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ARTICLE

Validation of next-generation sequencing for comprehensive chromosome screening of embryos

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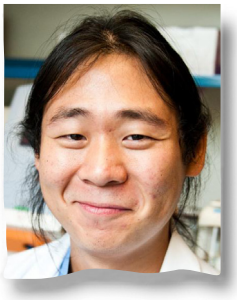

Allen Kung obtained his BSc degree in Biology at Pennsylvania State University. In 2011 he started clinical work in the field of preimplantation genetic diagnosis at Reprogenetics under the supervision of Santiago Munné. He has now moved onto becoming a clinical research associate for the company, being involved in many research projects as well as randomized clinical trials. His newest specialty is working on next-generation sequencing technology.

Abstract Massively parallel genome sequencing, also known as next-generation sequencing (NGS), is the latest approach for preimplantation genetic diagnosis. The purpose of this study was to determine whether NGS can accurately detect aneuploidy in human embryos. Low coverage genome sequencing was applied to trophectoderm biopsies of embryos at the blastocyst stage of development. Sensitivity and specificity of NGS was determined by comparison of results with a previously validated platform, array-comparative genomic hybridization (aCGH). In total, 156 samples (116 were blindly assessed) were tested: 40 samples were re-biopsies of blastocysts where the original biopsy specimen was previously tested for aCGH; four samples were re-biopsies of single blastomeres from embryos previously biopsied at the cleavage stage and tested using aCGH; 18 samples were single cells derived from well-characterized cell lines; 94 samples were whole-genome amplification products from embryo biopsies taken from previous preimplantation genetic screening cycles analysed using aCGH. Per embryo, NGS sensitivity was 100% (no false negatives), and 100% specificity (no false positives). Per chromosome, NGS concordance was 99.20%. With more improvement, NGS will allow the simultaneous diagnosis of single gene disorders and aneuploidy, and may have the potential to provide more detailed insight into other aspects of embryo viability.

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KEYWORDS: Aneuploidy, Comprehensive chromosome screening, Embryo, Next-generation screening, Preimplantation genetic diagnosis

Introduction

Chromosomal abnormalities are a major contributor to human reproductive failure. Embryonic aneuploidy is associated with spontaneous abortion, with up to 70% of first-trimester miscarriages being aneuploid (Carp et al., 2001; Daniely et al., 1998; Garrisi et al., 2009; Hassold et al., 1980; Hodes-Wertz et al., 2012; Menasha et al., 2005; Munne et al., 2005; Qumsiyeh et al., 2000). In addition, embryonic aneuploidy is also associated with implantation failure (Gianaroli et al., 1999; Munne et al., 1993, 1999, 2004; Scott et al., 2013a). Furthermore, there is a significant increase in embryonic aneuploidy with advanced maternal age. A 40% aneuploidy rate has been reported in blastocysts from egg donors, but this rises to 85% in blastocysts from women 43 and older (Ata et al., 2012). Therefore embryonic aneuploidy is likely to be the main factor responsible for the concomitant decrease in implantation rates with advancing maternal age.

Preimplantation genetic screening (PGS) was proposed as a method to improve assisted reproductive technology (ART) outcomes by distinguishing chromosomally normal embryos from those with potentially lethal forms of aneuploidy (Munne et al., 1993). The first generation of PGS strategies involved the use of fluorescent in-situ hybridization (FISH) to screen for chromosome abnormalities in polar bodies and cleavage stage biopsies (Colls et al., 2007; Gianaroli et al., 1999; Magli et al., 2001; Munne et al., 1993, 1998, 1999; Rubio et al., 2013a; Verlinsky et al., 2005). However, FISH is limited to screening 3 to 12 chromosomes in each embryo biopsy specimen (Colls et al., 2009; Munne et al., 2010b). A 12-probe assay is only capable of identifying ~90% of the chromosomally abnormal embryos detectable by comprehensive chromosome screening (CCS) technologies (24-chromosome aneuploidy testing technologies) (Munne et al., 2010a). In addition, FISH accuracy is affected by variability in cell fixation skills (Velilla et al., 2002). More importantly, recent studies have shown that the implantation potential of cleavage-stage embryos can be hampered by the biopsy procedure, especially if performed under suboptimal conditions or by insufficiently skilled personnel (Mastenbroek et al., 2007; Munne et al., 2010b; Scott et al., 2013b). A meta-analysis of clinical trials using FISH to screen cleavage stage biopsied embryos showed no benefit (Mastenbroek et al., 2011), although such meta-analysis did not take into consideration biopsy conditions.

Comparative genome hybridization (CGH) was the first technology to allow PGS of all 24 types of chromosome (Voullaire et al., 2000; Wells and Delhanty, 2000; Wells et al., 1999, 2002; Wilton et al., 2001) and the first to show improved ART outcome (Schoolcraft et al., 2010). Second-generation PGS is the combination of improvements in genetic testing and advances in embryological practice. These include extended embryo culture (Gardner and Lane, 2003; Gardner and Schoolcraft, 1999; Gardner et al., 1998), blastocyst biopsy following breach of the zona pelucida (McArthur et al., 2005) and, in some cases, vitrification of embryos, with transfer to the uterus taking place in a subsequent cycle (Cobo et al., 2012; Kuwayama, 2007; Kuwayama et al., 2005; Stehlik et al., 2005).

Currently, second-generation PGS can involve chromosomal testing using any of several alternative techniques. These PGS methods, including microarray comparative genomic hybridization (aCGH), single nucleotide polymorphism microarrays (SNP arrays), and quantitative polymerase chain reaction (qPCR) have been validated and applied clinically (Colls et al., 2012; Gutierrez-Mateo et al., 2011), (Schoolcraft et al., 2011), (Treff et al., 2012). Multiple randomized clinical trials (Forman et al., 2013; Rubio et al., 2013a, 2013b; Scott et al., 2013a; Yang et al., 2012) have shown a significant increase in implantation and ongoing pregnancy rates when second-generation PGS was used. Furthermore, the usual decline in the likelihood of embryo implantation with advancing female age is not observed when PGS is used and transfer is limited to euploid blastocysts. This confirms aneuploidy to be the principal cause of the well-documented age-related decline in embryo competence (Harton et al., 2013).

Next-generation sequencing (NGS) is the newest technique to be incorporated into second-generation PGS. NGS is fundamentally built around the ability to massively parallel sequence small DNA fragments, until the required depth of coverage (number of sequence reads covering a genome) is achieved. For CCS, only a low amount of coverage is needed to accurately assess aneuploidy (Wells et al., 2014).

Compared with the other PGS methods, NGS has the advantage that it could potentially test for both aneuploidy and monogenic diseases simultaneously. In contrast, the other PGS techniques only detect chromosome abnormalities. Even a very recent technique, karyomapping (Natesan et al. 2014), an SNP array-based technology, which can test for single gene defects and detects most aneuploidies of meiotic origin, fails to detect some mitotic chromosome abnormalities (Konstantinidis et al., 2014; Prates et al., 2014). Microarray CGH and SNP arrays are compatible with the processing of high sample volumes, but are constrained in terms of the number of samples that can be tested using an individual array slide, and each additional slide increases the cost of the test proportionally. While qPCR is the fastest technique, it is not ideally suited for high-throughput applications and is hampered by an extremely low resolution. For example, while aCGH can detect most imbalances caused by reciprocal translocations without any change of protocol (Alfarawati et al., 2012; Colls et al., 2012), application of qPCR for this purpose usually requires addition of extra sets of PCR primers, followed by costly re-validation of the test.

aCGH and SNP-arrays rely on whole-genome amplification (WGA) to generate sufficient DNA from biopsied cells for subsequent analysis, whereas qPCR utilizes a sequence-specific targeted approach. Any DNA amplification method applied to single cells risks the introduction of allele drop out (ADO: failure to successfully amplify both alleles of a locus). ADO is particularly problematic for preimplantation genetic diagnosis (PGD) of monogenic diseases, where failure to detect each copy of a mutant gene can potentially result in a misdiagnosis. Both WGA and targeted PCR approaches can be used to amplify sufficient DNA from the sample for subsequent PGD or PGS using NGS (Chen et al., 2014a, 2014b; Fiorentino et al., 2014a; Treff et al., 2013; Wang et al., 2014b; Yin et al., 2013).

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However, it should be noted that since current NGS methods used for embryo testing rely on DNA amplification (either WGA or targeted PCR), ADO continues to present a challenge for the diagnosis of individual mutations.

Simultaneous testing of biopsy specimens derived from several different embryos allows the costs of NGS to be shared across multiple samples, reducing the effective "per embryo" cost of analysis. Testing of several samples at the same time also has the effect of reducing the amount of sequence data obtained from each embryo. Some published protocols sequence considerably less than 1% of each embryo's genome, referred to as "low-pass genome sequencing", yet even this low level of genomic coverage provides ample data for aneuploidy detection (Wells et al., 2014). The lower reagent costs and higher throughput of NGS, relative to other methods used for the purpose of PGS, are important advantages of the technology. As an alternative to inexpensive low-pass NGS, the technology can be used for high-depth sequencing, which offers the possibility of more powerful and comprehensive analysis, simultaneously providing aneuploidy detection, analysis of chromosomal structural variations (e.g. copy number variations, translocations), and even detection of single gene defects, although at a much higher cost.

All new diagnostic technologies need to undergo preclinical validation to establish their sensitivity and specificity. Such studies should be followed by randomized clinical trials (RCT) to establish the value of the method in an actual clinical context. Currently, there are three ongoing RCTs utilizing a validated NGS assay. One trial (NCT02268786) compares standard IVF treatment (with embryo selection based upon traditional morphological criteria) with cycles in which NGS-based aneuploidy detection is used to assist embryo selection; a similar trial (NCT02032264) compares the traditional IVF treatment with double embryo transfer (DET) to an NGS-based CCS and DET. The third trial (NCT02000349) is a comparison of a frozen-thawed and fresh embryo transfer, both utilizing PGS by NGS.

In this present study, sensitivity and specificity have been investigated by comparing an NGS protocol, using the Ion Torrent PGM sequencing platform, to an established array CGH method. Unlike other NGS methods, this protocol provides CCS results in under 24 h.

Materials and methods

Experimental plan

The purpose of the study was two-fold. The first was to improve a previously validated NGS-based PGS protocol (Wells et al., 2014), streamlining the methodology to allow for routine < 24-h turnaround time for clinical samples. The second was to validate the resulting protocol and obtain an error rate for the overall technique.

Validation strategy

WGA products of blastocyst stage embryos, previously analysed by aCGH, were tested using the optimized NGS protocol. Additionally, non-transferred blastocyst and cleavage stage embryos donated for research and previously analysed by aCGH were re-biopsied, and the new biopsy specimen was blindly analysed with the optimized NGS protocol. Finally, single cells from cytogenetically well-characterized cell lines were tested using NGS. The NGS and previously obtained aCGH results were compared to determine the specificity and sensitivity of the NGS technique, according to the scoring criteria described below. In the case of blinded samples, results were decoded by a technician who was not involved in the study.

WGA strategy

The concept of utilizing NGS for aneuploidy detection in embryos depends upon WGA in order to provide enough DNA from embryo biopsies for subsequent library construction. After it has been generated the WGA product is fragmented into a library of small fragments (100 to 200 bp), which are sequenced in parallel. These sequences or "reads" are then aligned to the current human reference genome. The relative number of fragments of DNA sequence reads that map to each chromosome provides an indication of ploidy (e.g. trisomy is associated with an increase in the relative number of reads for the affected chromosome, while a monosomy results in a decrease).

Two different WGA methods were compared; Sureplex (Rubicon) and multiple displacement amplification (MDA) using Repli-G Midi (Qiagen). For comparison with NGS, embryo samples amplified using Sureplex were tested via aCGH (24Sure, Illumina). These embryos were then either re-biopsied and amplified using the Repli-G, or aliquots of the original Sureplex WGA were processed by NGS, all samples analysed both blinded and non-blinded.

WGA using MDA was performed according to the manufacturer's instructions, except for a modification of the incubation time (reduced to 2 h). The WGA using Sureplex was processed as directed by the manufacturer.

Array-CGH

Array-CGH was performed as described by (Gutierrez-Mateo et al., 2011) with modifications to the reference DNA to complement the current 24Sure V3 protocol (Illumina).

Library preparation for NGS

WGA products were enzymatically fragmented and processed following the Life Technologies Ion Xpress Plus Fragment Library preparation guide, size selecting at 200 bp using the gel option, and using the Ion Library Equalizer Kit. Samples were barcoded and pooled into 8–32 sample multiplexes. The sequencing runs were performed at 200 bp chemistry using the Ion PGM sequencing 200 kit v2, and samples were loaded onto 316 v2 chips.

Bioinformatics evaluation for NGS

The primary data generated from a Torrent server 4.0.2, was analysed using the platform's pipeline software 4.0.2 for read

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filtering, trimming and alignment to the hg19 human reference. The coverage analysis plug-in was also run to make sure that each sample had a sufficient number of reads. For data interpretation, the samples were processed through the Ion Reporter aneuploidy workflow to detect any chromosomal abnormalities with low genome coverage (0.01×). Analysis is based on a hidden Markov model to calculate confidence and precision metrics. The data set was later viewed in the Integrated Genome Viewer (IGV). The software combines corrected GC nucleotide bias read coverage and a set of male references as a baseline. Scoring of aneuploidy was based on the visualization of the IGV profile indicating the losses or gains of whole chromosomes coupled with the confidence and precision metrics. Confidence is defined as the log ratio between the observed ploidy value and the expected value. Precision is the log ratio of the seen ploidy value and the next possible ploidy value.

Scoring evaluation

The validation of NGS to detect whole chromosome abnormalities was based on the following criteria: (i) a result was classified as false negative when the original diagnosis was abnormal (aneuploid or complex abnormal) or chaotic (essentially degraded DNA) and re-testing was euploid; (ii) a result was classified as false positive when the original diagnosis was euploid and the re-testing revealed an abnormal or chaotic result; and (iii) results were classified as concordant when the original results and the NGS results were categorized in the same grouping, euploid, abnormal and chaotic.

An aneuploid result was defined as a full chromosomal loss (monosomy) or gain (trisomy) of a chromosome. A complex abnormal diagnosis was a combination of three or more trisomic or monosomic events. A chaotic profile was indicated by multiple gains or losses of chromosomes or chromosome fragments.

After NGS analysis, results were compared with those previously obtained with aCGH to determine sensitivity (false positives) and specificity (false negatives).

Consent and IRB approval

Patients included in this study had consented to PGS by aCGH and use of discarded embryos for research. The study was determined to be exempt from Institutional Review Board (IRB) approval. According to the common rule 45 CFR 46.101(b) (4), exemptions include "research, involving the collection or study of existing data, documents, records, pathologic specimens, if these sources are publicly available or if the information is recorded by the investigator in such manner that subjects cannot be identified, directly or through identifiers linked to subjects."

Results

Samples

The samples were collected from 35 PGS patients, four aneuploid and two euploid cell lines (all considered to be cytogenetically stable). The average maternal age was 38.0 ± 4.4 years. Twenty-three cases had at least two indications: advanced maternal age; repetitive pregnancy loss; spontaneous abortion; request; repetitive implantation failure; and previous aneuploidy pregnancy. There were 12 cases with one indication: patient request; advanced maternal age; and previous aneuploidy pregnancy. Two cases used egg donors and one PGS cycle involved a combined test for a single gene defect and aneuploidy.

There was a total of 138 embryo biopsies (three biopsies came from the same embryo) from 35 PGS cycles and 18 single cells from six cell lines, producing a total of 156 samples. Of the 138 embryo biopsies, 94 samples (from 94 embryos) were aliquots of trophectoderm WGA products from previous PGS cycles tested by aCGH. The other 44 embryo biopsies were trophectoderm re-biopsies; of these samples, 40 (from 38 embryos) were re-biopsies at blastocyst stage, and the remaining four samples were day-3 embryos rebiopsied at blastocyst stage. The 18 single cell samples came from six well-characterized cell lines and tested by G-Banding. During the initial WGA strategy, six samples were from re-biopsies of six of the 44 embryo re-biopsies and tested with both amplification methods (Sureplex and MDA). The details of the sample description are shown in **Table 1**.

NGS depth

Samples were simultaneously tested in multiplexes of varying size (8–32 samples) on 316 v2 chips with an average 84% loading density.

The average number of reads obtained depended on how many samples per chip were run. An eight-sample multiplex sequencing run produced an average of 362,611 filtered reads per sample. A 16-sample multiplex produced an average of 224,305 filtered reads per sample. A 32-sample multiplex produced an average of 109,671 filtered reads per sample. Overall, euploid and aneuploid samples had an average of 182,387 reads; the exceptions were samples with degraded DNA, which on average had only 8283 reads per sample. Examples of an NGS result shown in IGV are shown in **Figure 1**.

Aneuploidy analysis

In the initial assessment of the improved NGS protocol, 40 samples were analysed (not blindly); of these, six were recorded as euploid, 31 as aneuploid and three as chaotic. Six of these samples were re-biopsies. The remaining 116 samples were blindly assessed, 38 samples were re-biopsies, the details of the blinding allocation are shown in **Table 1**. The samples were recorded as: 38 as euploid, 77 as aneuploid and one as chaotic profile.

Overall, of the 156 samples analysed by NGS, 109 samples were originally recorded as aneuploid using aCGH (in the case of embryo samples) or G-banding (in the case of cell lines), 44 as euploid, and three as chaotic (degraded DNA) (**Table 2**). NGS produced results in 155/156 (99.4%) samples and four were recorded as chaotic (degraded DNA). For all the groups (normal, abnormal, chaotic) there was a >99% concordance rate (44/44; 108/109; 3/3, respectively). One sample, scored

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Table 1 Sample allocation and performance in NGS compared with aCGH (embryos) or G-banding (cell lines).

	Blinded				*Not blinded*			
Sample	*Correct diagnosis*	*False positive*	*False negative*	*Chromosomes with concordant result (%)*	*Correct diagnosis*	*False positive*	*False negative*	*Chromosomes with concordant result (%)*
Single cells from six cell lines	18/18	0	0	432/432 (100)	–	–	–	–
Trophectoderm WGA products[a]	59/60[b]	0	0	1328/1344 (98.81)[c]	34/34	0	0	808/816 (99.02)
Trophectoderm re-biopsies	38/38 [d]	0	0	908/912 (99.56)	6/6	0	0	143/144 (99.31)

aCGH, array-comparative genomic hybridization; NGS, next-generation sequencing; WGA, whole-genome amplification.
[a]Ninety-four biopsies from 94 embryos (amplified by Sureplex).
[b]One sample originally scored as complex abnormal by aCGH produced a chaotic profile on NGS.
[c]Four samples produced chaotic profiles by NGS; therefore those samples' chromosomes are not included in the calculations.
[d]Includes three biopsies from one embryo.

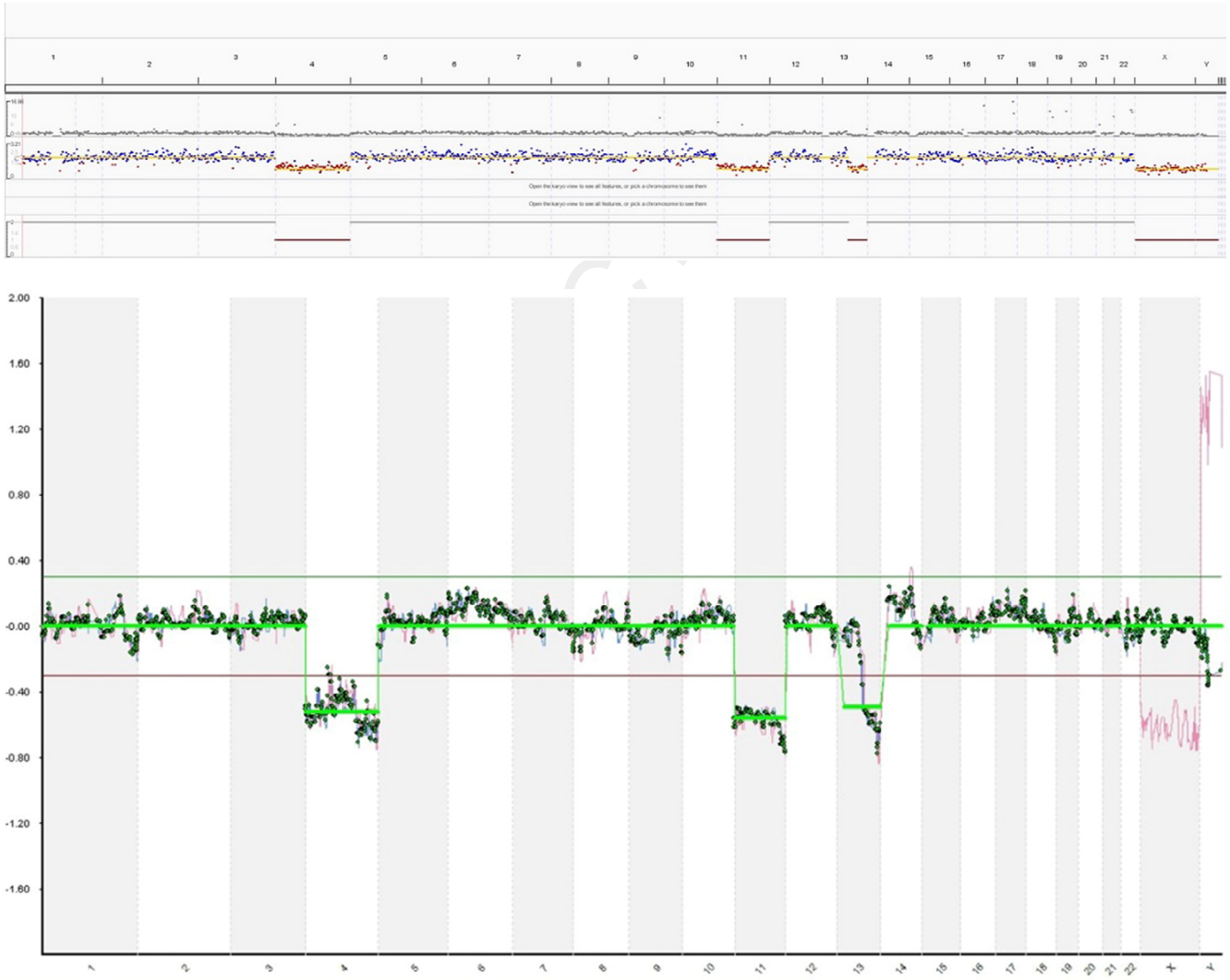

Figure 1 Comparison of a BlueGnome array-comparative genomic hybridization (aCGH) profile to next-generation sequencing (NGS)-produced Integrated Genome Viewer (IGV) profile. An example of a blastocyst sample processed using both aCGH and NGS (Illumina microarrays and the Ion Torrent PGM). The upper panel is the graphical visualization of the NGS analysis using IGV. The lower panel is the graphical representation of the array profile. Both samples presented are scored as an XY, monosomy 4, 11, and a partial monosomy on chromosome 13 from q21.32 to q34.

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Table 2 NGS whole sample concordance to aCGH or G-Banding results.

	Normal	*Abnormal*	*Chaotic*
Concordant	44	108	3
Discordant	0	1	0
Total	44	109	3
Concordance (%)	100	99.08	100
False positive/negative (%)	0.0	0.0	0.0

CGH, array-comparative genomic hybridization; NGS, next-generation sequencing.

as a complex abnormal by aCGH, was scored as a chaotic (degraded DNA) by NGS.

For the analysable samples there was a 100% concordance rate for euploid embryos (44/44) and 100% for aneuploid embryos (108/108). Therefore NGS sensitivity and specificity at the level of the embryo was 100%. From the 152 analysable samples (four chaotic samples excluded), a total of 3648 chromosomes was assessed, with 3619 producing concordant results and 29 discordant (missed or extra chromosome calls). The technical concordance measured per chromosome was therefore 99.20%; see **Table 1**.

Of the samples included, which are not necessarily representative of any specific population, indication or age group, single aneuploidy was the predominant anomaly, detected in 59% of abnormal embryos (43% were single monosomies, and 57% were single trisomies). The presence of two aneuploid chromosomes was seen in 25% of abnormal embryos, and complex abnormalities accounted for 16%.

There were 15 blastocysts classified as abnormal by aCGH, which showed some discrepancies affecting individual chromosomes when analysed by NGS, but other abnormalities within the same embryos were confirmed and therefore the embryos were still classified as abnormal (**Table 3**). Two samples showed a possible false negative when testing the re-biopsied samples with NGS subsequent to MDA; however, when the original Sureplex WGA product was re-tested using NGS, the results were found to be concordant with the aCGH analysis. This suggests that the discrepancy was not due to an inherent problem of NGS and might be explained by an artefact introduced during WGA.

Discussion

In this study a validation of an NGS platform, utilizing semiconductor sequencing (Ion Torrent PGM), and a clinically applicable protocol for PGS that requires less than 24 h from sample acquisition to diagnosis were performed. The samples were originally tested using well-validated techniques, aCGH or G-Banding, revealing the presence of major chromosomal anomalies. The samples assessed consisted of surplus WGA products from clinical PGS cycles, single cells from cytogenetically characterized cell lines, and re-biopsies of embryos previously screened for aneuploidy using aCGH. Currently, DNA amplification is essential for NGS of cells from preimplantation embryos. During this study, two different WGA technologies were evaluated in conjunction with NGS: Sureplex and MDA. The Sureplex WGA proved to be the most consistent, and ultimately this was the method taken forward and used in experiments aimed at determining the specificity and sensitivity of the NGS test.

The results obtained indicate that the NGS technique described is comparable to aCGH for the diagnosis of aneuploidy, with >99% concordance between NGS and aCGH. There were 15 instances in which differences were seen between results obtained using NGS and aCGH originating from either the same Sureplex sample or a re-biopsy sample (all biopsies taken at the blastocyst stage). Twelve of the embryos with some discordance in NGS and aCGH results had multiple (≥3) chromosomal aneuploidies, some of which were detected by both methods while others were only observed using one of the two techniques. It is typical for highly abnormal embryos to display mosaicism (Colls et al., 2007; Munne et al., 2002) and consequently some cytogenetic divergence between biopsy specimens is expected. Importantly, despite a degree of discrepancy in terms of the predicted karyotype, the actual clinical diagnosis (i.e. abnormal) was the same for these embryos regardless of whether NGS or aCGH was used.

A single possible false negative result was obtained. The embryo in question had been diagnosed euploid using aCGH, but a second biopsy sample, which was subjected to MDA and NGS, gave an aneuploid result. In order to determine whether the discrepancy was due to differences in the capacity of NGS and aCGH to detect chromosomal abnormality, the original Sureplex WGA product, generated from the first biopsy specimen and used aCGH, was re-tested with NGS. The result obtained was identical to the original aCGH analysis, correctly classifying the embryo as euploid. This suggests that the reason for the discordant result was embryo mosaicism or an artefact introduced during MDA or Sureplex amplification. It is also worth noting that the aCGH (Illumina) results were obtained using Bluefuse software, and the NGS (LifeTechnologies, Ion Torrent) results were obtained using a combination of Ion Reporter and IGV. Analysis using two different software tools introduces another source of variation between aCGH and NGS, unrelated to the underlying molecular genetic process.

It is encouraging that two very different techniques, aCGH and NGS, provide >99% concordance, suggesting that these CCS methods are accurate and can be considered equivalent for the diagnosis of aneuploidy. On this basis NGS is likely to have the same capacity to improve ART outcomes as other CCS methods of similar accuracy (e.g. qPCR, aCGH, etc).

The NGS protocol accurately detected a segmental aneuploidy in one sample, which was also detected by aCGH, as seen in **Figure 1**. However, this result, from a single sample, is insufficient to draw any conclusions about the general ability of NGS to diagnose duplication or deletion of chromosome fragments. It remains to be determined whether the number of reads obtained using the current method is sufficient to detect imbalance involving small translocated chromosomal segments. A very recent study, using a different NGS platform (HiSeq 2500) and sequencing approximately 5 million 36 bp single end reads, per sample, successfully detected terminal chromosome imbalances close to 1 Mb in size (Wang et al., 2014a). Most probably, small translocations of that type will require more reads than delivered by the protocol described here.

The total number of sequenced reads obtained per NGS experiment is a function of the type of chip used and also the

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Table 3 Discrepancies between NGS and aCGH results of the same embryo.

Sample	*aCGH results*	*NGS results*		*Re-biopsy or re-analysis*
	Sureplex	*Sureplex*	*MDA*	*Comments*
7198–31220–1	41, XY, −2, −5, −7, −10, −14, −19, +20	40, XY, −2, −5, −7, −10, −14, −19	N/A	Re-analysis using Sureplex
1–7183–31219–7	52, XY, +1, +5, +9, +10, +11, +12, −17, +20	56, XY, +1, +2, +5, +9 (4), +10, +11, +12, +16, +20	N/A	Re-analysis using Sureplex
2–0816–13	47, XY, +20	48, XY, +9, +20	46, XY	Re-analysis using Sureplex
2–4583–6	45, XY, +15, −17, −21	46, XY, +15, −17	N/A	Re-analysis using Sureplex
2–7497–2	47, XXY	47, XY, +9	N/A	Re-analysis using Sureplex
3–7648–2	49, XY, +3, −6, +9, +16, +19	52, XY, +1, −2, +3, −6, +9, +13, +14, +15, +18, +19, −20, +21	N/A	Re-analysis using Sureplex
3–7631–2	47, XY, +16	49, XY, +4, +16, +20	N/A	Re-analysis using Sureplex
4–0000–23	44, XY, +9, −10, −12, −22	46, XY, +1, +9, −10, +11, −12, −22	N/A	Re-biopsy using Sureplex
4–0000–26	45, XXY, −13, −15, +16, −17	45, XXY, −15, −17	N/A	Re-biopsy using Sureplex
2–5103–4	46, XY, +18, −22	47, XY, +18, +19, −22	45, XY, −22	Re-biopsy using MDA, reanalysis using Sureplex
2–5103–5	47, XY, −5, +10, +16	47, XY, −5, +10, +16	46, XY, −15, +19	Re-biopsy using MDA, reanalysis using Sureplex
2–5103–7	47, XY, +9, +17	47, XY, +9, +17	46, XY	Re-biopsy using MDA, reanalysis using Sureplex
3–6544–4	42, XY, -7, −15, −20, −21	N/A	Chaotic Profile	Re-analysis using MDA
3–6785–4	46, XY, −4, −7, +8, +21	48, XY, −4, −7, +8 (4), +21 (4)	N/A	Re-analysis using Sureplex
2–9153–2	45, XX, −7, +17, −21	43, XX, −6, −7, +17, −21, −22	N/A	Re-analysis using Sureplex

Ploidy value in parentheses if above 3.
aCGH, array-comparative genomic hybridization; MDA, multiple displacement amplification; NGS, next-generation sequencing.

amount of barcoding utilized. As well as affecting the number of reads, the extent of barcoding dictates the overall cost of the test by allowing multiple samples to be simultaneously analysed within one sequencing run. The number of reads required for genetic diagnosis depends on the type of question being asked. As demonstrated in the current investigation, aneuploidy detection in cells biopsied from preimplantation embryos requires relatively few reads. In contrast, the use of NGS for applications such as non-invasive prenatal diagnosis requires many more reads, since the changes in the amount of DNA associated with aneuploidy are much more subtle. The analysis of an entire genome requires even greater numbers of reads and is currently cost-prohibitive for routine application in PGD.

According to the manufacturer, the Ion Torrent PGM system allows the generation of 5.5×10^5, 3.0×10^6, and 5.5×10^6 reads per Ion 314v2, 316v2, and 318v2 chip, respectively. For the detection of aneuploidy using PGS (rather than PGD for translocations) 77,000 reads per sample, or 0.35% of the genome at 1X coverage, can be sufficient for aneuploidy detection (Wells et al., 2014). Therefore, theoretically, a 314 chip can process at least seven samples, a 316 chip can process 38 samples, and a 318 chip can process 71 samples. However, it is possible to sequence significantly more reads than the manufacturer's stated cap, therefore allowing more sequencing depth per sample or multiplexing of larger numbers of samples.

The principal advantage of NGS over other techniques used for CCS lies in its flexibility and the numerous applications it can potentially be applied to. Advanced equipment for DNA sequencing, such as the Ion Torrent Proton (LifeTechnologies), HiSeq, and Nextseq (Illumina), are now available and have the capacity to sequence an entire human genome relatively rapidly and at a substantially lower price than the previous sequencers.

Although still cost-prohibitive at present, rapid evolution of sequencing technologies means that the possibility of whole-genome sequencing of embryo biopsies, for the purpose of assisting embryo selection, may soon become a clinical reality. Data presented in this study and others (Fiorentino et al., 2014a, 2014b; Wells et al., 2014) suggest that current genome sequencing technologies are suitable for use at the preimplantation stage. Before application of such methods it is important that the ethics of preimplantation genome analysis are carefully considered and that strategies for the interpretation of variants of unknown clinical significance are defined.

For the moment, this study serves to demonstrate that NGS can be successfully applied to WGA material biopsied from human embryos, allowing an accurate determination of the copy number of all 24 chromosomes. The potential for high-throughput analysis offered by sequencing technology makes it ideally suited to large genetics laboratories that carry out testing for multiple IVF clinics (i.e. transport PGD reference centres). At present, NGS is likely to be an expensive and uncompetitive strategy for small laboratories or individual IVF clinics, but for the largest reference centres there may be cost benefits of using NGS in addition to the exciting possibility

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of being able to delve deeper into the embryonic genome in the future.

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