed than the model without leveraging this risk Also, Figure 11-B consistently showed the model with adjusting the artificial variant outperformed for the prediction of all confirmed causal variants.

3billion’s automated reanalysis system

It is reported that approximately 30% of exome-negative patients receive diagnoses through reanalysis service (interval: 2–3 years), with a considerable increase of 10–15% in the overall diagnostic rate [32, 33, 34, 35]. It also indicates an over five- and three-fold increase in the diagnostic rate compared with the chromosomal microarray technique and all genetic tests in clinical practice. Diagnosis through reanalysis reduces costs, as patients can avoid unnecessary redundant diagnostic testing. Moreover, patients and family members have a better chance of being involved in making the right treatment decisions.

3billion performs reanalysis of the exome sequencing data on all patients who did not receive a clear molecular diagnosis for their chief complaints. Patients have the option to opt-out from receiving the reanalysis. An updated report is generated at no cost if a clinically significant variant is identified or a previously reported variant is reclassified through the reanalysis.

3billion's reanalysis is performed through EVIDENCE using the latest supporting evidence downloaded by the automated database updating system. To estimate the molecular diagnostic rate from reanalysis, we tracked 1,064 patients with a neurodevelopmental delay between April, 2018-Feb, 2022 who were referred as part of a research project. 31 patients received a new diagnosis through reanalysis. The time interval between the initial analysis and the reanalysis that yielded a new diagnosis was 1.2 ± 0.9 years (from a minimum of 1 month to a maximum of 3.3 years, Figure 13). Most of the diagnosis from reanalysis were due to novel genes discovered in between the initial analysis and reanalysis.

*** At the moment, an automated daily reanalysis system is only applied for 3B-EXOME samples. Reanalysis of 3B-GENOME is performed per request.**

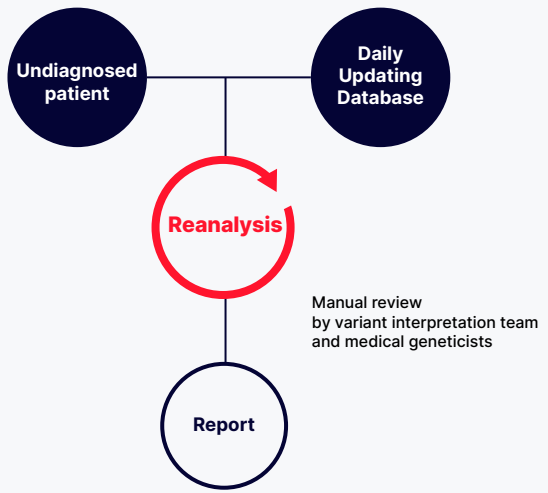


Figure 12. Daily reanalysis system. For patients with no clinically significant variants, EVIDENCE is run daily with the most recent annotation information. All variants reclassified as pathogenic or likely pathogenic in genes that could fit the patient's phenotype are reviewed by 3billion's medical geneticists.

3billion's reanalysis is performed through EVIDENCE using the latest supporting evidence downloaded by the automated database updating system. To estimate the molecular diagnostic rate from reanalysis, we tracked 1,064 patients with a neurodevelopmental delay between April, 2018-Feb, 2022 who were referred as part of a research project.

31 patients received a new diagnosis through reanalysis. The time interval between the initial analysis and the reanalysis that yielded a new diagnosis was 1.2 ± 0.9 years (from a minimum of 1 month to a maximum of 3.3 years, Figure 13)[36]. Most of the diagnosis from reanalysis were due to novel genes discovered in between the initial analysis and reanalysis.

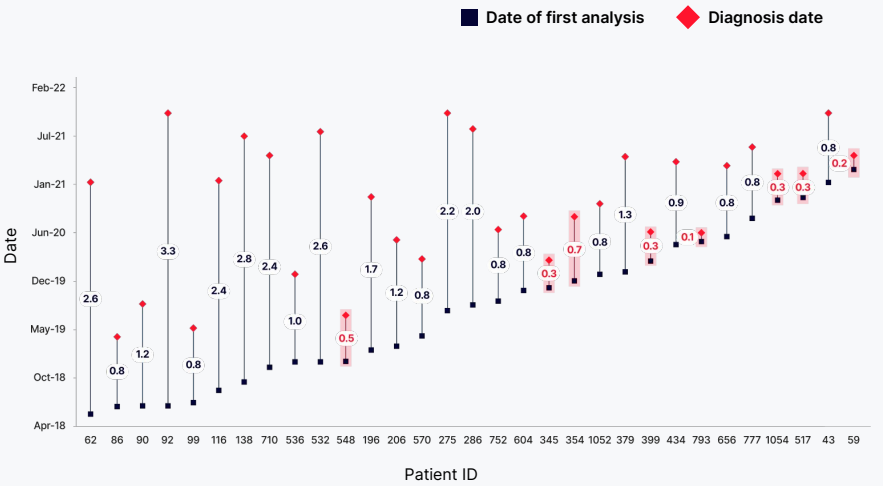


Figure 13. The time interval between the first analysis (blue dot) and the reanalysis yielded a molecular diagnosis (red dot) for 31 patients who received a diagnosis through reanalysis. X-axis: individual patients. Y-axis: Analysis date. The number shown for each patient: time interval in years

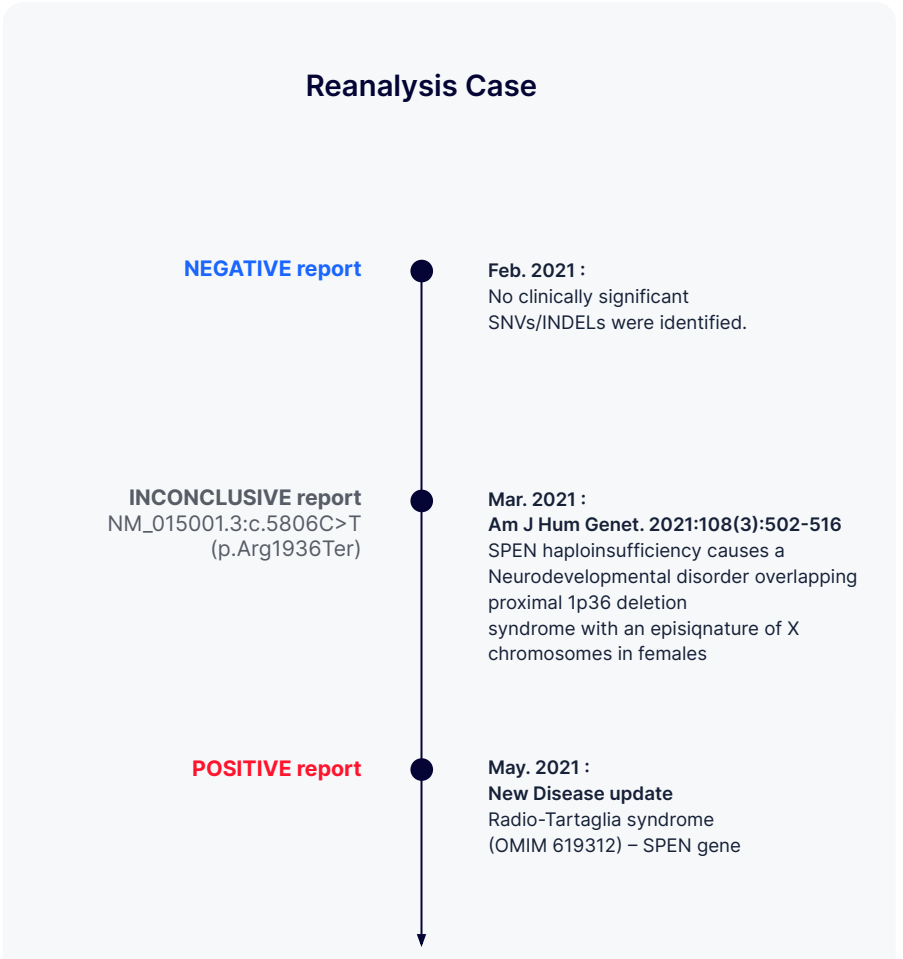


Figure 14. Case example of a patient's timeline from test order to diagnosis through reanalysis.

3billion’s reports

3B-EXOME/GENOME report

3billion’s NGS test report consists of 10 sections: Demographic/Clinical information; Results; Interpretation; Secondary findings (if opted in); Resources; References; Recommendations; Methods; Director’s Signature; Disclaimer. Variant information is mainly described in the Results, Interpretation and Secondary findings section as described below.

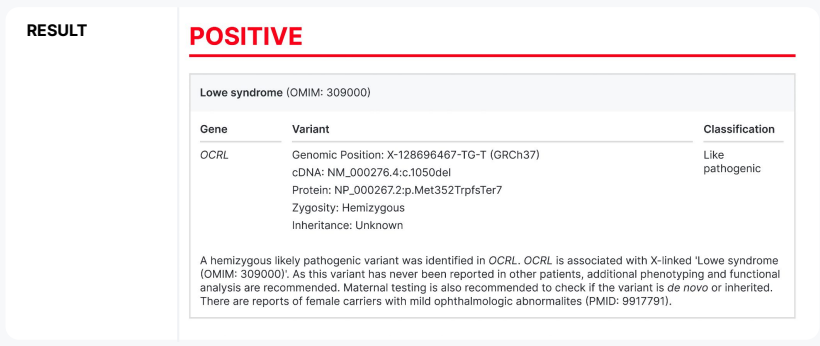
1. Results

Results can be positive, inconclusive, or negative. For positive and inconclusive reports, a variant table(s) is shown with the variant, gene and disease information as shown below.

POSITIVE:

Autosomal dominant (AD) or X-linked (XL) disease: one heterozygous or hemizygous P/LP variant in a known disease gene that would fit the patient's phenotype well will be described.

Autosomal recessive (AR) disease: one homozygous P/LP variant or two P/LP (potential) compound heterozygous variants in a known disease gene that would fit the phenotype well will be described.


RESULT

POSITIVE

Lowe syndrome (OMIM: 309000)

Gene	Variant	Classification
OCRL	Genomic Position: X-128696467-TG-T (GRCh37) cDNA: NM_000276.4:c.1050del Protein: NP_000267.2:p.Met352TrpfsTer7 Zygosity: Hemizygous Inheritance: Unknown	Like pathogenic

A hemizygous likely pathogenic variant was identified in *OCRL*. *OCRL* is associated with X-linked 'Lowe syndrome (OMIM: 309000)'. As this variant has never been reported in other patients, additional phenotyping and functional analysis are recommended. Maternal testing is also recommended to check if the variant is *de novo* or inherited. There are reports of female carriers with mild ophthalmologic abnormalites (PMID: 9917791).

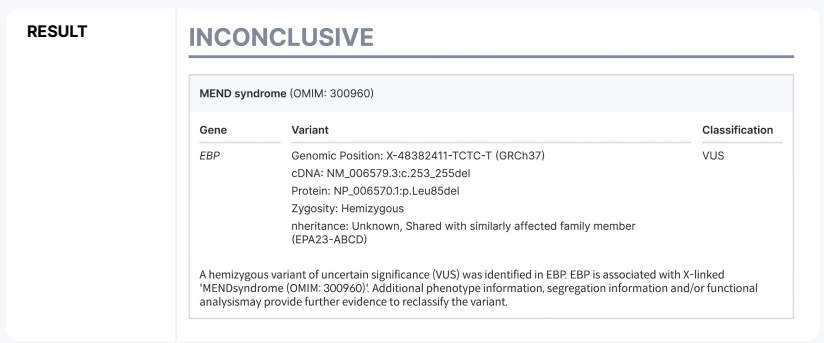
Figure 15. An example of a positive test result

INCONCLUSIVE:

AD or XL disease: one heterozygous or hemizygous VUS in a known disease gene that would fit the patient's phenotype well will be described.

AR disease:

- One homozygous VUS or two (potential) compound heterozygous VUS in a known disease gene that would fit the patient's phenotype well will be described.
- One P/LP variant and one VUS found as (potential) compound heterozygous in a known disease gene that would fit the patient's phenotype well will be described.
- One P/LP variant identified in a known disease gene that would fit the patient's phenotype well will be described. This is so that the ordering physician can perform additional tests on the reported gene as there are certain variant types that WES cannot detect.
- A P/LP variant(s) in a gene that is considered a novel gene that has sufficient evidence of being a disease gene and the patient's phenotype would fit well with the phenotype described for the gene. The gene may not be registered in gene-disease databases such as OMIM as a disease gene yet. In these cases, even though the variants are classified as P/LP, it is reported as an 'inconclusive' result.


RESULT

INCONCLUSIVE

MEND syndrome (OMIM: 300960)

Gene	Variant	Classification
EBP	Genomic Position: X-48382411-TCTC-T (GRCh37) cDNA: NM_006579.3:c.253_255del Protein: NP_006570.1:p.Leu85del Zygosity: Hemizygous nheritance: Unknown, Shared with similarly affected family member (EPA23-ABCD)	VUS

A hemizygous variant of uncertain significance (VUS) was identified in EBP. EBP is associated with X-linked 'MENDsyndrome (OMIM: 300960)'. Additional phenotype information, segregation information and/or functional analysismay provide further evidence to reclassify the variant.

Figure 16. An example of an inconclusive test result

NEGATIVE:

No clinically significant variant that would fit the patient's phenotype well is identified.

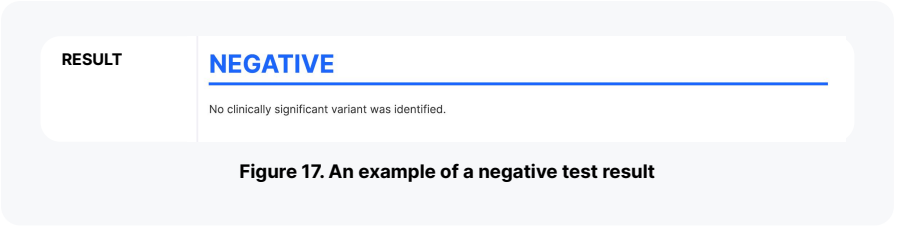


Figure 17. An example of a negative test result

For Copy number variants (CNVs) and Structural variants (SVs):

- 3B-EXOME: CNVs with 3 consecutive exons with sufficient evidence of loss or gain are reported as positive results with predicted intervals of the variant breakpoints. A disclaimer that the variant should be confirmed by an alternate clinical test is added to the report.
- 3B-GENOME: SVs with sufficient evidence are reported as positive. Most of the SVs are reported after Sanger confirmation with exact breakpoints. Occasionally, when the breakpoints cannot be determined, predicted intervals of the variant breakpoints will be reported with a disclaimer that the variant should be confirmed by an alternate clinical test.
- 3B-VARIANT: Only the variants with exact breakpoints are subject to 3B-VARIANT test.

2. Interpretation

As shown below in an example, the interpretation section provides detailed information of the variants being reported in the context of the ACMG guidelines: population data, predicted consequence and location of the variant, segregation data if family members were tested, computation and functional data from in silico prediction programs and literature, previous reports on the variant if available, disease association, Sanger validation results, and variant classification.

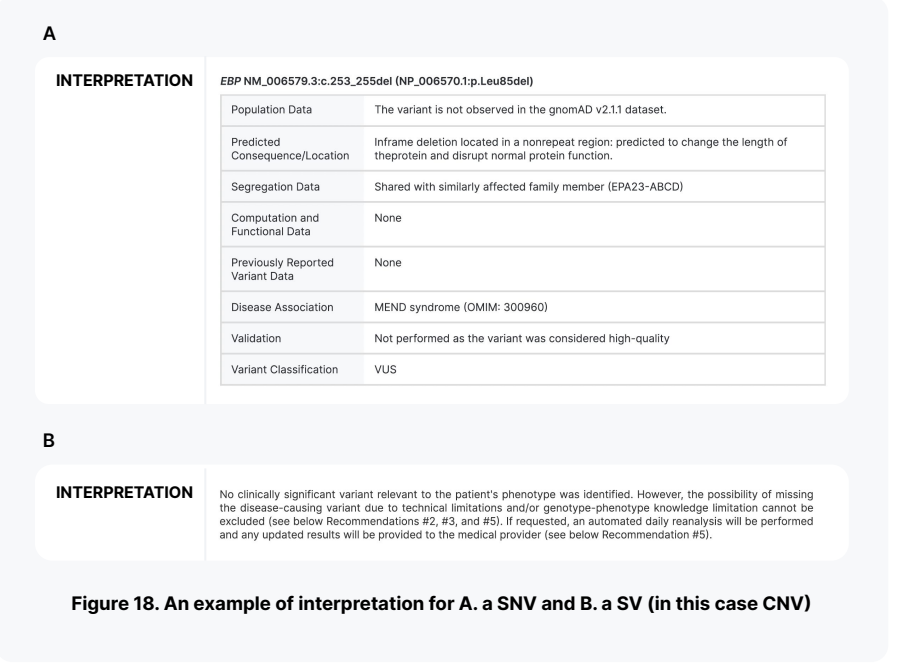
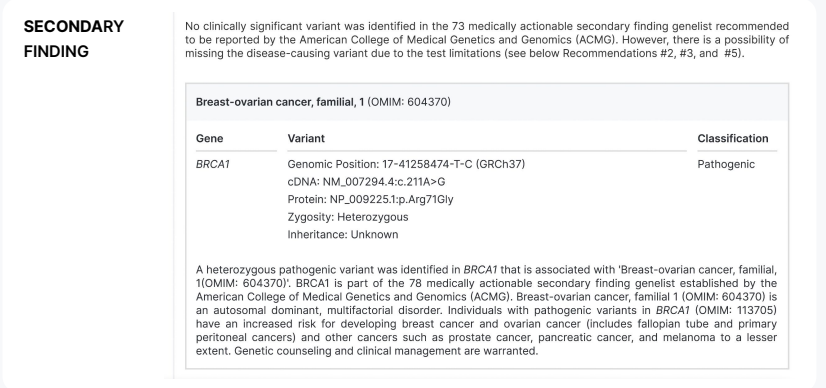


Figure 18. An example of interpretation for A. a SNV and B. a SV (in this case CNV)

3. Secondary finding (*if opted in*)

The secondary findings section describes the variant identified in one or more of the 78 genes that were selected by ACMG [37] as medically actionable and recommended to be reported if a pathogenic or likely pathogenic variant is found (details vary by gene). This section will be included only when the patient opts in to receive the information.



SECONDARY FINDING

No clinically significant variant was identified in the 73 medically actionable secondary finding genelist recommended to be reported by the American College of Medical Genetics and Genomics (ACMG). However, there is a possibility of missing the disease-causing variant due to the test limitations (see below Recommendations #2, #3, and #5).

Breast-ovarian cancer, familial, 1 (OMIM: 604370)

Gene	Variant	Classification
BRCA1	Genomic Position: 17-41258474-T-C (GRCh37) cDNA: NM_007294.4:c.211A>G Protein: NP_009225.1:p.Arg71Gly Zygosity: Heterozygous Inheritance: Unknown	Pathogenic

A heterozygous pathogenic variant was identified in *BRCA1* that is associated with 'Breast-ovarian cancer, familial, 1(OMIM: 604370)'. BRCA1 is part of the 78 medically actionable secondary finding genelist established by the American College of Medical Genetics and Genomics (ACMG). Breast-ovarian cancer, familial 1 (OMIM: 604370) is an autosomal dominant, multifactorial disorder. Individuals with pathogenic variants in *BRCA1* (OMIM: 113705) have an increased risk for developing breast cancer and ovarian cancer (includes fallopian tube and primary peritoneal cancers) and other cancers such as prostate cancer, pancreatic cancer, and melanoma to a lesser extent. Genetic counseling and clinical management are warranted.

Figure 19. An example of secondary findings

3billion’s reports

3B-VARIANT report

3B-VARIANT report consists of 4 sections: order information, result, methods, and references. Test result is described in the result section, consisting of 3 types of results; Positive, Negative and Inconclusive.

POSITIVE:

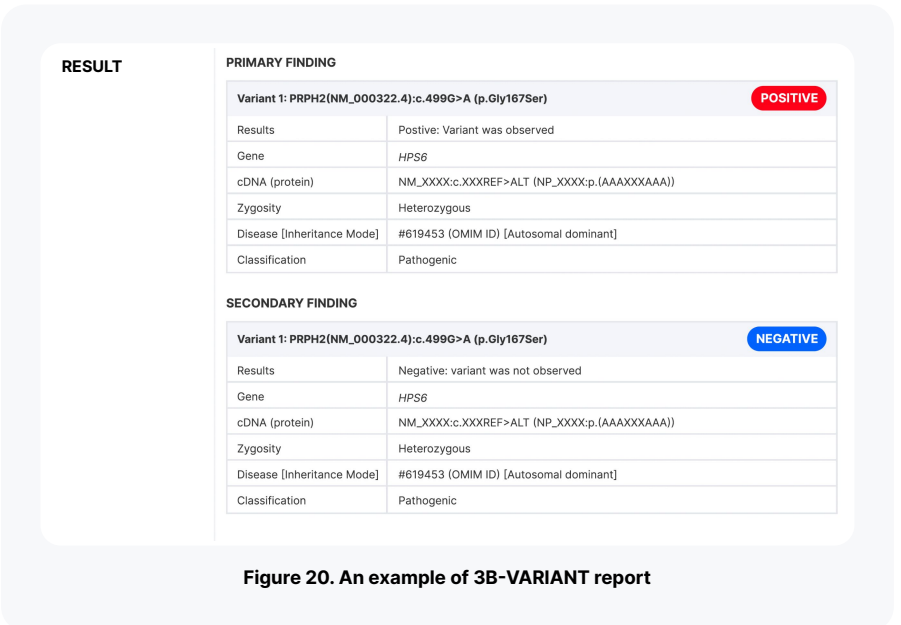
Positive result is delivered when the variant previously identified by 3B-EXOME or 3B-GENOME is also found in the sample ordered for 3B-VARIANT.

INCONCLUSIVE:

Inconclusive result means that sanger sequencing result was not clear, requiring additional testing by another method.

NEGATIVE:

Negative result is delivered when the sample does not carry the variant of interest.



RESULT

PRIMARY FINDING

Variant 1: PRPH2(NM_000322.4):c.499G>A (p.Gly167Ser)	POSITIVE
Results	Postive: Variant was observed
Gene	*HPS6*
cDNA (protein)	NM_XXXX:c.XXXREF>ALT (NP_XXXX:p.(AAAXXXAAA))
Zygosity	Heterozygous
Disease [Inheritance Mode]	#619453 (OMIM ID) [Autosomal dominant]
Classification	Pathogenic

SECONDARY FINDING

Variant 1: PRPH2(NM_000322.4):c.499G>A (p.Gly167Ser)	NEGATIVE
Results	Negative: variant was not observed
Gene	*HPS6*
cDNA (protein)	NM_XXXX:c.XXXREF>ALT (NP_XXXX:p.(AAAXXXAAA))
Zygosity	Heterozygous
Disease [Inheritance Mode]	#619453 (OMIM ID) [Autosomal dominant]
Classification	Pathogenic

Figure 20. An example of 3B-VARIANT report

For more information, check sample reports at our Resources page.

Conclusions

Over 255+ medical institutions across 55+ countries have used our service to diagnose 35,200+ suspected rare genetic disease patients (Figure 21).

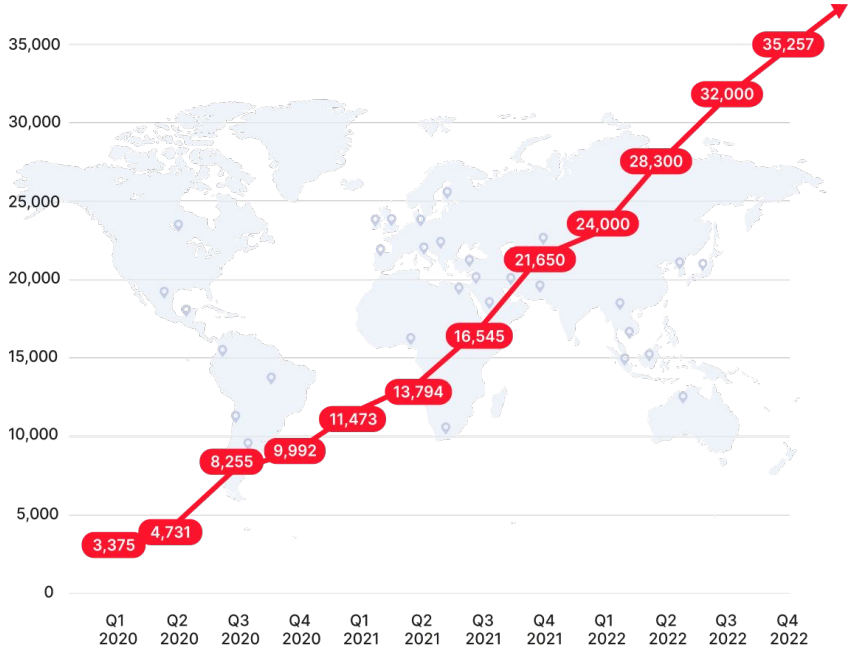


Figure 21. Accumulated number of patient between 2020–2022

The overall diagnostic rate of all tested patients is approximately 30%. The diagnostic rate varies among different disease categories as shown in Figure 22.

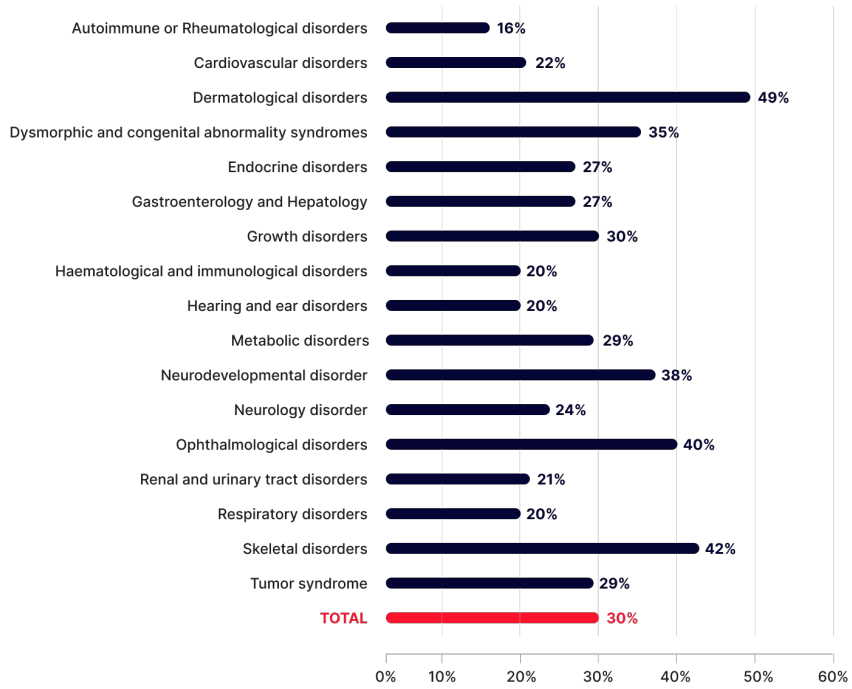


Figure 22. Diagnostic rate for different disease categories

The accumulated genomic and clinical data are invaluable sources to make the more accurate diagnosis achievable, for which we do research collaborations with physicians and investigators worldwide. 3billion is also committed to contribute in discovering drug targets using artificial intelligence and genomic data, which paves the path to a new drug for various rare diseases yet immedicable.

3billion is always here to help patients suffering from an undiagnosed rare genetic disorder until their diagnostic odyssey ends. We vision that no undiagnosed patient is left behind without access to genetic testing. Join us to work together to explore the world of rare genetic disorders.

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