Non-invasive prenatal testing: Embracing low-input methodologies

Watchmaker DNA Library Prep Kit

Pilot and verification studies and routine implementation of the Watchmaker kit were performed under the supervision of Gabriel Minárik, Ph.D., Head of Trisomy Test Laboratories at Trisomy test Ltd, Slovakia. Results from this study were originally presented at the annual European Society for Human Genetics (ESHG) conference, held from 1 – 4 June 2024 in Berlin, Germany.

INTRODUCTION

Circulating DNA in maternal plasma consists of a mixture of maternal and fetal (placental) cell-free DNA (cfDNA). Low-coverage whole genome sequencing (WGS) of such samples enables early and highly sensitive, non-invasive prenatal screening for genomic abnormalities such as chromosomal aneuploidies and copy number variation (CNV) that are associated with genetic disorders or birth defects.

Fetal cfDNA fragments are ~143 bp in length and are distinguishable from slightly longer, ~166-bp maternal fragments.[1] The proportion of fetal DNA (fetal fraction, FF) in a cfDNA sample from a pregnant woman is critical in the ability to obtain an informative sequencing-based NIPT result. The average FF is approximately 10% at 11 – 13 weeks of gestation (the time frame in which NIPT is traditionally performed),[2] but is impacted by both maternal and fetal factors.[1,3] A fetal fraction of 4% is the lower limit for obtaining an informative WGS-based NIPT result. Approximately 0.5% of pregnancies fall below this limit, but this percentage may be much higher depending on the cohort. For instance, the proportion of samples with FF <4% increases from 0.7% at 60 kg maternal weight to 7.1% at 100 kg.[2]

Given these challenges, genomic library preparation for NGS-based NIPT must be highly efficient, robust, and consistent. In addition, turnaround time can be critical in providing feedback for clinical decision making. When establishing their NIPT pipeline, Trisomy test Ltd identified ten key criteria for their library preparation reagents and supplier (see text box to the right).

Their original, validated pipeline utilized the Illumina® TruSeq™ DNA Nano kit, which was regarded as the gold standard for library preparation from low-input samples at the time — despite a recommended input (50 ng – 200 ng) that is well above the amount of DNA typically available for cfDNA library preparation.

Key Criteria for NIPT Genomic Library Preparation Reagents and Supplier

- ☑ Low DNA input (mass, volume, and concentration)
- ☑ Compatibility with a variety of commercially available indexing kits
- ☑ Minimal sample handling (to mitigate potential errors, sample degradation and contamination)
- ☑ Speed
- ☑ Robustness
- ☑ Consistency
- ☑ Overall performance (success rates, specificity, PPV, sequencing economy)
- ☑ Support for IVD-R procedures[4] (incl. OEM option)
- ☑ Sales and technical support
- ☑ Competitive price

The Watchmaker DNA Library Prep kit was selected for evaluation due to its compatibility with ultra-low-input (down to 0.5 ng) samples[5] combined with Watchmaker's Custom Genomic Solutions capabilities[6] and competitive pricing. Here, we present data from a pilot study and subsequent verification of the Watchmaker DNA Library Prep Kit for Trisomy's NIPT pipeline. In addition to satisfying all Trisomy's key criteria, the Watchmaker chemistry resulted in an improvement in the percentage of informative results and enabled a 24-hour turnaround time from plasma extraction to test result.

EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

This study was conducted in two phases, namely a pilot study and a verification study. Sample details and key criteria for each phase are given in Figure 1.

Blood samples are routinely collected in EDTA or Cell-Free DNA BCT (Streck) tubes, depending on whether clinician-to-lab transportation time is expected to be less or more than 24 hours, respectively. All samples are archived in the form of plasma, stored at -15°C to -25°C for at least one year. Every sample that tested positive for an abnormality is stored indefinitely in this format for use in future verification/validation studies with the appropriate signed consent.

Cell-free DNA extraction was performed using Trisomy's validated protocol, starting from 680 µL of plasma. This typically yields cfDNA extracts with a concentration distribution ranging from >0 ng/µL (essentially unquantifiable) to 0.30 ng/µL (Figure 2). As 30 µL of extracted cfDNA is used for input into library preparation, this translates to an input mass range of unquantifiable to 9 ng. Samples processed with the Watchmaker DNA Library Prep Kit had a slightly lower mean concentration (0.171 ng/µL; 5.13 ng) than the mean for samples processed with the reference workflow (0.181 ng/µL; 5.43 ng).

In the cohort of samples collected in EDTA tubes, those with a concentration >0.30 ng/µL were excluded from further processing as these have historically been found to contain a higher than desired fraction of maternal DNA — presumably as a result of sample degradation. Since this reduces the fetal cfDNA fraction and, consequently, confidence in the test result, a second blood draw is requested in such cases. This concentration cutoff is not applied to samples collected in Streck tubes.

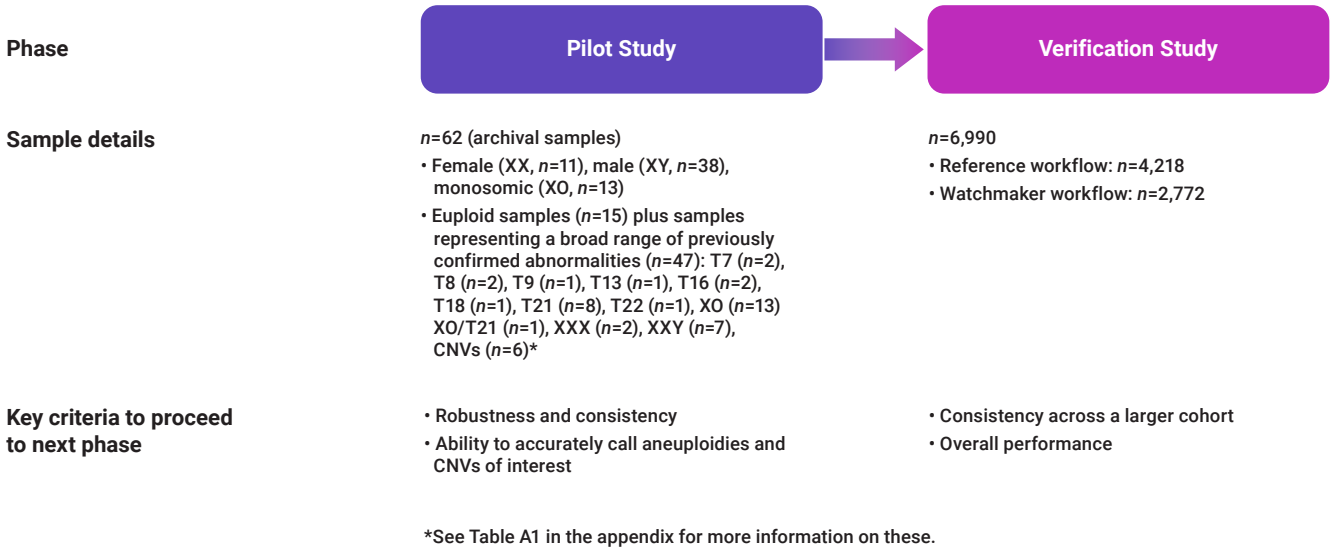


Figure 1. Experimental design. A set of 62 samples, representing a range of previously confirmed aneuploidies or other abnormalities, as well as normal genotypes (euploid samples), was selected for the pilot study. These archival samples were processed with the Watchmaker DNA Library Prep Kit and data were compared to historical data generated with Trisomy's existing, validated ("reference") workflow. Note that 63 structural variant calls were reported from 62 samples due to the complex phenotype of one of the samples (X0/T21). During the verification study, both workflows were run in parallel. Genotyping results for samples processed with both workflows were compared, whereas overall performance was assessed based on the total number of samples processed with each of the two workflows.

Library preparation workflows assessed are outlined in Figure 3. Thirty microliters of isolated cfDNA were used for both workflows, with no attempt to normalize for input mass. The Watchmaker protocol (bottom) starts with a size selection step, aimed at enriching input DNA for fetal fragments of approximately 150 bp, whereas this enrichment step is performed between end repair and adapter ligation in the reference workflow. Eight cycles of library amplification were performed for both workflows. Library QC was performed and sequencing pools prepared using Trisomy's existing, validated protocols. Low-coverage WGS was performed using an Illumina® NextSeq® 500 or 550 instrument. Data analysis was performed with Trisomy's validated, in-house developed analysis pipeline, which calculates a number of key performance parameters, including an estimate of fetal fraction in each library based on sequencing data.

It is important to note that library construction (from extracted cfDNA to sequencing-ready pool) takes 5 hours and 20 min with the reference workflow, with almost 3.5 hours of hands-on time. The streamlined Watchmaker protocol requires less than 3 hours of hands-on time, and can be completed in 4 hours and 20 minutes. Not only does this meet the key criterion of less sample handling, but — more importantly — enables a truly single-day pre-analytical process (from plasma separation to sequencer setup). With the Watchmaker kit, this process can be completed in 8 hours and 30 minutes. In contrast, the existing workflow requires an hour longer (9 hours and 30 minutes) and can therefore not be completed in a standard work day (Figure 3).

RESULTS

Pilot study

Robustness and consistency. The broad range of input DNA concentrations shown in Figure 2 translates to a cfDNA input of >0 – 10 ng into genomic library construction. This presents a challenge for optimal library amplification and the preparation of sequencing pools.

Electropherograms from the pilot study (Figure 4) indicated higher consistency in final library concentrations across samples processed with the Watchmaker DNA Library Prep Kit vs. the reference workflow.

Aneuploidy and CNV detection. The Trisomy pipeline delivers a Z-score for each chromosome in a sample, based on the relative abundance of specific chromosomal biomarkers. Additionally, the NIPT pipeline is designed to detect CNVs across the entire genome involving duplications/deletions of ≥3 Mb. With only minor optimization, the Watchmaker DNA Library Prep workflow yielded results that correlated well with historical data (Figure 4) and enabled clear interpretation for all chromosomes of interest (see Table A1 in the Appendix).

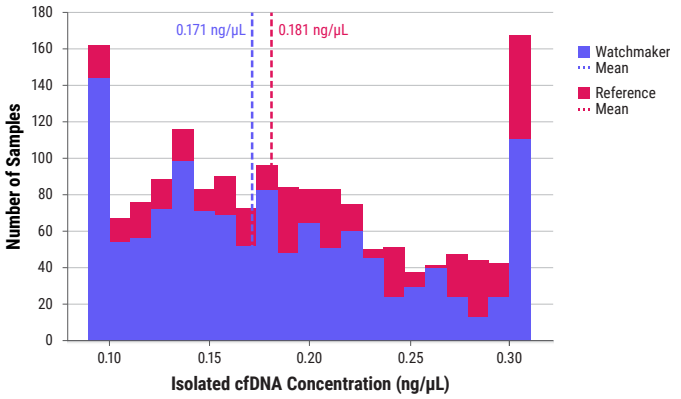


Figure 2. Concentration distribution of cfDNA extracts, determined using a Qubit™ fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). *n*=1,654 and 1,229 for reference and Watchmaker samples, respectively. All samples were collected from clinicians in Slovakia using EDTA collection tubes and processed in the Trisomy laboratory in Bratislava.

The set of 62 samples selected for the Watchmaker pilot study comprised a broad range of previously confirmed aneuploidies, namely:

- Trisomies: chromosome 7 (*n*=2), chromosome 8 (*n*=2), chromosome 9 (*n*=1), chromosome 13 (*n*=1), chromosome 16 (*n*=2), chromosome 18 (*n*=1), chromosome 21 (*n*=8), chromosome 22 (*n*=1)
- Sex chromosomes aneuploidies: monosomy (X0, *n*=13 and X0/T21, *n*=1) or trisomy (XXX, *n*=2 and XXY, *n*=7).

In addition, six samples contained other CNVs, whereas fifteen normal, euploid samples were included as a control group. Chromosome profiles for all samples processed with the Watchmaker kit correlated to those generated with the reference workflow (see Figure 5 for representative examples). This paved the way for a more extensive verification study, with a view toward implementation of the Watchmaker DNA Library Prep Kit in Trisomy's routine NIPT pipeline.

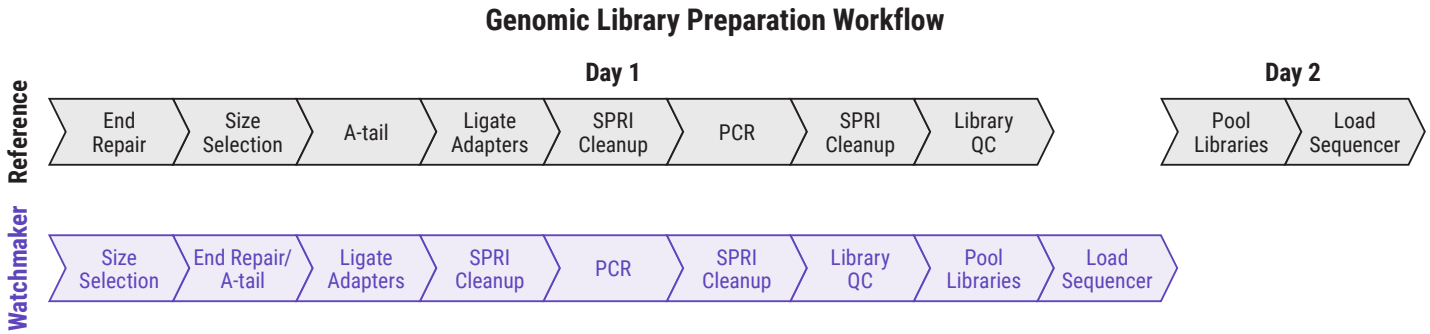


Figure 3. Genomic library preparation workflow from isolated cfDNA fragments to sequencer loading. The flexible, streamlined Watchmaker protocol (bottom) allows for upfront enrichment for ~150 bp fetal cfDNA fragments, requires fewer reagent additions than the reference workflow, and is compatible with all commercially available Y-shaped adapters.

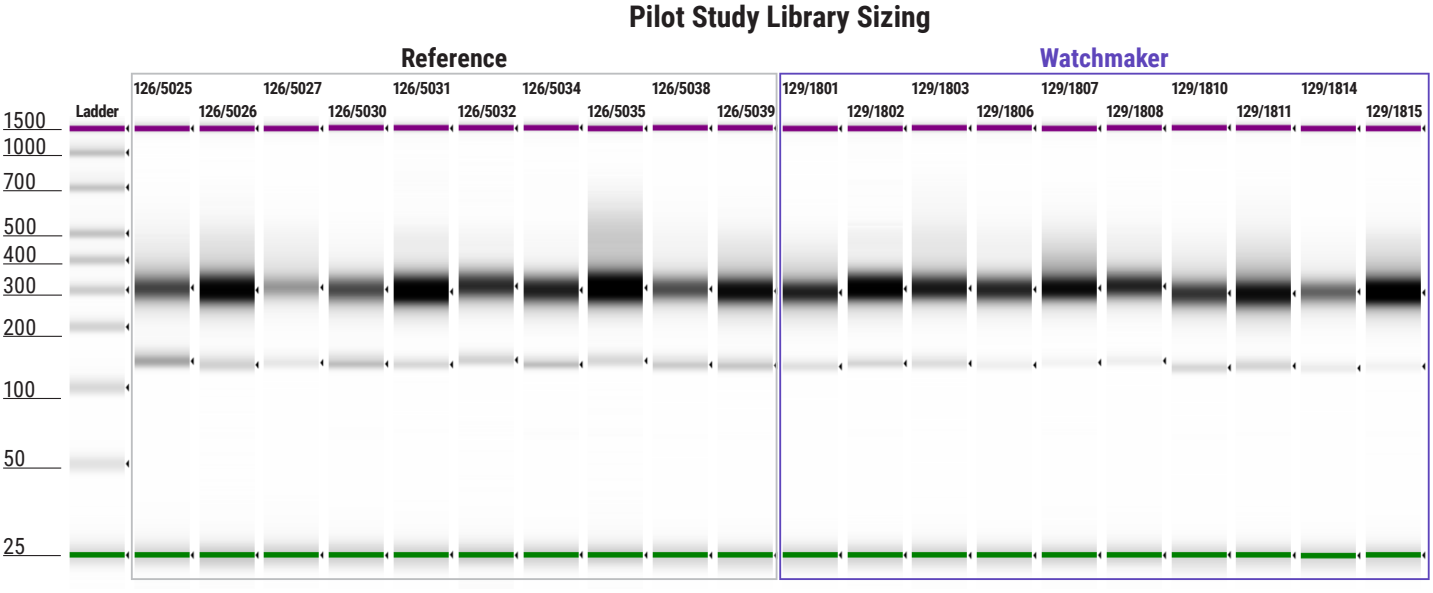
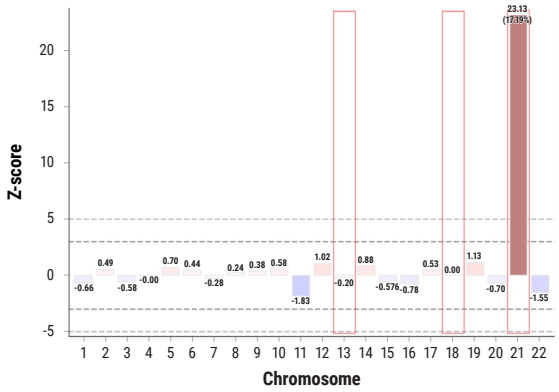
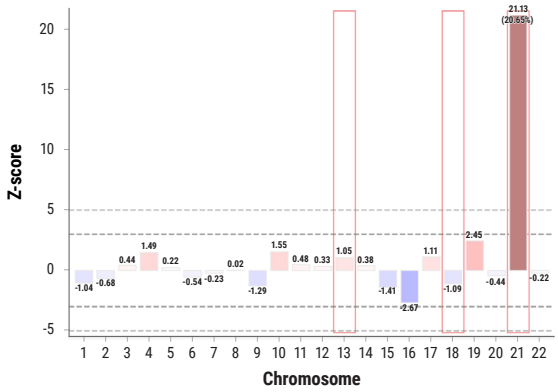


Figure 4. Electropherograms for representative reference libraries (left) and representative libraries from the Watchmaker pilot study (right). Electropherograms were generated with a 4200 TapeStation System and High Sensitivity D1000 DNA ScreenTape assays (Agilent Biotechnologies). Electropherograms indicate higher consistency in final library concentrations across samples processed with the Watchmaker DNA Library Prep Kit vs. the reference workflow.

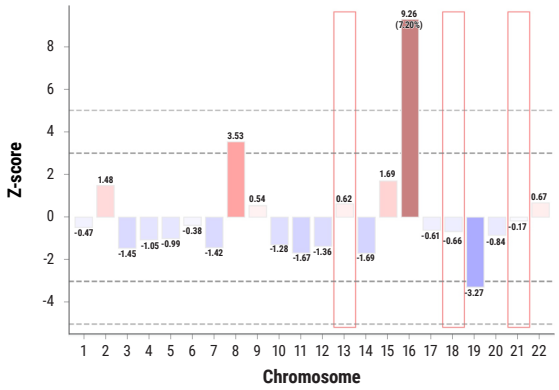
Reference	Watchmaker DNA Library Prep Kit

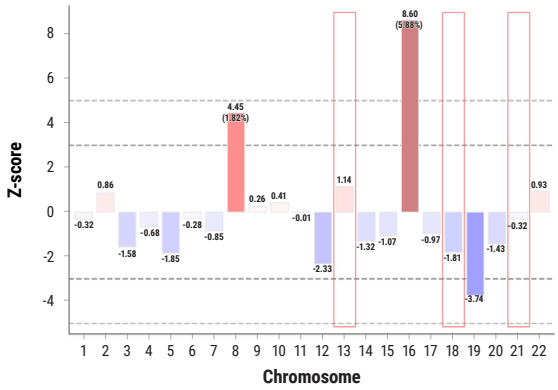
A. Previously confirmed T21 (Down syndrome) sample



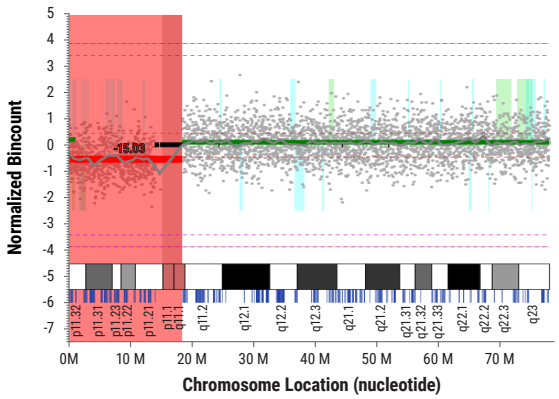


B. Previously confirmed Mosaic T16 sample





C. Sample with previously confirmed CNV – 18p deletion



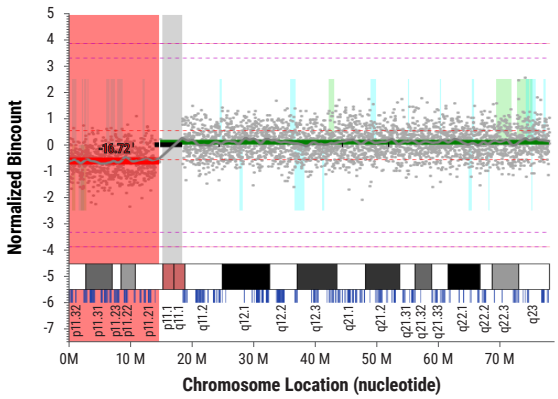


Figure 5. Representative examples of accurate aneuploidy and CNV detection in samples processed with the Watchmaker DNA Library Prep Kit during the pilot study. In **(A)** and **(B)**, the bars (chromosomal Z-score) indicate a high risk for trisomies of chromosomes 21, and 16. Z-scores ≥4 are indicative of a potential trisomy, whereas a Z-score ≤-4 suggests a potential monosomy. In **(C)**, a short arm deletion in chromosome 18 is designated by the area highlighted in red.

Verification study

Consistency. Improvements in the robustness and consistency of library preparation with the Watchmaker kit observed in the pilot study were confirmed during the verification study (Figure 6). The Watchmaker kit yielded a lower mean final library concentration (4.158 ng/µL) as compared to the reference workflow (7.024 ng/µL), which was at least partially attributed to the lower mean cfDNA input into library construction (refer to Figure 2). The library concentration distribution was, however, significantly narrower for the Watchmaker workflow. This facilitates the preparation of sequencing pools (eliminating the need to make additional dilutions for more concentrated libraries) and mitigates the risk of over- or under-sequencing libraries as a result of dilution errors

Overall performance. Overall performance metrics for the verification study (*n*=4,218 and 2,772 for the reference and Watchmaker workflows, respectively) are given in Table 1.

Library construction with the Watchmaker kit in the Trisomy workflow therefore enables two critical improvements in overall performance:

1. A 1% improvement in the total number of informative test results (from samples that would have failed as a result of low FF or other reasons). Given Trisomy's current annual test volume of approximately 9,300 samples, this translates to 93 pregnancies for which an informative result could be provided.
2. A reduction in overall turnaround time (as outlined in Figure 3). Since the Watchmaker kit enables a single-day pre-analytical workflow, sequencing can be completed and data analysis initiated overnight. This enables Trisomy to deliver a test result within 24 hours in urgent cases.

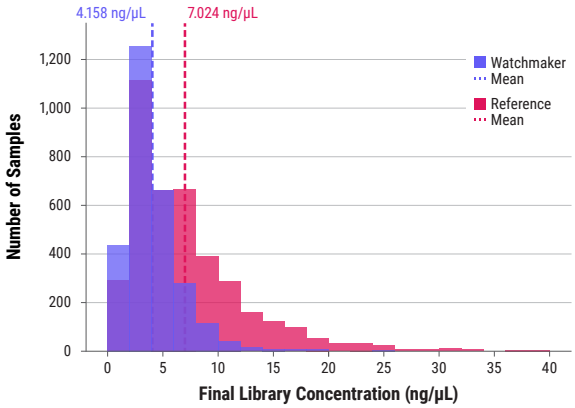


Figure 6. Final library concentration distributions for samples processed during the Watchmaker verification study, determined using a Qubit™ fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). *n*=4,218 and 2,772, for reference and Watchmaker samples, respectively. The lower mean library concentration obtained with the Watchmaker workflow did not impact library construction success (i.e., the percentage of libraries with sufficient yield for sequencing), whereas the narrower distribution facilitated the preparation of sequencing pools.

Table 1. Overall performance metrics

	Metric	Watchmaker	Reference
A	**Number of samples processed**	**2,772**	**4,218**
B	Number of samples with FF below 4% threshold	87	137
C	**Percentage of processed samples with FF below threshold (B ÷ A x 100%)**	**3.14%**	**3.25%**
D	Number of samples with acceptable FF (A – B)	2,685	4,081
E	Percentage of samples with acceptable FF (D ÷ A x 100%)	96.86%	96.75%
F	Number uninformative test results (for reasons other than below threshold FF)	24	74
G	**Percentage of uninformative results for samples with acceptable FF (F ÷ A x 100%)**	**0.87%**	**1.75%**
H	Number of informative test results	2,661	4,007
I	**Percentage of informative test results from samples with acceptable FF (H ÷ D x 100%)**	**98.19%**	**99.11%**
J	**Percentage of informative test results from all samples (H ÷ A x 100%)**	**96.00%**	**95.00%**
K	Total number of uninformative tests (all reasons)	111	211
L	**Percentage of samples for which an informative result could not be provided (all reasons) (K ÷ A x 100%)**	**4.00%**	**5.00%**

CONCLUSIONS

Trisomy test Ltd offers high-performance, non-invasive prenatal screening based on low-coverage WGS in Slovakia, the Czech Republic, Hungary, and through partnerships in several other countries in Eastern Europe. In a limited pilot (*n*=62) and extensive verification (*n*=2,772) study, the Watchmaker DNA Library Prep Kit was shown to outperform the library construction kit previously used in Trisomy's validated pipeline in terms of turnaround time, robustness, consistency, and overall success rates. Watchmaker satisfied all of Trisomy's key reagent performance and supplier criteria and has been adopted as Trisomy's preferred, validated library preparation chemistry. Future efforts include validating the test for larger-capacity Illumina® instruments, such as the NextSeq 1000 and 2000, as well as non-Illumina platforms.

APPENDIX

Table A1. Key chromosomes assessed using the Trisomy NIPT pipeline

Chr	Aneuploidy	Associated condition	Prevalence	Ref.
7	Trisomy	(Mosaic) trisomy 7	Rare (<1/106); generally considered lethal in embryogenesis. Surviving children are mosaics with highly variable phenotypes.	7,8
8	Trisomy	Mosaic trisomy 8	Rare with highly variable phenotypes	9
9	Trisomy	Mosaic trisomy 9	Rare (<1 in 1 million live births) with highly variable phenotypes	10
13	Trisomy	Patau syndrome	1 in 10,000 to 20,000 live births with antenatal mortality of over 95% of gestations	11
16	Trisomy	Mosaic trisomy 16	Rare with highly variable phenotypes	12
18	Trisomy	Edwards syndrome	1 in 3600 to 1 in 10,000 live births; positive correlation with maternal age	13
21	Trisomy	Down syndrome	1 in 319 to 1 in 1000 live births; varying in different populations and with positive correlation with maternal age.	14
22	Trisomy	Mosaic trisomy 22	Rare with highly variable phenotypes	15
X (X0)	Monosomy	Monosomy X, Turner syndrome	At least 1 in 2,000 to 2,500 live female births	16
X (XXY)	Extra chromosome X	XXY syndrome, Klinefelter syndrome	1:500 to 1,000 live male births	17

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For Technical Support, please contact support@watchmakergenomics.com.

5744 Central Avenue, Suite 100
Boulder, CO 80301
www.watchmakergenomics.com

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