

Single Cell Whole Genome Amplification Technique Impacts the Accuracy of SNP Microarray Based Genotyping and Copy Number Analyses

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ABSTRACT

Methods of comprehensive microarray based aneuploidy screening in single cells are rapidly emerging. Whole genome amplification (WGA) remains a critical component for these methods to be successful. A number of commercially available WGA kits have been independently utilized in previous single cell microarray studies. However, direct comparison of their performance on single cells has not been conducted. The present study demonstrates that among previously published methods, a single cell GenomePlex WGA protocol provides the best combination of speed and accuracy for SNP microarray based copy number analysis when compared to a REPLI-g or GenomiPhi based protocol. Alternatively, for applications that do not have constraints on turn-around time and that are directed at accurate genotyping rather than copy number assignments, a REPLI-g based protocol may provide the best solution.

KEYWORDS: whole genome amplification; SNP microarray; copy number; single cell genotyping; aneuploidy screening

INTRODUCTION

Many groups have developed whole genome microarray based methods to assess chromosome copy number in order to diagnose aneuploidy in human embryos from a single cell (Treff *et al.* , 2010a, Handyside *et al.* , 2009, Johnson *et al.* , 2010a, Vanneste *et al.* , 2009, Gutierrez-Mateo *et al.* , 2010). These developments are in large part due to the failure of fluorescence *in situ* hybridization (FISH) based methods to result in the expected clinical benefit of aneuploidy screening for treatment of infertility (reviewed in Fritz, 2008). Genome wide approaches are certainly more comprehensive than FISH (24 compared to ≤ 12 chromosomes, respectively) and some microarray based methods have shown significantly improved consistency (Treff *et al.*, 2010a, Treff *et al.* , 2010b) and predictive value for aneuploidy diagnosis (Northrop *et al.* , 2010, Scott *et al.* , 2008).

Some methods of 24 chromosome copy number have also demonstrated accuracy of blinded predictions in single cells from a variety of cell lines with previously well characterized karyotypic abnormalities (i.e. Treff *et al.*, 2010a). Unfortunately, other studies have considered a method to be accurate by only establishing that two different methods of analysis both indicate that an embryo is abnormal even if the results of the two tests indicate that the abnormalities involved completely different chromosomes (Gutierrez-Mateo *et al.*, 2010). This may be inadequate to establish the accuracy of a test for single cell 24 chromosome aneuploidy diagnosis. Even more troubling is the lack of any accuracy calculations after analysis of single cells from cell lines with known abnormalities by technologies such as comparative genomic hybridization (CGH) or array-CGH. Some microarray based studies have performed testing of single cells from cell lines (Johnson *et al.*, 2010a, Vanneste *et al.*, 2009). However, one study suggested that the method was accurate after evaluating only a small number of single cells (n=7) from cell lines and was unable to obtain an interpretable result in 41% of blastomeres evaluated, and required analysis by two arrays; BAC and SNP (Vanneste *et al.*, 2009). This

may not represent sufficient validation, reliability, or feasibility for routine clinical application. While the second study involving cell lines (Johnson *et al.*, 2010a) did evaluate a large sample size (n=459), only a single type of abnormality (trisomy 21) was represented. This may also be inadequate to determine the accuracy of predicting aneuploidy for all 24 chromosomes.

Unfortunately, these and other preclinical validation considerations have gone overlooked during the development and implementation of many new technologies for 24 chromosome aneuploidy screening. Clinical studies have also been limited. For example, case control and observational studies may not represent sufficient strength of evidence to determine the clinical validity of new technologies. This is particularly true in light of the experiences with FISH based aneuploidy screening which was suggested to be clinically beneficial based on case control studies (Gianaroli *et al.*, 1997, Munne *et al.*, 2003, Munne *et al.*, 1999, Munne *et al.*, 2006, Kahraman *et al.*, 2000) despite failing to show a meaningful benefit in all randomized controlled clinical trials (reviewed in Fritz, 2008). While similar case control and observational clinical studies using comprehensive methods of aneuploidy screening have been reported (Munne *et al.*, 2010, Wells *et al.*, 2009, Rabinowitz *et al.*, 2010, Schoolcraft *et al.*, 2010), randomized controlled trials have been limited. Indeed, class I strength of evidence for a significant improvement in clinical pregnancy and embryo implantation rates (i.e. Scott *et al.*, 2010) should be made standard for any new aneuploidy screening technology prior to routine implementation. An equally important clinical trial involves a prospective blinded non-selection design (i.e. Scott *et al.*, 2008) in which the negative predictive value of the test is determined. In other words, it is critical to know if the test produces false positive abnormal diagnoses in embryos that are otherwise capable of developing into chromosomally normal pregnancies. Such a study is important to confirming whether the test can be used to safely discard human embryos.

WHOLE GENOME AMPLIFICATION

A critical step in every single cell 24 chromosome aneuploidy screening method is whole genome amplification (WGA). Single cells possess approximately 6-7 picograms of genomic DNA (Dolezel *et al.*, 2003) and microarrays typically require nanogram amounts of DNA to proceed as recommended. This necessitates amplification of the genome by more than 1000 fold. Moreover, since these technologies have commonly been applied to quantitatively evaluate chromosomal copy number, the WGA procedure must result in unbiased amplification to maintain relative quantities of DNA across the entire genome. Some methods of WGA and microarray based molecular karyotyping rely upon interpretation of qualitative genotypes rather than quantitative copy number assignments (Handyside *et al.*, 2009, Johnson *et al.*, 2010a). In these situations and in applications where single gene disorders may be evaluated directly (Burlet *et al.*, 2006, Lledo *et al.*, 2007, Lledo *et al.*, 2006, Panelli *et al.*, 2006, Ren *et al.*, 2007, Renwick *et al.*, 2007, Hellani, 2005, Hellani *et al.*, 2008a) or through microarray based haplotype inheritance analyses (Handyside *et al.*, 2009), genotyping fidelity is also a critical component of WGA.

There are a variety of commercially available reagents to perform single cell WGA that have aided in wide-spread utilization (Table 1). For example, some groups have used a multiple displacement amplification (MDA) approach using QIAGEN's "RepliG" technology (Handyside *et al.*, 2009, Handyside *et al.*, 2004, Sher *et al.*, 2007, Sher *et al.*, 2009) or GE Healthcare's "GenomiPhi" technology (Vanneste *et al.*, 2009, Le Caignec *et al.*, 2006, Hellani *et al.*, 2008b). MDA involves the use of a bacteriophage (Φ 29) DNA polymerase that employs rolling circle amplification during incubation at a single temperature (isothermal) (Dean *et al.*, 2002). Other groups have employed PCR based amplification strategies using Sigma's "GenomePlex" technology (Treff *et al.*, 2010a, Gutierrez-Mateo *et al.*, 2010, Fiegler *et al.*, 2007). PCR based WGA involves use of a DNA polymerase from the thermophilic bacterium *Thermus aquaticus*

and repeated cycling between temperatures appropriate to sequentially denature and elongate the DNA (Saiki *et al.*, 1988). Interestingly, comparison studies of commercially available MDA and PCR based WGA methods have only evaluated performance on input DNA quantities that exceed those found in a single cell (Park *et al.*, 2005, Barker *et al.*, 2004, Lovmar *et al.*, 2003). The present study performs the first direct comparison of commercially available single cell WGA methodologies for amplification reliability, fidelity, and accuracy by SNP microarray analysis.

MATERIALS AND METHODS

Experimental design

This study was designed to evaluate 3 commercially available methods of whole genome amplification (WGA) on single cells. The evaluation was conducted using a SNP microarray platform with genomic DNA extracted from large amount of cells serving as a benchmark for genotyping and copy number accuracy on single cells from the same cell line.

Single cell isolation

Four human fibroblast cell lines were obtained from the Coriell Cell Repository (Camden, NJ). The karyotype of each cell line was different in the copy number of the X chromosome, and included a 46,XY cell line (GM00323) representing a chromosome X copy number of 1, a 46,XX cell line (GM00321) representing a chromosome X copy number of 2, a 47,XXX cell line (GM04626) representing a chromosome X copy number of 3, and a 49,XXXXY cell line (GM00326) representing a chromosome X copy number of 4. Cells were cultured in Eagle's Minimum Essential Medium with 15% Fetal Bovine Serum, 2x Non-Essential Amino Acid and 1% Penicillin-Streptomycin-Glutamine (Invitrogen Corp., Carlsbad, CA) at 37°C and 5% CO₂. Single cells were isolated following treatment with trypsin/EDTA (Invitrogen) to detach the adherent fibroblast cultures as recommended. Single cells were then picked

up in 1 μ l of media using a 100 μ m stripper tip (Midatlantic Diagnostics, Mount Laurel, NJ) under a dissecting microscope and placed in the bottom of a 0.2ml PCR tube (Ambion Inc., Austin, TX) holding WGA method-specific solutions as described below. Thirty single cells were picked up from each cell line; 10 single cells for each WGA method. One μ l of media was removed to serve as negative controls for each WGA method. Genomic DNA was also extracted from each cell line immediately after single cells were obtained using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) as described by the manufacturer.

Single cell whole genome amplification

The GenomiPhi DNA amplification kit (GE Healthcare, Piscataway, NJ) was used on single cells according to a previous publication (Le Caignec *et al.*, 2006). One μ l of culture media containing a single cell was loaded into 0.2 ml PCR tubes containing 2.5 μ l alkaline lysis buffer (200 mM KOH and 50 mM DTT (Cui *et al.*, 1989)). The samples were stored at -80°C for at least 30 minutes and then incubated at 65°C for 10 minutes. Two and a half μ l of neutralization buffer (0.9 M Tris-HCl, pH 8.3, 0.3 M KCl and 0.2 M HCl (Cui *et al.*, 1989)) was then added to the sample to neutralize the lysis buffer. Nine μ l of GenomiPhi sample buffer containing the random hexamer primers was added to the neutralized cell lysate, followed by 9 μ l of GenomiPhi reaction buffer, and 1 μ l of GenomiPhi Enzyme Mix. The isothermal amplification was performed at 30°C for 3 hours and the reaction was stopped upon incubation at 65°C for 10 min.

The RepliG Midi Kit (Qiagen) was used on single cells according to a previous publication (Handyside *et al.*, 2004). Single cells in 1 μ l of culture media were loaded into 0.2 ml PCR tubes containing 2.5 μ l PBS buffer. Three and a half μ l of buffer D2 was added followed by a 10 minute incubation on ice and a 5 minute incubation at 65°C. Three and a half μ l of stop solution was added to stop the lysis reaction. A WGA master mix containing 10 μ l nuclease free water, 29 μ l reaction buffer,

and 1 μ l DNA polymerase was added to the cell lysate followed by the isothermal amplification at 30°C for 16 h and inactivation at 65°C for 3 min.

The GenomPlex Single Cell Whole Genome Amplification Kit (WGA4; Sigma Aldrich, St. Louis, MO) was used on single cells as described in a previous publication (Fiegler *et al.*, 2007). Single cells in 1 μ l of culture media were loaded into 0.2 ml PCR tubes containing 7 μ l of nuclease free water. One μ l of alkaline lysis buffer was added followed by incubation at 65°C for 10 min to lyse the cell. One μ l of neutralization buffer was added to neutralize the lysis buffer. Whole genome amplification was performed following the manufacturer's instructions (Sigma Aldrich).

WGA DNA from each of the 3 methods described above was purified using the GenElute PCR Cleanup Kit (Sigma Aldrich) as described in manufacturer's instructions.

Single cell WGA reliability

The concentration of purified WGA DNA and gDNA was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE) and DNA yield was calculated. One hundred ng of WGA DNA and gDNA was loaded to 2% E-Gel electrophoresis system (Invitrogen) and visualized with a Kodak Gel Logic 100 system (Kodak, Rochester, NY). Successful WGA was defined as a single cell sample that yielded more than the required input WGA DNA amount for SNP microarray based analysis (250ng). For each method, reliability was defined as the percentage of samples that met this definition.

Single cell WGA genotyping fidelity

Three representative WGA DNA samples from each WGA method and each cell line were evaluated by SNP microarray analysis. Two hundred and fifty ng of WGA DNA or gDNA was processed with the GeneChip 250K Nspl SNP microarray as instructed by the supplier (Affymetrix, Santa Clara, CA). Genotypes of each SNP were obtained using the Dynamic Model Mapping Algorithm of the GeneChip

Genotyping Analysis Software (GTYPE) 4.1 (Affymetrix). Genotyping coverage was defined as the percentage of SNPs which were successfully assigned a genotype. As such, the SNPs given a “no call” assignment would contribute to reduced genotyping coverage. Genotyping accuracy was defined as the percentage of SNPs assigned a genotype that was equivalent to the genotype assigned to purified genomic DNA from the same cell line. Allele dropout (ADO) was defined as the number of SNPs that were assigned a homozygous genotype despite being assigned a heterozygous genotype in the purified genomic DNA profiles from the same cell line.

Single cell WGA copy number accuracy

The same data used to evaluate genotyping accuracy above was also evaluated for copy number accuracy by using the Copy Number Analysis Tool (CNAT) 4.0.1 (Affymetrix). The copy number (CN) assignments of each sample were compared with that of the purified genomic DNA from the same cell line and to the known karyotype of each cell line as reported by the Coriell Cell Repository. Results were evaluated for accuracy at 3 levels of analysis; each individual SNP, each individual chromosome, and each individual cell's 23 chromosome molecular karyotype. The overall copy number assignment for a single chromosome was determined based on the SNP copy number that represented the majority of the assignments within that chromosome (Treff *et al.*, 2010a). Diagnostic accuracy was defined as the percentage of single cells given the correct whole chromosome specific gain, loss, or euploid assignments.

Statistics and data repository

A Student's t-test was used to evaluate significance. Alpha was set at 0.05. Variation was reported as ± 1 standard error of the mean (S.E.M.). The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE24690 (<http://www.ncbi.nlm.nih.gov/geo>).

RESULTS AND DISCUSSION

Both GenomiPhi and REPLI-g methods produced WGA DNA that was equivalent in molecular weight to that of the genomic DNA (Figure 1A). However, a similar sized DNA smear was detected from the no template controls amplified with REPLI-g. As a result, gel electrophoresis of REPLI-g WGA DNA alone was insufficient to determine whether amplification was successful. GenomePlex WGA DNA product size ranged from 100 to 1000 basepairs (Figure 1A). The average WGA DNA yield from the GenomiPhi protocol was $0.57 \pm 0.1 \mu\text{g}$, and significantly less ($P < 0.01$) than the $7.63 \pm 0.4 \mu\text{g}$ from GenomePlex or the $18.93 \pm 0.8 \mu\text{g}$ from REPLI-g (Figure 1B). Similar quantities of DNA were detected from the no template controls amplified using the REPLI-g protocol ($15.62 \pm 2.3 \mu\text{g}$). As a result, DNA quantitation by UV spectroscopy of REPLI-g WGA DNA was also insufficient to determine whether specific amplification was successful. This is consistent with previous studies which have found non-specific primer-directed DNA amplification with no template control MDA reactions (Lage *et al.*, 2003, Brukner *et al.*, 2005). Eighty-eight percent (35/40) of the single cells successfully amplified with the GenomiPhi method by yielding greater than 250 ng of WGA DNA. REPLI-g and GenomePlex methods yielded greater than 250ng WGA DNA from 100% of the single cells (Figure 1C). No significant difference in reliability of obtaining sufficient quantities of DNA for microarray analysis was observed between the 3 methods.

Single cell WGA DNA provided an average of 74% genotyping coverage with the GenomiPhi protocol and 78% with GenomePlex, which were both significantly lower than the 88% obtained with REPLI-g (Figure 2A). Single cell WGA DNA genotypes provided an average of 86% accuracy with the GenomiPhi protocol, which was significantly less than the 89% accuracy obtained with GenomePlex (Figure 2B). Both the GenomiPhi and GenomePlex protocols' genotyping accuracy was significantly lower than the 96% obtained with REPLI-g (Figure 2B). There was an average ADO rate of 14% using

GenomiPhi and 11% using GenomePlex, both of which were significantly higher than the 4% obtained using REPLI-g (Figure 2C). These results are applicable to performance of methods that require accurate genotyping and qualitative analysis of aneuploidy, such as those described by Johnson et al. (2010a) and Handyside et al. (2010), or in situations where one might consider using WGA DNA to genotype specific genes of interest (i.e. for single gene disorder screening).

Similarity of single cell copy number assignments with assignments made on genomic DNA and as expected from the conventional karyotype data for each cell line were evaluated at 3 levels. For individual SNPs, 62% similarity was obtained using the GenomiPhi protocol, which was significantly less than the 95% similarity obtained using REPLI-g or the 99% similarity obtained using GenomePlex (Figure 3A). For individual chromosomes, 75% similarity was obtained using the GenomiPhi protocol, which was significantly less than the 97% similarity obtained using REPLI-g or the 99% similarity obtained using GenomePlex (Figure 3B). For single cell molecular karyotyping diagnosis, 0% accuracy was obtained using the GenomiPhi protocol, which was significantly less than the 83% similarity obtained using REPLI-g or the 100% similarity obtained using GenomePlex (Figure 3C). A comprehensive view of the genomic DNA and single cell copy number assignments are also displayed in Figure 4 and reflects the levels of accuracy reported above. These results are of particular importance to the performance of methods that require accurate quantitative analysis of copy number such as those reported by Le Caignec et al. (2006) and Vanneste et al. (2009), which used GenomiPhi technology, and Feigler et al. (2007), Treff et al. (2010a), and Gutierrez-Mateo et al. (2010), which used GenomePlex technology.

Duration of amplification is also important when considering application of single cell WGA technology to clinical PGD. WGA is only one step necessary to generate a diagnosis for the amplified sample, which also involves downstream microarray processing and analysis. For example, the most typical PGD application requires completion of single cell analysis within 24 hours of initiating the

procedure in order to avoid embryo cryopreservation. Therefore, while the REPLI-g protocol may be suitable for single cell applications that do not have time constraints, the 16 hour turn-around time may not allow for its routine use in PGD for aneuploidy screening. A more rapid turn-around time with isothermal MDA was represented in this study by the GenomiPhi protocol. Unfortunately, this shortened MDA protocol performed with the least reliability, fidelity, and accuracy of all methods tested. In contrast, the GenomePlex protocol provided a more rapid turn-around time (4 hours) which could be suitable for application to PGD and produced the highest copy number assignment accuracy of all methods tested. Therefore, for applications requiring accurate and rapid copy number analysis, such as PGD for aneuploidy screening, the GenomePlex protocol may be more appropriate than REPLI-g or GenomiPhi MDA based protocols. However, for those applications requiring accurate genotyping analysis without time constraints, the REPLI-g protocol may be more appropriate than the GenomePlex or GenomiPhi protocols.

In summary, this study represents the first direct comparison of commercially available single cell WGA method performance, a necessary step in all 24 chromosome aneuploidy screening technologies. Clinically relevant measurements of reliability, fidelity and accuracy were evaluated for each method. In general, a longer MDA protocol was better for genotyping accuracy than PCR, and PCR was better and faster than MDA for copy number accuracy. Clinicians and laboratory directors should consider these and other critical pieces of evidence (presented in Table 1 and reviewed in Scott and Treff, 2010) when evaluating new technologies that intend to predict the chromosomal status and reproductive potential of human embryos.

AUTHOR CONTRIBUTIONS

N.R.T and R.T.S designed the study, N.R.T., J.S., and L.E.N. wrote the manuscript, J.S., X.T. and L.E.N. performed the experiments and prepared the microarray data for publication.

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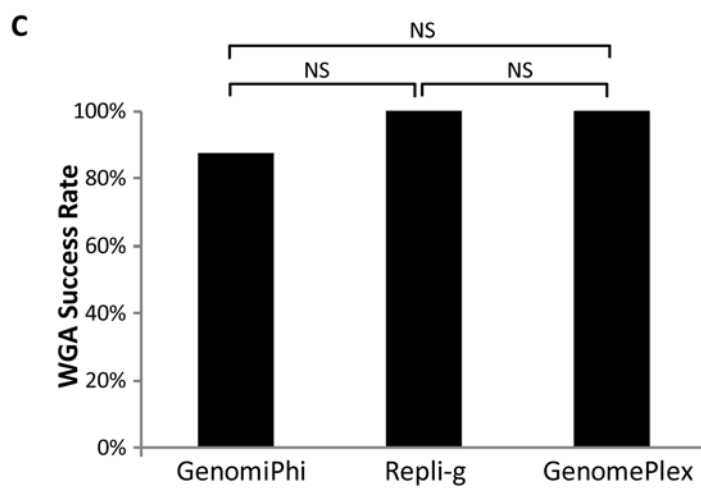
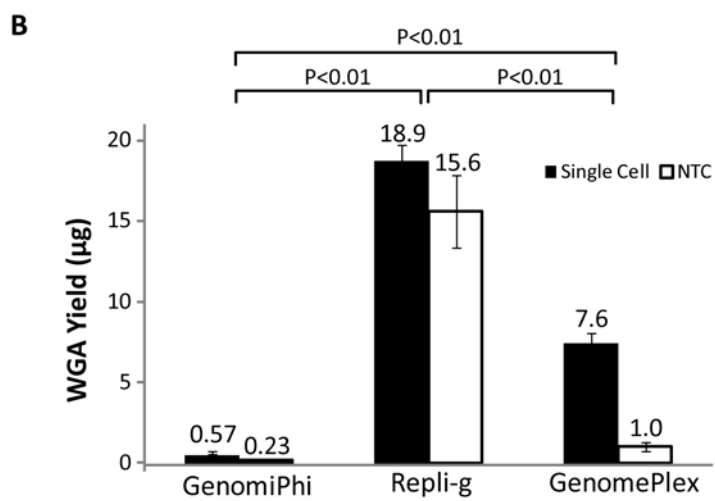
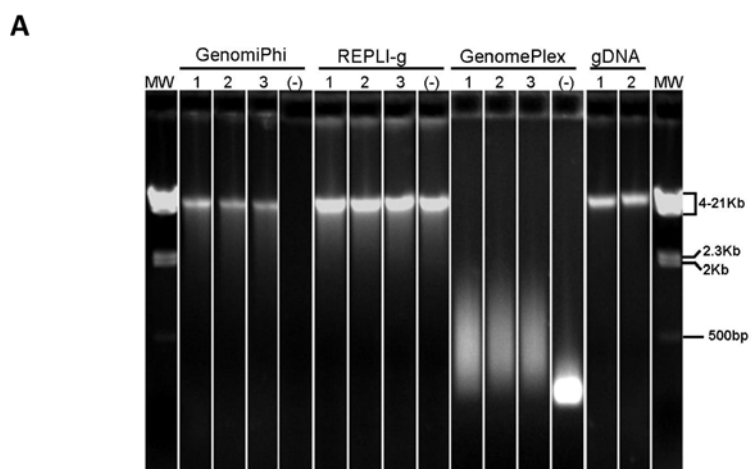
FIGURE LEGENDS

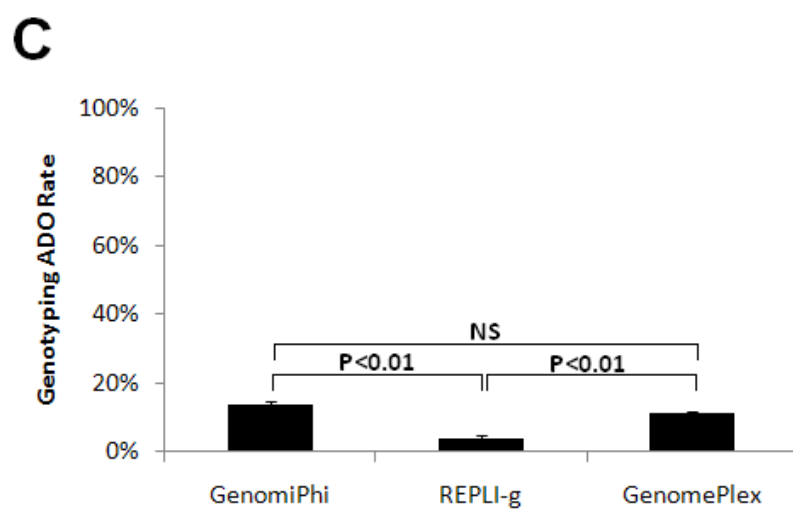
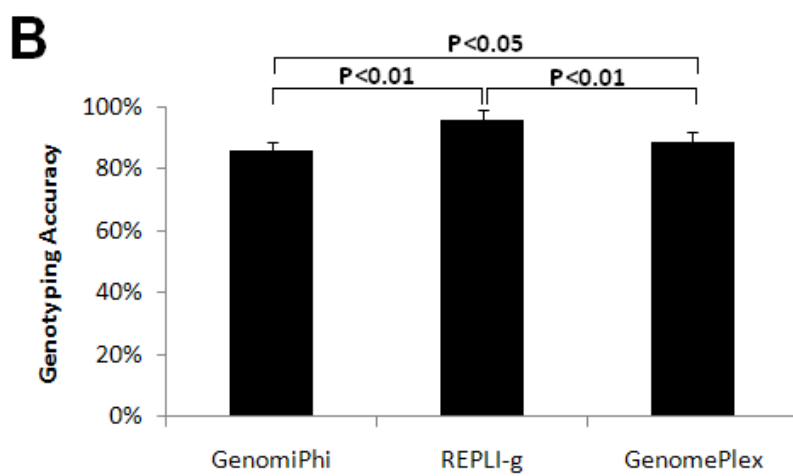
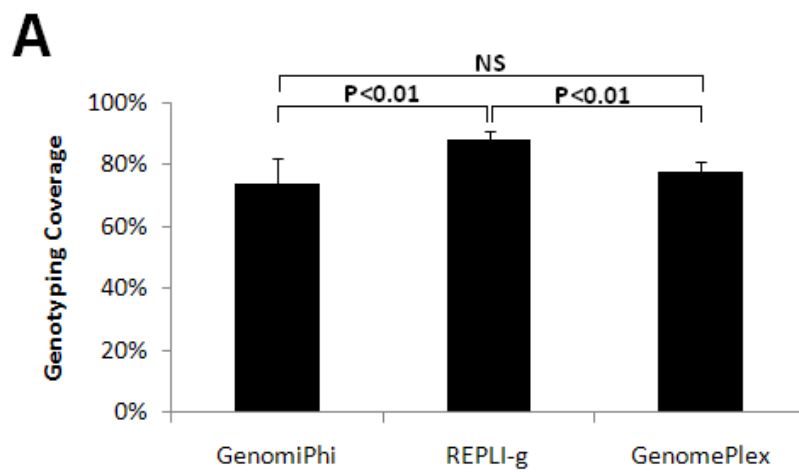
Figure 1. Reliability of single cell WGA. **(A)** Gel electrophoresis of purified reaction products of 3 representative samples and 1 no template control (-) from each of 3 single cell WGA methods (GenomiPhi, REPLI-g, and GenomePlex). Representative purified genomic DNA (gDNA) and molecular weight markers (MW) are included for size references. **(B)** The mean yield of amplification (\pm s.e.m.) of 40 single cells (black bars) or 4 no template controls (NTC; white bars) from each of 3 single cell WGA methods. **(C)** The rates of successful amplification of 40 single cells from each of 3 single cell WGA methods.

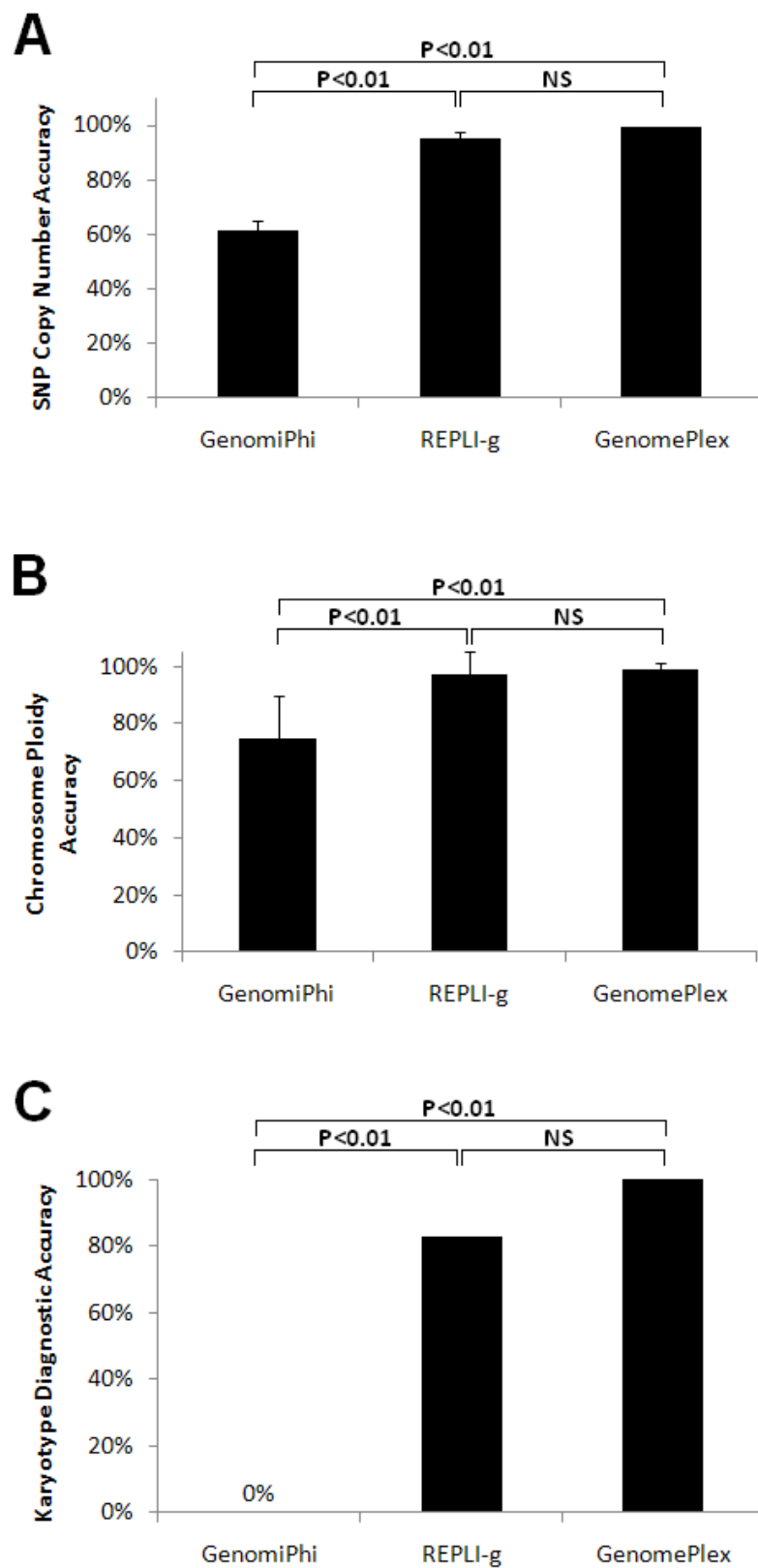
Figure 2. Genotype fidelity of single cell WGA. **(A)** The percentage of SNPs evaluated that were successfully assigned a genotype (genotyping coverage) for each of 3 single cell WGA methods. **(B)** The percentage of SNPs assigned a genotype identical to the purified genomic DNA assignments (genotyping accuracy) for each of 3 single cell WGA methods. **(C)** The percentage of SNPs assigned a homozygous genotype in the single cells but also assigned a heterozygous genotype in the purified genomic DNA samples (Genotyping ADO rate) for each of 3 single cell WGA methods.

Figure 3. Copy number assignment accuracy of single cell WGA. **(A)** The percentage of SNPs evaluated that were assigned the expected copy number (SNP copy number accuracy) for each of 3 single cell WGA methods. **(B)** The percentage of chromosomes evaluated that were assigned the expected copy number (chromosome ploidy accuracy) for each of 3 single cell WGA methods. **(C)** The percentage of cells that were assigned the expected chromosome loss, gain, or euploidy (karyotype diagnostic accuracy) for each of 3 single cell WGA methods.

Figure 4. SNP microarray based copy number (CN) graphs of **(A)** purified genomic DNA, or single cells amplified with **(B)** GenomiPhi, **(C)** REPLI-g, or **(D)** GenomePlex protocols. Each panel includes analyses of each of 4 cell lines possessing one to four X chromosome copies.







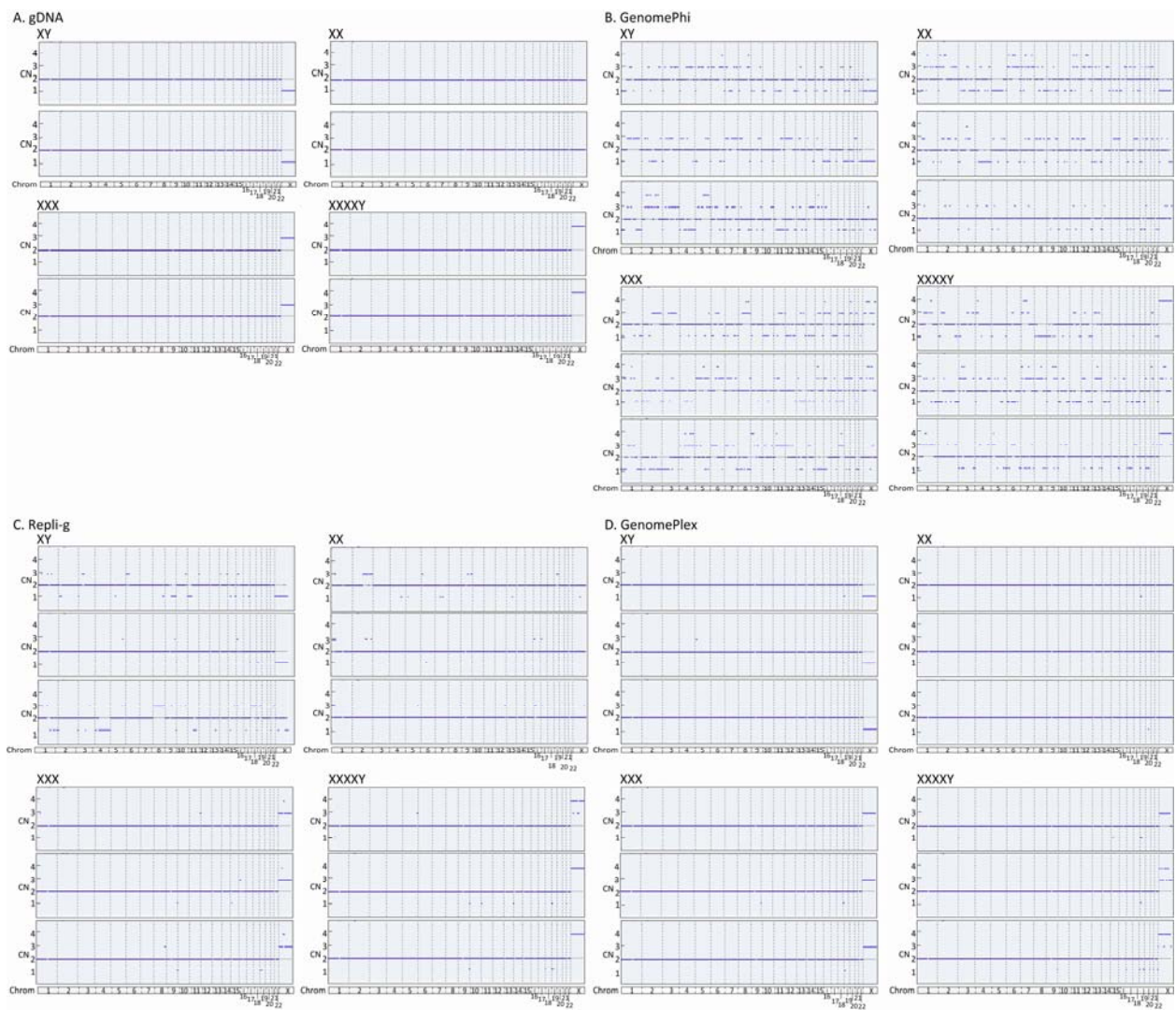


TABLE 1

Comparison of notable 24 chromosome aneuploidy screening technologies

Characteristic	CGH		SNP array			array-CGH		SNP array + array-CGH
	Wells et al., 1999	Sher et al., 2007	Johnson et al., 2010a	Treff et al., 2010a	Handyside et al., 2009	Gutierrez-Mateo et al., 2010	Hellani et al., 2008b	Vanneste et al., 2009
WGA method	PCR (custom DOP-PCR)	MDA (RepliG)	MDA (undisclosed)	PCR (GenomePlex)	MDA (RepliG)	PCR (GenomePlex/Sureplex)	MDA (GenomiPhi)	MDA (GenomiPhi)
Array method	NA	NA	370K SNP	250K SNP	370K SNP	2K CGH (BAC)	44K oligonucleotide	4K CGH (BAC) and 250K SNP
2 day turn-around-time	-	-	+	Treff et al., 2009a	-	+	-	-
Cell line study ^A	-	-	+	+	-	-	-	+
Consistency study ^B	Wells and Delhanty, 2000	-	+	+	-	-	-	+
FISH comparison study	Fragouli et al, 2008	Keskinetepe et al., 2007	-	Treff et al., 2010b; Northrop et al., 2010	-	+	+	-
Single gene disorder detection ^C	-	-	Rabinowitz et al., 2009	Treff et al., 2009b	Handyside et al, 2010	-	-	-
Chromosome translocation detection	-	-	Johnson et al., 2010b	Treff et al., 2010c	-	Escudero et al., 2010	-	-
Observational or case control study	Schoolcraft et al., 2010a	+, Sher et al., 2009	Rabinowitz et al., 2010	Schoolcraft et al., 2010b	-	Munne et al., 2010	-	-
Non-selection study	-	-	-	Scott et al., 2008	-	-	-	-
Randomized controlled study	-	-	-	Scott et al., 2010 ^D	-	-	-	-
Deliveries reported	Wells et al., 2009	Sher et al., 2009	-	Treff et al., 2009a	-	+	-	-

Note: A "+" symbol refers to the reference cited in the header of each respective column

^Aanalysis of accuracy on single cells with known karyotypes

^Banalysis of multiple blastomeres from within the same embryos

^Cdemonstrated ability to evaluate a monogenic disorder from the same biopsy

^Dthis study included demonstrating equivalence of a real-time PCR protocol (Treff et al., 2009c) to the SNP microarray protocol (Treff et al., 2010a) prior to using it in a randomized controlled trial