

Development and validation of a next-generation sequencing–based protocol for 24-chromosome aneuploidy screening of embryos

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Objective: To validate a next-generation sequencing (NGS)–based method for 24-chromosome aneuploidy screening and to investigate its applicability to preimplantation genetic screening (PGS).

Design: Retrospective blinded study.

Setting: Reference laboratory.

Patient(s): Karyotypically defined chromosomally abnormal single cells and whole-genome amplification (WGA) products, previously analyzed by array comparative genomic hybridization (array-CGH), selected from 68 clinical PGS cycles with embryos biopsied at cleavage stage.

Intervention(s): None.

Main Outcome Measure(s): Consistency of NGS-based diagnosis of aneuploidy compared with either conventional karyotyping of single cells or array-CGH diagnoses of single blastomeres.

Result(s): Eighteen single cells and 190 WGA products from single blastomeres, were blindly evaluated with the NGS-based protocol. In total, 4,992 chromosomes were assessed, 402 of which carried a copy number imbalance. NGS specificity for aneuploidy call (consistency of chromosome copy number assignment) was 99.98% (95% confidence interval [CI] 99.88%–100%) with a sensitivity of 100% (95% CI 99.08%–100%). NGS specificity for aneuploid embryo call (24-chromosome diagnosis consistency) was 100% (95% CI 94.59%–100%) with a sensitivity of 100% (95% CI 97.39%–100%).

Conclusion(s): This is the first study reporting extensive preclinical validation and accuracy assessment of NGS-based comprehensive aneuploidy screening on single cells. Given the high level of consistency with an established methodology, such as array-CGH, NGS has demonstrated a robust high-throughput methodology ready for clinical application in reproductive medicine, with potential advantages of reduced costs and enhanced precision. (Fertil Steril® 2014;101:1375–82. ©2014 by American Society for Reproductive Medicine.)

Key Words: Comprehensive chromosome screening, preimplantation genetic screening, array comparative genomic hybridization, next-generation sequencing, embryo aneuploidy

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Chromosomal aneuploidy is recognized to be a significant contributing factor in implantation failure and spontaneous miscarriage (1) and is likely to be responsible for the majority of IVF failure. Preim-

plantation genetic diagnosis for aneuploidy screening of embryos derived from subfertile patients undergoing IVF, also termed preimplantation genetic screening (PGS), enables the assessment of the numeric chromo-

somal constitution of embryos before transfer. PGS offers IVF couples an additional selection tool, over simple morphologic and developmental characteristics (2), for choosing the most competent embryo(s) for transfer (3). It aims to enhance embryo selection, identifying and selecting for transfer chromosomally normal (euploid) embryos to increase the implantation and ongoing pregnancy rate for IVF patients, reduce the time to pregnancy, lower the incidence for miscarriage, and reduce the risk of an aneuploidy condition at term (4).

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Most of the initial studies of PGS, involving biopsies of single blastomeres from cleavage-stage embryos and the use of the fluorescence in situ hybridization (FISH) technique, provided disappointing clinical results. In fact, a large number of prospective randomized clinical trials (RCTs) have consistently failed to show any improvement in delivery rates with the use of FISH-based PGS (5), although one recent RCT has reported a significant increase in live birth rates in patients with advanced maternal age (6). One of the possible reasons for this poor clinical performance has been attributed to the well known limitations of the FISH technique, which screens for a minority of chromosomes, those most commonly observed in pregnancy loss and aneuploid deliveries, which are not necessarily the most relevant for early embryos. This may have led to the transfer of reproductively incompetent embryos with aneuploidy for chromosomes that were not analyzed. Thus, the reduced diagnostic accuracy of the FISH technology could have compromised any potential benefit of screening (7, 8). Therefore, the focus has now shifted to new technologies that allow for comprehensive screening of chromosomes or full karyotyping to provide a more accurate assessment of the reproductive potential of embryos.

A variety of methodologies for 24-chromosome analysis have been developed and are currently available for clinical use, including array comparative genomic hybridization (array-CGH) (9, 10), metaphase comparative genomic hybridization (11–13), single-nucleotide polymorphism microarrays (14, 15), and quantitative polymerase chain reaction (16).

Array-CGH was the first technology to be widely available for 24-chromosome copy number analysis (17) and is now used extensively around the world. This method uses microarray technology to deliver comprehensive aneuploidy screening through its ability to detect imbalances in any of the 24 chromosomes rather than the limited chromosome assessment achievable by FISH (9, 10, 17).

The first data from the clinical application of comprehensive chromosome screening techniques showed that aneuploidies may occur in preimplantation embryos in any of the 24 chromosomes, indicating that comprehensive aneuploidy screening is necessary to determine whether an embryo is chromosomally normal (9, 10, 14). Initial studies have also documented significantly improved consistency (9, 10, 14, 15, 18) and predictive value for aneuploidy diagnosis (19, 20), as well as high pregnancy outcomes following transfer of screened embryos (10, 13, 21–24).

The rapid development of next-generation sequencing (NGS) technologies has generated an increasing interest in determining whether NGS could be reliably applied for PGS purposes and if the technique may offer any improvements for the detection of chromosomal aneuploidy in preimplantation embryos compared with current comprehensive aneuploidy screening technologies. NGS may ultimately provide a number of advantages, including reduced costs and enhanced precision as well as parallel and customizable analysis of multiple embryos in a single sequencing run.

For chromosome copy number analysis by NGS, the principle involves fragmenting the amplified embryonic DNA into small fragments (100–200 base-pairs). Hundreds of thousands of these fragments are sequenced in parallel until a suf-

ficient sequencing depth (i.e., the number of sequence reads covering a given position in the genome) is acquired. The sequence data from chromosomes across the genome are first compared with the reference genome and then counted with the use of specialist software. Because the number of sequences from a specific chromosome should be proportional to the copy number, trisomy or monosomy will result in greater or lower numbers of reads, respectively (25, 26). With the use of this approach with single blastomeres or trophectoderm samples from blastocyst biopsies, both whole chromosome aneuploidy and segmental chromosome imbalances would be detected (27).

However, new comprehensive technologies need thorough validation determining the preclinical accuracy against a different and more established method before they might be considered within the standard of care in reproductive medicine. The present study investigated the accuracy of an NGS methodology for comprehensive chromosome screening as a preclinical step toward its application in the diagnosis of chromosomal aneuploidy on embryos at cleavage stage or blastocyst stages of development.

MATERIALS AND METHODS

Experimental Design

This study was organized into two steps of analysis. The first step involved a blind evaluation of karyotypically defined chromosomally abnormal single cells derived from cultured amniotic fluids or products of conception (POCs). The second step involved a retrospective blinded assessment of whole-genome amplification (WGA) products, selected from 68 consecutive clinical PGS cycles performed on single blastomeres biopsied from cleavage-stage embryos in the period of May–December 2010. The indications for PGS included: advanced maternal age ($n = 48$; mean age 40.7 ± 2.1 years, range 38–45); repeated implantation failure ($n = 16$; mean age 33.6 ± 2.7 years, range 29–37); and recurrent miscarriages ($n = 4$; mean age 28.8 ± 5.5 years, range 25–37).

Consistency of NGS-based 24-chromosome copy number assignments was evaluated with both previously established cytogenetic karyotypes (single cells) and array-CGH-based diagnoses (WGA products) at the level of individual chromosome copy numbers for the entire 24 chromosomes of each sample tested and for the overall diagnosis of aneuploidy or euploidy. Discordant samples were subsequently reevaluated by a third methodology, quantitative fluorescent polymerase chain reaction (QF-PCR), following the protocol described elsewhere (28). When QF-PCR confirmed one of the initial methods, the remaining discordant method was considered to have delivered an erroneous result.

Single-cell Lysis and Whole-genome Amplification

Karyotypically defined single cells and blastomeres were first lysed and genomic DNA amplified with the use of the Sureplex DNA Amplification System (Bluegenome), according to the manufacturer's protocol. One nanogram of genomic DNA and one reagent-negative control (amplification mixture only) were also subjected to WGA.

Array-CGH Analysis

WGA products were processed according to the Bluegenome 24sure V3 protocol (available at www.cytochip.com). These products were fluorescently labeled and competitively hybridized to 24sure V3 arrays (Bluegenome) with a matched control sample in an array-CGH experiment format, as described elsewhere (10).

Library preparation. Whole-genome amplification products were purified with the use of the Zymo DNA Clean and Concentrator (Zymo Research) and quantified with the use of the Qubit dsDNA HS Assay Kit (Life Technologies).

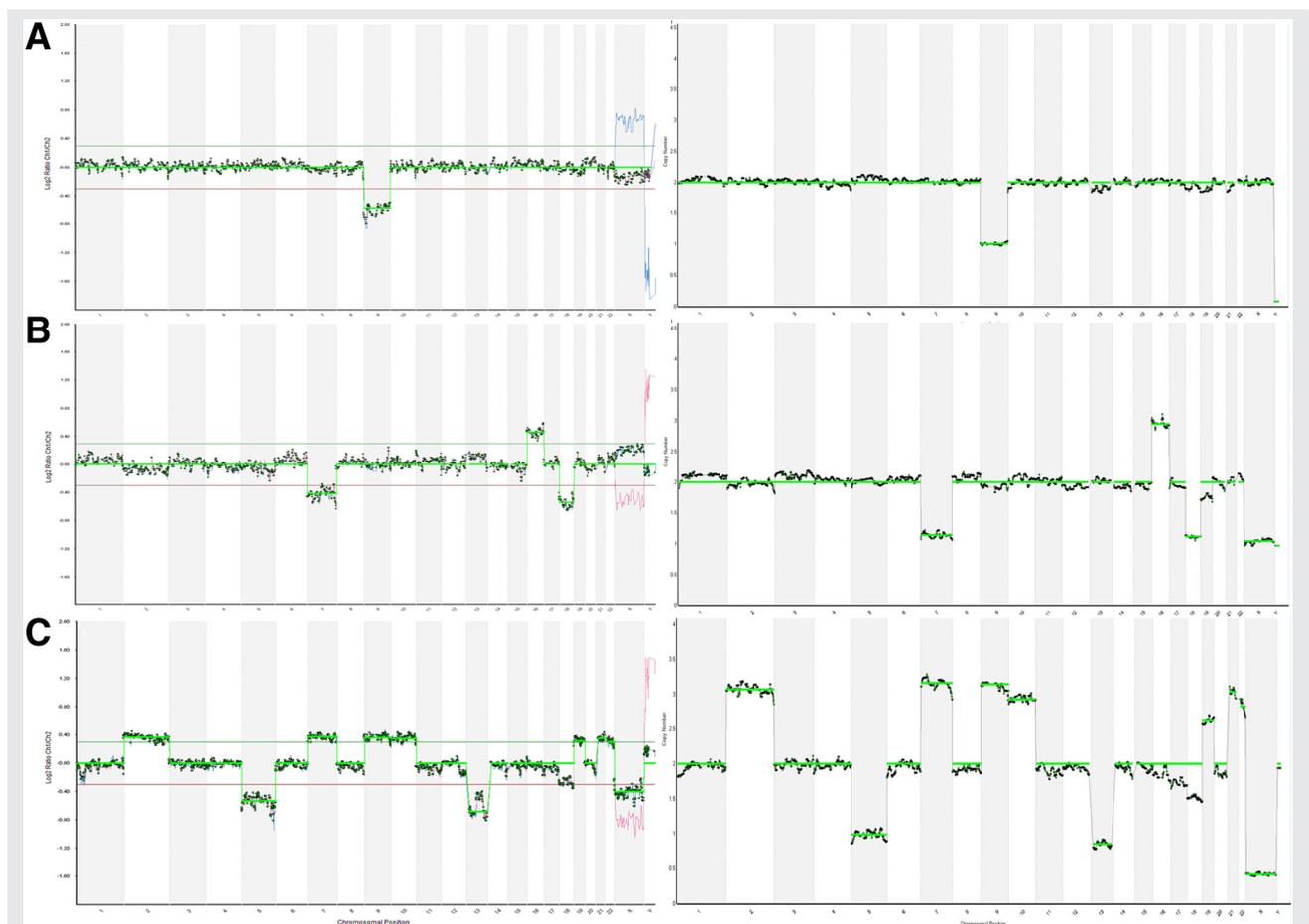
Libraries were prepared with the use of the Nextera XT DNA Sample Preparation (Illumina). DNA “barcoding” (29) was performed to simultaneously analyze embryos from different patients, with the use of the Nextera XT 96 Index Kit (Illumina). The quality of a subset of 32 libraries was assessed with the use of the Agilent High-Sensitivity DNA Kit

(Agilent Technologies) and by sequencing with the Miseq Reagent Kit v2 (Illumina).

Sequencing and sequence analysis. Paired-end dual index 2×36 bp sequencing was performed following the Illumina workflow on a HiSeq 2000 (Illumina) with the use of the Truseq PE Cluster Kit v3-cBot-HS (Illumina) and the cBot instrument (Illumina). Up to 96 barcoded samples were run on a single lane. The pooled DNA library were bound to the complementary adapter oligos on the surface of the flow cell (provided within the PE cluster kit). Afterward, the Truseq SBS kit v3-HS (Illumina), which contains the ready-to-load reagents, was used for sequencing on the HiSeq 2000.

Reads were aligned to the human genome hg19 using iSAAC (30) within the HiSeq analysis software. Bash scripting, BEDtools (31), and SAMtools (32) were used to remove unmapped reads, duplicate reads, reads with low mapping

FIGURE 1



Graphic representation of copy number changes observed in various aneuploid whole-genome amplification (WGA) products selected from clinical preimplantation genetic screening cycles with embryo biopsy at cleavage stage. Left: results from array-CGH analysis; right: results from next-generation sequencing (NGS)-based 24-chromosome aneuploidy screening analysis derived from the same WGA product as shown in the left panel. Each NGS graph in the right panel indicates the copy number assignments (0, 1, 2, 3, or 4) on the y-axis and the chromosome number on the x-axis. Gains (copy number state >2) and losses (copy number state <2) are seen as horizontal green bars above and below, respectively, the copy number state of 2. (A) Embryo showing monosomy 9. (B) Embryo showing monosomy 7, 18; trisomy 16. (C) Embryo showing trisomy 2, 7, 9, 10, 19, 21, 22; monosomy 5, 13, X.

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TABLE 1

Characteristics of samples investigated.

	n (%)
Type of samples analyzed	
Karyotypically defined single cells	18
WGA products from single blastomeres	190
No. of samples analyzed	208
Euploid	67 (32.2)
Aneuploid	141 (67.8)
No. of chromosomes assessed	4,992
Euploid	4,590 (91.9)
Aneuploid	402 (8.1)
Trisomies	191 (47.5)
Monosomies	193 (48.0)
Segmental imbalances	18 (4.5)

Note: WGA = whole-genome amplification.

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scores, and reads with an edit distance greater than one, resulting in an average of 3.2 million (M) (median 2.9 M, standard deviation 1.5 M) filtered read positions per sample. The following bioinformatics analysis was accomplished with the alpha version of Bluefuse Multi for NGS (Blue-gnome). Each chromosome was divided into intervals, each covering ~1 Mb of sequence. Filtered reads from each sample were then mapped into the corresponding chromosome interval or bin. The count data in each bin was normalized with the use of GC content and in silico reference data to remove bias. The normalized bin counts were smoothed with a 13-bin sliding median and reexpressed as copy numbers by assuming that the median autosomal read count corresponds to copy number 2. Copy number status for each chromosome was finally determined with the use of a combination of a gaussian probability function (PDF; with copy number states 0–4 and a standard deviation of 0.33) and thresholding. The copy number state with the highest probability for a chromosome was used unless the distance to the next most probable copy number was >0.011. In that case, the median value of the most likely copy number states of all bins of a chromosome was used, set to a gain when >2.5 and to a loss when <1.5 (all values are listed in Supplemental Table 1, available online at www.fertstert.org). The probability distance as well as thresholds and calling strategy were established in previous experiments using cell lines and applied blindly to all samples.

Miseq sequencing was performed with the use of dual index single-end 36 bp reads. Alignment was performed using Bwa (33) within the Miseq Reporter software. Filtering and analysis was performed as described for the Hiseq data.

Classification of Results

Chromosomal aneuploidies were detected as copy number imbalances. The analysis pipeline expected a default copy number of 2 for autosomes; the sample sex and sex chromosome copy numbers were determined by an initial calling algorithm. Embryos were diagnosed as “aneuploid” if the median chromosomal copy number measures deviated

from the default copy number. Gains (copy number state >2) and losses (copy number state <2) are seen as horizontal green bars above and below, respectively, the copy number state of 2 in Figure 1. The method also allows a specific copy number (0, 1, 2, 3, or 4) to be directly assigned. Embryos were diagnosed as “euploid” if the generated plot showed no gain or loss.

Concordance analysis. Copy number calls automatically generated by the NGS pipeline and Bluefuse Multi (Blue-gnome) were assessed manually and compared for sample ploidy status, sample karyotype, and chromosome ploidy status. Concordance of the NGS results regarding the array results was calculated with the use of the classifications true positive (TP; gain or loss detected), true negative (TN; euploidy status confirmed), false negative (FN; gain or loss missed), and false positive (FP; additional gain or loss called).

Evaluation of Sensitivity and Specificity of Aneuploidy Screening by NGS

To assess the reliability of NGS for aneuploidy detection, the sensitivity, specificity, and positive and negative predictive values of the test were calculated as follow:

Specificity: $\text{no. of true negatives} / (\text{no. of true negatives} + \text{no. of false positives})$

Sensitivity: $\text{no. of true positives} / (\text{no. of true positives} + \text{no. of false negatives})$

Positive predictive value: $\text{no. of true positives} / (\text{no. of true positives} + \text{no. of false positives})$

Negative predictive value: $\text{no. of true negatives} / (\text{no. of false negatives} + \text{no. of true negatives})$

Sensitivity defines the probability that the aneuploidy call will be positive when aneuploidy is present (true positive rate). Specificity defines the probability that the aneuploidy call will be negative when aneuploidy is not present (true negative rate). The positive predictive value defines the probability that an embryo is aneuploid when the test detects aneuploidy. The negative predictive value defines the probability that an embryo is euploid when the test do not detect aneuploidy.

Miseq/Hiseq NGS Data Concordance

To assess NGS data concordance between Hiseq and Miseq sequencing machines, 32 samples previously sequenced on a Hiseq were also analyzed on the Miseq platform. Concordance was assessed as described above.

Ethical Approval

The material used in this study was obtained with patient consent and Institutional Review Board approval from the Genoma center.

RESULTS

A total of 18 karyotypically defined chromosomally abnormal single cells and 190 WGA products from biopsied single-cell

embryos with consistent array-CGH diagnosis from 68 consecutive PGS cycles, were blindly assessed with the NGS-based 24-chromosome aneuploidy screening protocol. Successful results were obtained by NGS in 208/208 samples (100%) included in the experiment. The results were compared for consistency with those obtained by previously established cytogenetic karyotypes and array-CGH. Sixty-seven WGA samples, diagnosed as euploid by array-CGH, were selected from embryos whose transfer resulted in viable pregnancies followed by the birth of chromosomally normal children. In 141 samples, one or more aneuploidies were detected, accounting for a total of 402 different aneuploid chromosomes, 18 of which were segmental aneusomies (Table 1). The details of karyotype predictions are included in Supplemental Table 2 (available online at www.fertstert.org).

Examples of NGS results are shown in Figures 1 and 2.

A single sample produced discordant results, consisting in a false positive call by NGS for trisomy 18 (Supplemental Fig. 1, available online at www.fertstert.org). All of the remaining chromosomes for all of the remaining samples

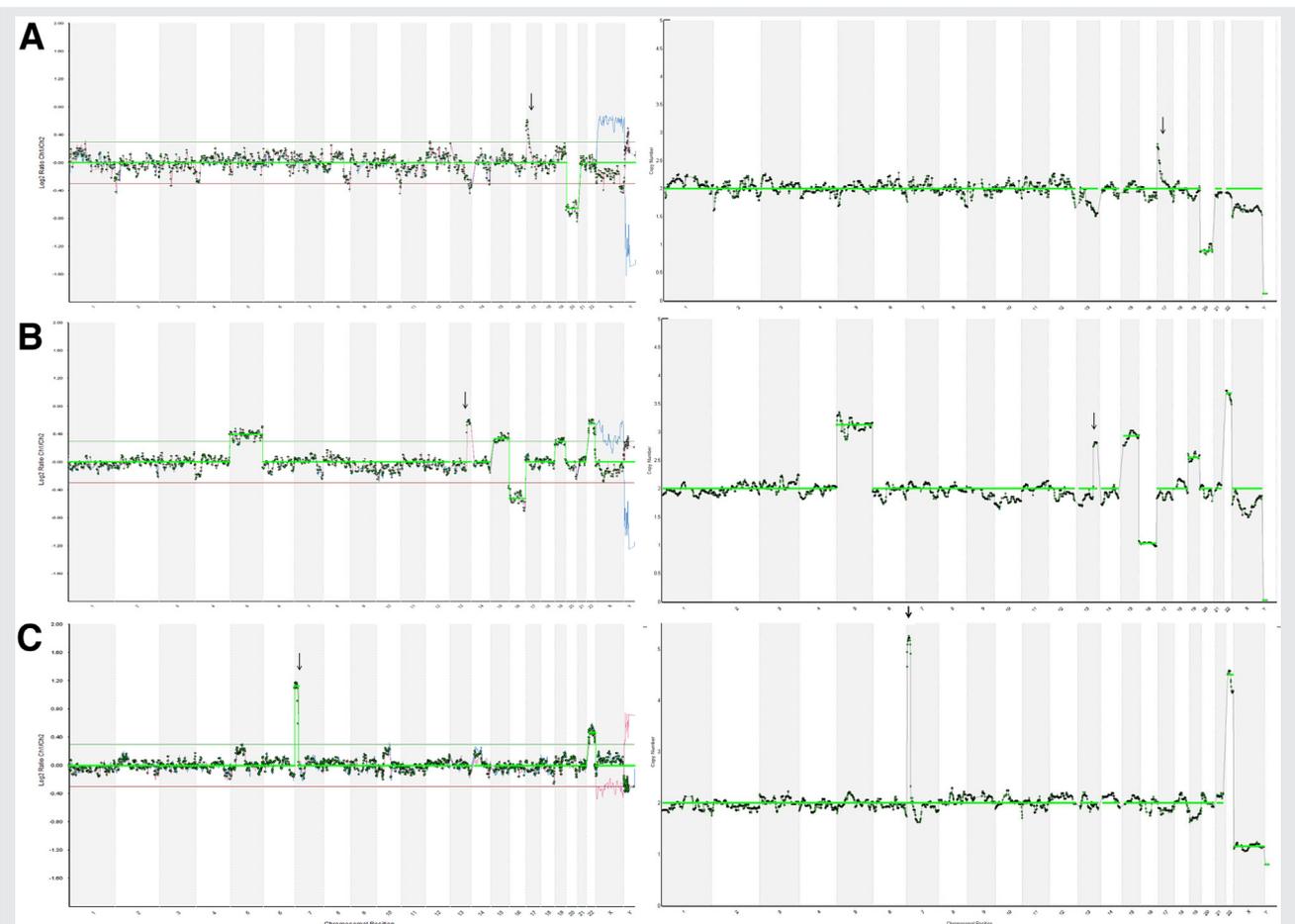
were consistent between NGS and array-CGH, including segmental aneusomies, which were reliably identified with a segmental imbalance as small as 14 Mb in size (Fig. 2). There were no false negative diagnoses for aneuploid chromosomes or embryos, nor inaccurate predictions of sex.

The discordant sample was reevaluated by QF-PCR, which confirmed the array-CGH diagnosis and thus the false positive call by NGS.

NGS specificity for aneuploidy call (consistency of chromosome copy number assignment) was 99.98% (95% CI 99.88%–100%) with a sensitivity of 100% (95% CI 99.08%–100%). NGS specificity for aneuploid embryo call (24-chromosome diagnosis consistency) was 100% (95% CI 94.59%–100%) with a sensitivity of 100% (95% CI 97.39%–100%). Both positive and negative predictive values of the NGS-based 24-chromosome aneuploidy screening protocol were 100% (Table 2).

Concordance between Hiseq and Miseq sequencing was evaluated with 32 samples sequenced in parallel. The obtained

FIGURE 2



Examples of partial aneusomy detection by next-generation sequencing compared with array comparative genomic hybridization. Arrows indicate partial chromosomal imbalances. (A) Embryo showing a 14-Mb segmental duplication on the short arm of chromosome 17. (B) Embryo showing a 20-Mb segmental gain of chromosome 13q. (C) Embryo showing a 17-Mb segmental duplication on the short arm of chromosome 7.

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TABLE 2

Next-generation sequencing performance.

Concordance analysis	n or % (95% CI)
Chromosome calling comparison	
Euploid chromosomes (true negatives)	4,590
Aneuploid chromosomes (true positives)	402
Missed chromosome calls (false negatives)	0
Extra chromosome calls (false positives)	1
Aneuploidy call performance	
Sensitivity	100% (99.08%–100%)
Specificity	99.98% (99.88%–100%)
Whole-sample aneuploidy/euploidy status comparison	
Euploid embryo (true negatives)	67
Aneuploid embryo (true positives)	141
Missed aneuploid embryo calls (false negatives)	0
Extra aneuploid embryo calls (false positives)	0
Aneuploid embryo call performance	
Sensitivity	100% (97.39%–100%)
Specificity	100% (94.59%–100%)
Positive predictive value	100% (97.39%–100%)
Negative predictive value	100% (94.59%–100%)

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data demonstrated an exact overlapping of the results, indicating that the NGS-based method for 24-chromosome screening may be used with both instruments ([Supplemental Fig. 2](#), available online at www.fertstert.org). The copy number assessment method described here is therefore independent from platform and alignment software.

DISCUSSION

Next-generation sequencing is an emerging technology that provides high throughput with parallel analysis of multiple embryos and high-resolution data for chromosomal analysis, but it has yet to be validated for PGS application.

Clinical validation of new technologies to be applied for embryo diagnostics, such as comprehensive aneuploidy screening, is particularly challenging when dealing with single-cell analysis. A key issue is to determine the predictive value of the technique. In fact, it is critical to know whether the test produces false positive chromosomally abnormal diagnoses in embryos that are actually normal and have normal reproductive potential, and vice versa.

We performed a large preclinical validation study to determine the accuracy of an NGS-based 24-aneuploidy screening protocol. NGS ability to accurately identify aneuploidy was assessed by testing, in a blinded manner, multiple single cells in which the expected karyotype had been previously defined by a well established independent methodology.

In this study, the technical accuracy of the NGS approach was measured in two phases. The first phase involved the use of single cells of known abnormal genetic complements, with

results demonstrating 100% consistency of the NGS-based comprehensive aneuploidy screening. The second phase of the study involved the assessment of WGA products selected from previously performed clinical PGS cycles with biopsy of single blastomeres from cleavage-stage embryos. The results achieved clearly demonstrated the ability of the NGS-based method to predict chromosome copy number for the direct diagnosis of aneuploidy. In fact, NGS analysis of the above samples resulted in a 99.98% chromosome copy number assignment consistency with the highly validated method of aneuploidy screening, array-CGH. Importantly, all embryos diagnosed as euploid by array-CGH were confirmed as euploid with NGS and all embryos diagnosed as aneuploid by array-CGH were confirmed as aneuploid by NGS (100% 24-chromosomes diagnosis consistency).

Although this study was designed to validate the performance of NGS in the detection of whole-chromosome aneuploidies, the NGS protocol presented here has also shown accurate detection of segmental changes (as small as 14 Mb in size), indicating that diagnosis of partial aneuploidies is well within the ability of this technology. It is therefore reasonable to assume that patients with balanced translocations will also benefit from the NGS-based approach, with the added advantage of allowing comprehensive chromosome screening in addition to detection of the unbalanced derivatives. However, further studies with the use of cell lines or products from parents carrying known translocation breakpoints are required to assess accuracy and resolution limits of the approach for this purpose.

There are numerous advantages to using NGS for 24-chromosome aneuploidy screening. The parallel nature of NGS data provides a unique opportunity to evaluate multiple samples for multiple different indications on the same sequencing chip, with the use of DNA barcoding methodologies (29). This feature of NGS is very useful because of its potential to substantially increase throughput by analyzing DNA sequences of embryos from different patients simultaneously. In fact, with the use of an NGS instrument at high capacity (e.g., HiSeq), it is possible to evaluate sequence information for up to 96 samples in a single run.

In addition, NGS technique has the advantage not only of screening for aneuploidies, but it may also allow for simultaneous evaluation of single-gene disorders (34), translocations (27), and abnormalities of the mitochondrial genome from the same biopsy without the need for multiple unique technologic platforms. The additional sequence data obtained with the use of the NGS approach may also provide unprecedented amounts of genetic information from human embryos which could be useful for diagnostic and research purposes, with the potential to revolutionize preimplantation diagnosis. A further advantage, compared with array-CGH, is that cohybridization of a control sample is not necessary. NGS methods may ultimately lead to reduced costs per patient, allowing IVF couples a wider use of PGS for choosing the most competent embryo(s) for transfer. However, these predictions need to be validated by further studies with specific design objectives.

Although there are many advantages regarding the use of the NGS technology, the limitations must also be considered. Similarly to other technologies currently used for PGS, NGS

can not directly detect balanced chromosomal rearrangements, because there is no imbalance in the total DNA content. Moreover, although NGS has the potential to detect haploidy and some polyploidies with the use of allele ratios, the sequence coverage of the protocol is insufficient to enable allele detection, which requires higher read depths. It is also important to note the cost of NGS instruments. Potential cost benefits may not be achieved if there are insufficient samples available to fully utilize the available sequencing capacity in every run.

This is the first study reporting extensive preclinical validation and accuracy assessment of NGS-based comprehensive chromosome screening of single cells. Given the high degree of concordance between NGS and array-CGH, NGS-based aneuploidy screening appears to be a robust methodology ready to find a place in routine clinical application. Subsequent efforts need to be directed toward performing prospective studies investigating embryos from clinical PGS cycles with the use of the NGS-based aneuploidy screening method. The results of these trials will be critical when considering this new technology in a clinical setting. A prospective study involving a parallel evaluation of embryos at blastocyst stage with both NGS and array-CGH is currently being conducted by our lab and will help to outline the potential for routine clinical use of NGS-based preimplantation embryo assessment.

In conclusion, evidence of accuracy indicates that NGS provides a reliable high-throughput methodology for 24-chromosome aneuploidy screening. This approach has the potential to represent a useful strategy in reproductive medicine.

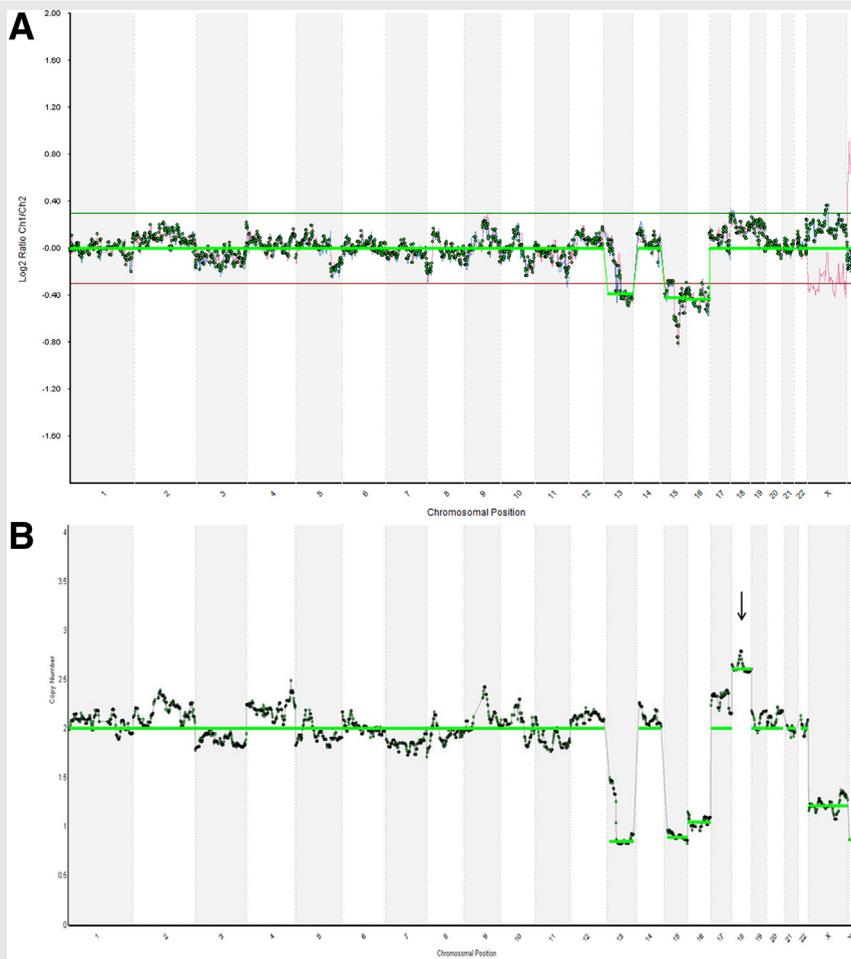
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REFERENCES

- Lathi RB, Westphal MD, Milki AA. Aneuploidy in the miscarriages of infertile women and the potential benefit of preimplantation genetic diagnosis. *Fertil Steril* 2008;89:353–7.
- Ebner T, Moser M, Sommergruber M, Tews G. Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review. *Hum Reprod Update* 2003;9:251–62.
- Munne S, Wells D, Cohen J. Technology requirements for preimplantation genetic diagnosis to improve assisted reproduction outcomes. *Fertil Steril* 2010;94:408–30.
- Wilton L. Preimplantation genetic diagnosis for aneuploidy screening in early human embryos: a review. *Prenat Diagn* 2002;22:512–8.
- Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation genetic screening: a systematic review and meta-analysis of RCTs. *Hum Reprod Update* 2011;17:454–66.
- Rubio C, Bellver J, Rodrigo L, Bosch E, Mercader A, Vidal C, et al. Preimplantation genetic screening using fluorescence in situ hybridization in patients with repetitive implantation failure and advanced maternal age: two randomized trials. *Fertil Steril* 2013;99:1400–7.
- Harper J, Coonen E, De Rycke M, Fiorentino F, Geraedts J, Goossens V, et al. What next for preimplantation genetic screening (PGS)? A position statement from the ESHRE PGD Consortium Steering Committee. *Hum Reprod* 2010;25:821–3.
- Harper JC, Harton G. The use of arrays in PGD/PGS. *Fertil Steril* 2010;94:1173–7.
- Gutierrez-Mateo C, Colls P, Sanchez-Garcia J, Escudero T, Prates R, Ketterson K, et al. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. *Fertil Steril* 2011;95:953–8.
- Fiorentino F, Spizzichino L, Bono S, Birick A, Kokkali G, Rienzi L, et al. PGD for reciprocal and Robertsonian translocations using array comparative genomic hybridization. *Hum Reprod* 2011;26:1925–35.
- Fragouli E, Wells D, Whalley KM, Mills JA, Faed MJ, Delhanty JD. Increased susceptibility to maternal aneuploidy demonstrated by comparative genomic hybridization analysis of human MII oocytes and first polar bodies. *Cytogenet Genome Res* 2006;114:30–8.
- Wilton L, Voullaire L, Sargeant P, Williamson R, McBain J. Preimplantation aneuploidy screening using comparative genomic hybridization or fluorescence in situ hybridization of embryos from patients with recurrent implantation failure. *Fertil Steril* 2003;80:860–8.
- Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertil Steril* 2010;94:1700–6.
- Treff NR, Su J, Tao X, Levy B, Scott RT Jr. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. *Fertil Steril* 2010;94:2017–21.
- Johnson DS, Gemelos G, Baner J, Ryan A, Cinnioğlu C, Banjevic M, et al. Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24 h protocol. *Hum Reprod* 2010;25:1066–75.
- Treff NR, Tao X, Ferry KM, Su J, Taylor D, Scott RT Jr. Development and validation of an accurate quantitative real-time polymerase chain reaction-based assay for human blastocyst comprehensive chromosomal aneuploidy screening. *Fertil Steril* 2012;97:819–24.
- Wells D, Alfarawati S, Fragouli E. Use of comprehensive chromosomal screening for embryo assessment: microarrays and CGH. *Mol Hum Reprod* 2008;14:703–10.
- Treff NR, Levy B, Su J, Northrop LE, Tao X, Scott RT Jr. SNP microarray-based 24 chromosome aneuploidy screening is significantly more consistent than FISH. *Mol Hum Reprod* 2010;16:583–9.
- Scott RT Jr, Ferry K, Su J, Tao X, Scott K, Treff NR. Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: a prospective, blinded, nonselection study. *Fertil Steril* 2012;97:870–5.
- Northrop LE, Treff NR, Levy B, Scott RT Jr. SNP microarray-based 24 chromosome aneuploidy screening demonstrates that cleavage-stage FISH poorly predicts aneuploidy in embryos that develop to morphologically normal blastocysts. *Mol Hum Reprod* 2010;16:590–600.
- Forman EJ, Tao X, Ferry KM, Taylor D, Treff NR, Scott RT Jr. Single embryo transfer with comprehensive chromosome screening results in improved ongoing pregnancy rates and decreased miscarriage rates. *Hum Reprod* 2012;27:1217–22.
- Fragouli E, Katz-Jaffe M, Alfarawati S, Stevens J, Colls P, Goodall NN, et al. Comprehensive chromosome screening of polar bodies and blastocysts from couples experiencing repeated implantation failure. *Fertil Steril* 2010;94:875–87.
- Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, et al. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. *Mol Cytogenet* 2012;5:24.
- Scott RT Jr, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertil Steril* 2013;100:697–703.
- Handyside AH, Wells D. Single nucleotide polymorphisms and next generation sequencing. In: Gardner DK, Sakkas D, Seli E, Wells D, editors. *Human gametes and preimplantation embryos: assessment and diagnosis*. New York: Springer Science Business Media; 2013:135–46.
- Handyside AH. 24-chromosome copy number analysis: a comparison of available technologies. *Fertil Steril* 2013;100:595–602.
- Yin X, Tan K, Vajta G, Jiang H, Tan Y, Zhang C, et al. Massively parallel sequencing for chromosomal abnormality testing in trophectoderm cells of human blastocysts. *Biol Reprod* 2013;88:1–6.

28. Fiorentino F, Kokkali G, Biricik A, Stavrou D, Ismailoglu B, De Palma R, et al. Polymerase chain reaction-based detection of chromosomal imbalances on embryos: the evolution of preimplantation genetic diagnosis for chromosomal translocations. *Fertil Steril* 2010;94:2001–11.
29. Knapp M, Stiller M, Meyer M. Generating barcoded libraries for multiplex high-throughput sequencing. *Methods Mol Biol* 2012;840:155–70.
30. Raczy C, Petrovski R, Saunders CT, Chorny I, Kruglyak S, Margulies EH, et al. iSAAC: ultra-fast whole-genome secondary analysis on Illumina sequencing platforms. *Bioinformatics* 2013;29:2041–3.
31. Quinlan AR, Hall IM. BEDtools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010;26:841–2.
32. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* 2009;25:2078–9.
33. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;25:1754–60.
34. Treff NR, Fedick A, Tao X, Devkota B, Taylor D, Scott RT Jr. Evaluation of targeted next-generation sequencing–based preimplantation genetic diagnosis of monogenic disease. *Fertil Steril* 2013;99:1377–84.

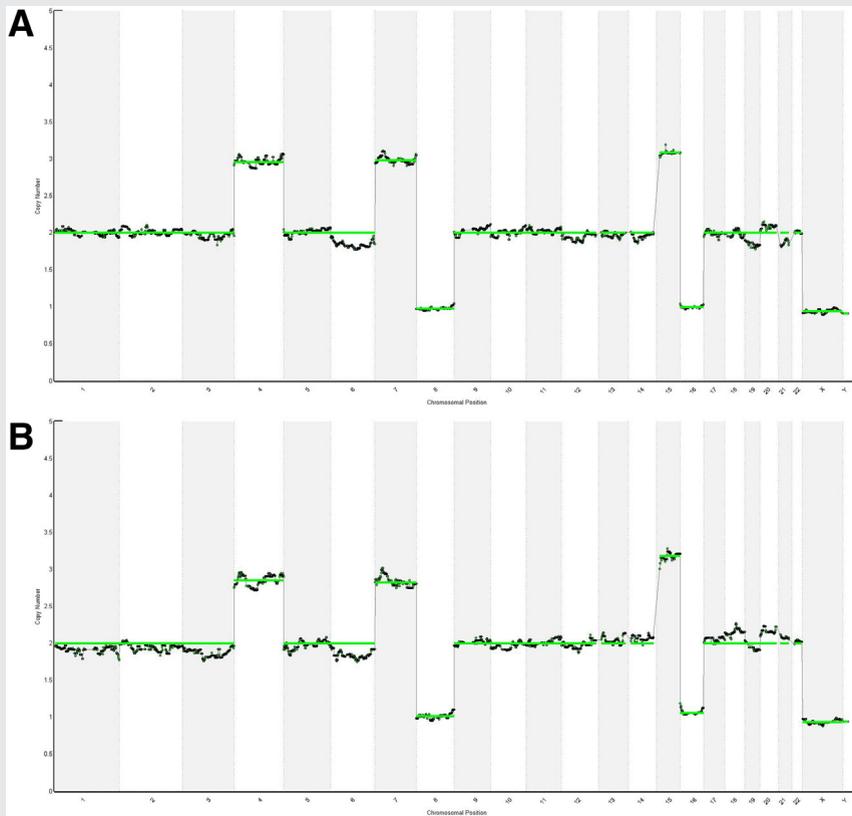
SUPPLEMENTAL FIGURE 1



Sample with discordant result showing a false positive call by next-generation sequencing for trisomy 18 (*arrow*) in addition to true positive calls of loss of chromosomes 13, 15, and 16. (A) Profile from array-CGH analysis. (B) Profile from NGS-based 24-chromosome aneuploidy screening analysis derived from the same WGA product.

Fiorentino. Validation of NGS for PGS. Fertil Steril 2014.

SUPPLEMENTAL FIGURE 2



Example of 24-chromosome aneuploidy screening result with complex karyotype obtained from whole-genome amplification products sequenced on (A) a HiSeq and (B) a Miseq instrument, showing concordance between the platforms.

Fiorentino. Validation of NGS for PGS. Fertil Steril 2014.