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Analytical validation to determine performance of a whole genome sequencing test and implications of testing in the NICU environment

INTRODUCTION

Approximately 3.5 million babies are born each year in the United States, with over 400,000 of these infants admitted to the NICU.[1,2] Underlying genetic etiologies occur in approximately 1/3 of these babies, and in the past, diagnoses were most often made post-mortem. Regardless, infants with genetic disorders or congenital malformations indicating a possible genetic syndrome not only comprise a substantial proportion of NICU admissions, but disproportionately contribute to neonatal mortality, responsible for about 30–50% of neonatal and infant deaths.[3] Identifying the underlying etiology quickly for these critically ill infants and children can lead to life-saving treatment, information for the family about what to expect, or the initiation of palliative care.

Whole Genome Sequencing (WGS) is a comprehensive genetic test that uses state-of-the-art technology to analyze 98% of a person's genome using a single NGS assay and is now recommended as a first tier test for children with congenital anomalies, developmental delay, or intellectual disability by the American College of Medical Genetics and Genomics (ACMG).[4] Prior to WGS, a step-wise approach to testing was required for individuals with rare disease, congenital anomalies, or developmental delay/intellectual disability including multiple tests such as chromosome microarray (CMA), NGS panels, and whole exome sequencing (WES), which is time-consuming and often delays diagnosis and thus medical management or treatment.[5] WGS has a higher diagnostic yield and allows for a comprehensive analysis at a fast turn-around time to provide an accurate diagnosis much earlier than the traditional stepwise approach.

The WGS test sequences every nucleotide in the human genome of an individual to a depth of coverage necessary to build a highly accurate consensus sequence. This consensus sequence is then compared to normal reference sequences and the results are interpreted by board-certified laboratory directors and clinicians. Knowledge of the underlying genetic cause for an individual's symptoms can be used to determine diagnosis, prognosis, guide treatment, plan surveillance, and take preventative action. This is a retrospective analytical validation study to determine the performance of this WGS assay.

METHODS

A series of studies were performed to validate and refine a WGS test to determine the performance and clinical application of this diagnostic test. The WGS workflow is validated for various postnatal sample types (blood, buccal swab, saliva, cultured cell lines, purified DNA).

WORKFLOW

Figure 1 describes the general workflow for the WGS test. The current version of the test, and the most recent validation performed is described here. After genomic DNA extraction, the sample DNA underwent a fragmentation step (by shearing), followed by further library preparation including a probe-capture enrichment step. Paired-end next-generation sequencing (NGS) was subsequently performed on the Illumina NovaSeq 6000 sequencer with a PCR-free protocol, obtaining a minimum of 40X average coverage, with 2X150bp read length and 550bp average insert size. Data is then processed and analyzed with the Illumina Dragen pipeline version 3.8.4 and the Emedgene platform mapping to the GRCh38 human reference genome, with variant callers consisting of a haplotype based

small variant caller, a read depth based CNV caller, and the Manta SV caller. Variant calls are then post-processed with custom scripts. Prior versions of the validation used the same workflow, but earlier versions of the Dragen pipeline and Emedgene platform.

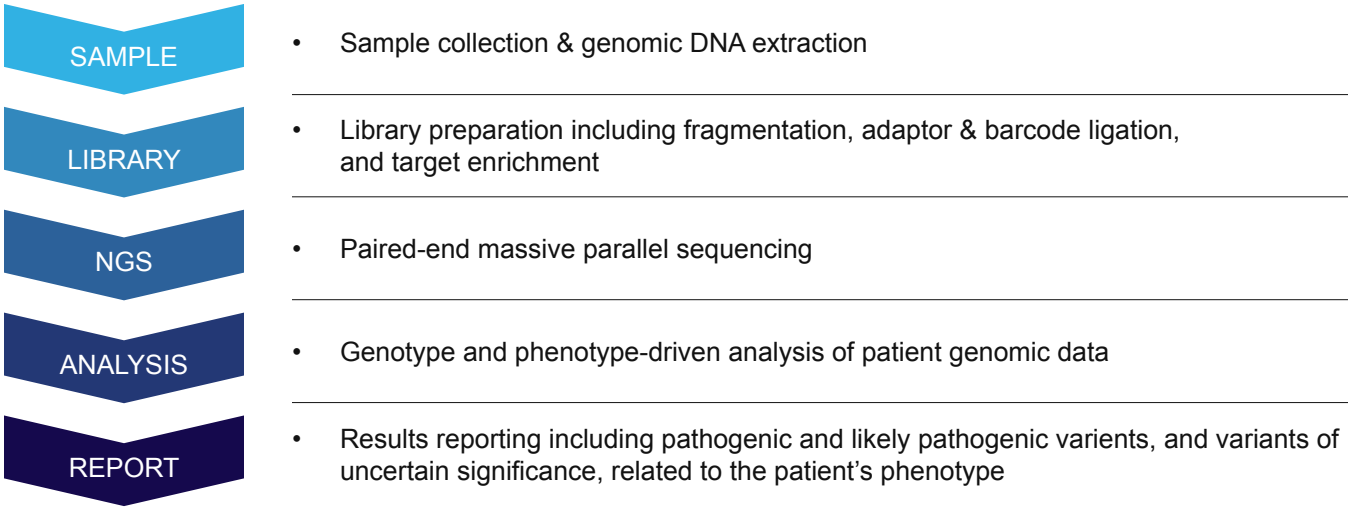

Figure 1. General whole genome sequencing workflow

The initial validation for the clinical WGS test included seventy-four unique clinical samples which were chosen for their richness of variant types. Importantly, variants that challenged the expected limit of detection/resolution and/or variants that are commonly detected in diagnostic testing were included to determine the reporting parameters for clinical testing. For reproducibility studies, four samples (three samples from a trio and one singleton sample) were run three times in one batch and in two additional batches. Sixty-three samples were whole blood, nine were extracted DNA, one cell line sample, and one amniotic fluid sample were included. Ten samples were analyzed together with their parent samples as complete trios. This initial validation and launch of the clinical WGS test occurred in 2019. In 2021, a follow-up validation study (WGSv2) was performed, to validate the test with the introduction of tagmentation. The number and types of positive variants for both validations are found in Table 1. All validation studies were performed at Baylor Genetics Laboratories.

Variant type	Number of Variants in 2019 validation	Number of Variants in 2021 validation
SNV	196	26
indel	12	11
CNV	94	7
Total	302	44

Note: Some samples had more than one variant.

Analytical and diagnostic performance of this assay was evaluated, including sensitivity, specificity, and accuracy (as described below).

$$\text{Sensitivity} = \frac{\text{True positive calls}}{\text{[True positive calls + False negative calls]}} * 100$$

$$\text{Specificity} = \frac{\text{True negative calls}}{\text{[True negative calls + False positive calls]}} * 100$$

$$\text{Accuracy} = \frac{\text{[True positive calls + True negative calls]}}{\text{All calls}} * 100$$

RESULTS

Analytical Sensitivity, Specificity, Accuracy for SNV and indel detection

As part of determining the analytical sensitivity, specificity, and accuracy for SNV and indel calling, one of the positive controls was a well-characterized Coriell sample. This sample includes high-confidence SNVs and indels previously validated by other sequencing techniques and is considered a gold-standard. Additional clinical samples previously analyzed at Baylor Genetics were included as positive (confirmed SNV and/or indel) or negative (no variants of clinical interest) controls.

The analytical sensitivity, specificity, and accuracy were determined to be >99.9% for SNV/indels in the initial validation of the clinical WGS test. Similarly, in the WGSv2 validation, specificity and accuracy were also >99.9%. While the sensitivity appeared to be slightly lower at 98.7% for SNVs and 95.2% for indels out of the WGSv2 validation, it was primarily due to the update of the high confidence regions and gold standard variants based on progress made in the research community. The assay performance in essence remained at the same level.

Copy Number Variant detection

Similarly, as part of determining the analytical sensitivity, specificity, and accuracy for CNV calling, two positive controls were high-confidence Coriell samples, each containing >90 previously validated CNVs (both gains and losses) considered gold standard positive findings. CNV analysis is based on a gene-centric backbone, therefore the analytical validation studies are focused on the CNVs overlapping the backbone (genic CNVs). We used percentage of overlapping between query CNVs and true CNVs to determine if a query CNV is truly positive.

The initial performance, prior to the manual review step, for CNVs was broken down by the size and type of the CNV. It is known that CNV calling is vulnerable to variable factors including data quality, regional complexity, and filtering criteria. Adjustments to filtering criteria can be made to increase sensitivity, however, that may also decrease specificity, thus manual CNV review using interactive visualization and curations tools was required to address this. As with SNVs, samples for CNV validation were chosen which challenged the expected limit of detection/resolution. Analytical sensitivity for all copy number gains was 75%. For gains >100Kb, the analytical sensitivity is 100% in this validation cohort. Analytical sensitivity for all copy number losses was 81%, and for losses > 100Kb the sensitivity in this validation cohort was 100%. Analytical specificity for all copy number gains was 91%. For gains >100Kb, the analytical specificity was 94% secondary to 1 false positive call in this cohort. Analytical specificity for all copy number losses was 85%, and for losses >100Kb, the specificity in this validation cohort was 100%.

In this validation, manual review was performed to determine if a CNV call was truly positive or a false positive given that some included CNVs were at the expected limit of detection/resolution. After manual review the analytical specificity was as follows: Analytical specificity for all copy number gains was 97%. For gains >100Kb, the analytical specificity was 100% (One false positive call was corrected after manual review). Analytical specificity for all copy number losses was 89%, and for losses >100Kb, the specificity in this validation cohort was 100%.

Finally, accuracy for all gains was 84% and gains >100Kb was 97%. Accuracy for all losses was 84% and losses >100Kb was 100% in this validation cohort.

CLINICAL PERFORMANCE CHARACTERISTICS

Clinical Sensitivity and Specificity of SNV/indels

Clinical sensitivity and specificity of SNV/indels were determined by using 209 reported clinical variants previously detected by WES or Sanger sequencing methods from the validation cohort. Sensitivity is 97.1% (202/208 variants detected). There were 7 variants not detected in this analysis, all with a variant allele fractions (VAF) <25% (range 2.7%-22%). Sanger confirmation was performed to evaluate the 7 discordant variants, showing that 6 of the 7 are true variants. When considering the six variants with low VAF, one variant CYP21A22 c.955C>T (p.Q319*) is embedded in a pseudogene that is known to be challenging for NGS-based calling. The other five variants with low VAF may have been missed secondary to lower sequencing depth. For variants with VAF above 25%, WGS can reach clinical sensitivity >99.9%. The clinical specificity was found to be 100% in this valdiation cohort.

Among the clinically positive variants, one intronic variant in the *MSANTD4* gene, c.463-2A>G, was reported by WES as a homozygous variant. However, the WES data has low coverage at this position. WGS correctly detected this variant as heterozygous, as was also confirmed by Sanger sequencing. To assess specificity, all samples not previously reported to carry the variant were tested and negative in the current analysis. Therefore, the clinical specificity for SNV/indels in this cohort was 100%.

Clinical Sensitivity and Specificity of CNVs

Clinical sensitivity and specificity of CNVs were determined by using the clinically reported variants from 48 samples previously analyzed by CMA or MLPA. The 94 selected true positive CNVs included 33 losses from 2Kb to 3.66Mb, and 61 gains ranging from 20 kb to 19.432 Mb. The clinical sensitivity of the copy number gains is 95% with three false negatives, and for the copy number losses the clinical sensitivity is 100% in this validation cohort. Manual review of the Dragen CNV call file revealed that all three false negatives were in gene desert regions with low coverage. This was addressed with an improved CNV analysis design in current versions of this test, even though there is low likelihood that these variants would be clinically significant. Including these variants in the validation was done to test the limits of detection. In practice, clinical sensitivity is likely higher, as variants in these desert regions are not expected to be clinically signficant. Clinical specificity for CNV calling is 98% for gains and 84% for losses. The overall clinical specificity for all CNV calling is 93%.

For the WGSv2 test, historical cases were used to determine clinical concordance. Previously identified variants of interest were matched with the current sample runs. Concordance was 100% for both SNVs/indels and CNVs.

Confirmation of Results

Sanger confirmation is rarely needed for SNV/indels for trio genome sequencing analysis. In the rare cases where the quality of the variant is questioned, and Sanger is required, it is usually when the variant occurs in a pseudogene. Conversely, when a CNV is detected and selected to be reported, it may need confirmation by an orthogonal method.

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Reportable Range

The reportable range for the current WGSv2 test encompasses all exonic regions in the transcript universe of GRCh38. Intronic regions extend into 35 bp from the exon-intron boundary as well as any region reported in HGMD padded by 20 bp. Non-coding transcripts that have never been reported to be associated with human disease (reported in HGMD version 2018.v4) are excluded. UTR regions of all the transcripts are included in the bed file.

DISCUSSION

These results demonstrate successful validation of a WGS test that provides a higher diagnostic yield than other genetic tests such as CMA and WES combined. There is a clear body of literature that supports WGS as the best genetic test for the sickest babies. Performing the most comprehensive test, with rapid results has an increased yield over WES of 17%.[6] Case examples from Baylor Genetics are included in the table below.

Clinical Indication	Molecular Diagnosis	Result - Gene/Variant/Type
Clinical diagnosis of Comelia de Lange syndrome, gastritis, esophageal stricture, vomiting, and seizures	Cornelia de Lange syndrome	HDAC8: NM 018486.3: c,738-1619G>A (deep intronic variant predicted to alter splicing)
Premature birth, macrocephaly, abnormality of the external nose, skin rash, skin plaque, purpura, cardiomyopathy. pulmonary venous hypertension, respiratory failure, lactic acidosis, hyperammonemia, overgrowth, maternal diabetes, increased mean corpuscular volume, anemia, neonatal hypotonia, tachycardia, tachypnea, macular purpura, hyperkalemia, antalgic gait. pallor, ecchymosis, protuberant abdomen, disseminated intravascular coagulation	Costello syndrome	HRAS:NM_005343.4:c.450+132_450+141del (deep intronic variant)
FTT, motor delay. generalized non-motor (absence) seizure, ataxia, abnormality of the palpebral fissures, abnormality of the philtrum, abnormality of the external nose, dermal translucency, gastroesophageal reflux, hypoglycemia, lactic acidosis, hyperammonemia, hypotonia, low-set ears, abnormality of the pinna	Congenital Myotonic Dystrophy Type1	TNR Pathogenic Expansion in the 3' UTR of DMPK (CTG). 1023 trinucleotide repeats detected. Maternal expansion -400-500 repeats
IUGR retrognathia, apnea, feeding difficulties, cryptorchidism, small scrotum, hypotonia, prominent nasal bridge, edema	Prader-Willi syndrome	SNORD116-1,UBE3A,GABRA5. HG38chr15:?22650251-27043625DEL 4393.37 (kb) Genome sequencing also showed the CNV may be secondary to a chromosome rearrangement event between chromosomes 15 and 17
Polyhydramnios, corneal opacity, narrow mouth, abnormality of the basal ganglia, hydronephrosis, flexion contracture, metabolic acidosis, anemia, macrocephaly, anasarca	MELAS	MT-TL1:ENST00000386347.1:n.14A>G (mitochondrial)
	Mandibuloacral dysplasia with type B lipodystrophy/ Restrictive dermopathy, lethal	ZMPSTE24:NM 005857.5: c.1085dup.p.L362Ffs*19/ c.11331137del.p.F378*

It should be noted that like all genetic tests, WGS has limitations. Not all regions of the genome can be covered sufficiently by WGS to detect genetic variation. Regions with a particularly 'difficult' genomic content (repetitive sequences, pseudogenes, etc.) may not be accurately evaluated. Small CNVs or CNVs that are in repetitive sequences may not accurately be detected. Nevertheless, WGS is the single, most comprehensive genetic test currently available. It is recommended as a first-tier test for children with congenital anomalies, developmental delay, or intellectual disability by the ACMG.[4] There is a clear increase in diagnostic yield as compared to WES, CMA and/or multi-gene panels, with the ability to shorten the diagnostic odyssey for children who can benefit from treatment sooner.

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CONCLUSION

Baylor Genetics offers a high-performing whole genome sequence test to families who need accurate diagnoses in a variety of settings. There is a clear increase in diagnostic yield when offering WGS as a first-tier test. Particularly important in the NICU setting, rapid WGS can be performed with a written result report issued in five days. This allows for life-saving treatment, information for the family about what to expect, or the initiation of palliative care for critically ill infants and children.

ORDERING INFORMATION

More information on ordering one of our WGS tests, including requisition forms and sample requirements, can be found on the Baylor Genetics website:

https://www.baylorgenetics.com/whole-genome-sequencing/

The requisition form includes the consent to opt in or opt out for several other types of findings:

- ACMG secondary findings
- Other medically actionable incidental findings known to be associated with disease but not known to be associated with the patient's phenotype
- Potentially clinically significant findings in genes unrelated to the patient's phenotype or in genes with no known disease associations.

Test code	Rapid TAT (5 days)	Regular TAT (6 weeks)
Proband only analysis	1829	1810
Duo analysis (proband + 1 parent)	1823	1803
Trio analysis (proband + both parents)	1822	1800

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