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Sensitivity and specificity of a novel preimplantation genetic test for aneuploidy (PGT-A) test detecting the presence and origin of the error: creation of haploid embryos as controls

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ABSTRACT

Introduction Conventional PGT-A methods using NGS sequencing can not detect polyploidy or haploidy, embryo parental origin, or differentiate between mitotic and meiotic abnormalities. To be detected, SNP analysis is needed.

Methods Here, we validated a novel preimplantation genetic test for aneuploidy (PGT-A) approach combining next-generation sequencing (NGS) with ad hoc use of single-nucleotide polymorphism (SNP) genotyping to detect aneuploidy, haploidy, triploidy and embryo parental origin, by using NGS complemented by an SNP panel, in which data were grouped into B-allele frequency categories and analysed using proprietary scoring algorithms to classify ploidy status. In the absence of haploid cell lines for validation purposes, haploid human embryos were generated by artificially activating unfertilised oocytes and we also focused our attention on analysing embryos with normal and abnormal number of pronuclei.

Results All diploid and triploid control samples were correctly identified and all embryos derived from parthenogenetically activated eggs showed haploid results (with one 'inconclusive' exception). Among the 254 embryos assessed for pronuclear origin, 64% of single pronucleus, 42% of three pronuclei and 99% of two pronuclei embryos were found to be diploid. Parental origin and relationship analysis was found to be 100% concordant with genomic DNA and 99% with the embryo biopsies.

Conclusions This approach can prevent the misdiagnosis of triploidy and haploidy, while verifying embryo-parent matching when required. The creation of haploid embryos for validation purposes represents a significant advance in the absence of haploid cell lines and this study thus represents the first time that 'true control' samples for haploidy have been used to validate a PGT-A test. Establishing that >50% of zygotes with abnormal pronuclear numbers were diploid and potentially transferable opens up that possibility for PGT-A to increase cumulative pregnancy rates.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Standard preimplantation genetic test for aneuploidy (PGT-A) methods using next-generation sequencing (NGS) are not capable of detecting triploidy or haploidy unless supplemented by single-nucleotide polymorphism (SNP) analysis.

WHAT THIS STUDY ADDS

⇒ Combining NGS-based PGT-A, with ad hoc use of SNPs, successfully detected triploidy and haploidy; validating the approach with created haploid embryos is a significant advance.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This approach can 'rescue' many single pronucleus and three pronuclei embryos that are euploid and our novel approach to validation may find widespread use.

INTRODUCTION

During the in vitro fertilisation (IVF) process, embryos that do not display two pronuclei (PN) after fertilisation and before syngamy are discarded by the embryologist. Of these, in conventionally inseminated oocytes, up to 7% have a single pronucleus (1PN) and up to 5% in those fertilised by intracytoplasmic sperm injection (ICSI).[1 2] Moreover, 3%–6% of conventionally inseminated oocytes and 4%–6.5% of ICSI oocytes result in three pronuclei (3PN) zygotes.[3 4] It is becoming increasingly apparent, however, that estimating ploidy levels solely by the number of pronuclei (PN) observed at the zygote stage is unreliable as events such as asynchronous pronuclear formation or pronuclear fusion may obscure the true fertilisation status.[5] The limitations of PN morphology assessment, whether by conventional microscopy or

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time-lapse imaging, have recently been comprehensively reviewed by Capalbo *et al*.[6] Indeed, the initial chromosomal studies of early human development using fluorescence in situ hybridisation demonstrated that many 1PN zygotes were diploid and fertilised.[5 7 8] That is, 1PN following IVF usually arises when the male and female chromosome sets are enveloped in a single pronuclear envelope, whereas 1PN following ICSI typically results from the embryo not being fertilised but activated.[5 9 10]

The most contemporary preimplantation genetic test for aneuploidy (PGT-A) methodology, next-generation sequencing (NGS), applies comparative chromosome copy number ratios to detect abnormalities but cannot differentiate diploidy from triploidy or haploidy. Several techniques can overcome this limitation by analysing single-nucleotide polymorphism (SNP) that are heterozygous, obtaining the minor allele ratio or 'B-allele frequency' (BAF), which can detect the presence of one, two or three different haploid genomes.[11–16] Recent studies employing SNP-based genotyping have reported that 30%–70% of 1PN zygotes are diploid,[6 15 17] and several investigations have documented that 1PN embryos can give rise to viable pregnancies.[12 17–19] In contrast, true haploid embryos have no implantation potential.[17] Molecular analyses have further shown that 50%–87% of 0PN zygotes are diploid,[15 17 18] and that approximately 90% of 2PN zygotes with an additional small pronucleus (2.1PN) are diploid.[20] With respect to 3PN embryos, 46%–50% are diploid or aneuploid, whereas ~50% are triploid.[6 17] Notably, even morphologically normal 2PN zygotes may be triploid, either due to asynchronous pronuclear formation or the presence of diploid gametes; molecular analyses indicate that ~0.8% of 2PN embryos are triploid and ~0.2% are haploid (reviewed in Capalbo *et al*[6]). Despite these observations, most embryology society guidelines until recently recommended discarding embryos derived from zygotes that are not 2PN.[6 21–24]

This study was designed with three primary objectives: first, to validate a novel PGT-A approach based on next-generation sequencing (NGS) that integrates ad hoc genotyping, enabling simultaneous detection of aneuploidy, triploidy and haploidy, while also allowing embryo paternity testing. Second, because haploid cell lines are not available to generate haploid embryos that can serve as controls for this validation. Third, to examine, prospectively, 1PN and 3PN embryos to determine the frequency at which they are diploid.

MATERIALS AND METHODS

Study design

We developed a method to create haploid embryos to be used as controls for haploidy. Together with diploid and triploid cell lines, these were used to validate a new PGT-A test capable of detecting aneuploidy, triploidy and haploidy. After validation, 1PN and 3PN embryos were analysed prospectively by this method to determine their true ploidy.

Validation metrics

The validation of the Previda Plus test was conducted in compliance with Clinical Laboratory Improvement Amendments (CLIA) guidelines to evaluate three core parameters: accuracy, sensitivity and specificity. In this study, triploid and haploid outcomes were classified as 'positives', whereas diploid outcomes were classified as 'negatives'. Definitions applied were as follows:

Accuracy=(true positives+true negatives)/total samples.

Sensitivity=true positives/(true positives+false negatives).

Specificity=true negatives/(true negatives+false positives).

Control samples for ploidy assessment

A total of 20 control samples were analysed, comprising both established cell lines and whole-genome amplified (WGA) DNA from previously genotyped embryo biopsies from routine PGT-A procedures performed at Progenesis and obtained from consenting patients. Human diploid and triploid cell lines were obtained from the Coriell Institute (Camden, New Jersey, USA), and their chromosomal status was verified before inclusion in the study (see online supplemental table 1).

Triploid WGA controls were derived from 3PN embryos identified in prior PGT-A analyses and were selected based on their chromosomal profile consistent with monospermic ICSI-derived triploidy (ie, failure to extrude the second polar body). These samples showed a distinctive 69,XXY pattern on copy number plots, distinguishable from 47,XXY trisomy by the 3:2:1 autosome:X:Y chromosomal ratio.

Creation of haploid embryos as reference controls

To ensure accurate haploid classification, embryos generated by parthenogenetic activation were used, rather than morphologically identified 1PN zygotes, as the latter are frequently diploid (see 'Introduction' section). We used 99 human donor oocytes obtained from Eugin Clinic (Barcelona, Spain). The donor oocytes used in this study were retrieved and vitrified initially for clinical use. However, these oocytes were subsequently deemed unsuitable for donation by the clinic. The main reasons were that in some cases the stimulation cycle did not yield the minimum number of oocytes required for allocation to a recipient, and in other cases the donor did not meet the updated genetic screening requirements. These oocytes were therefore not used clinically but were consented for research use purposes.

These vitrified oocytes were warmed using the Kitazato protocol and incubated at 37°C, 7% CO_2 and 5% O_2. Parthenogenetic activation was induced by ionomycin (5 µM) treatment for 5 min, followed by a 10 min culture, a second ionomycin exposure and a 4-hour incubation in cycloheximide (10 µg/mL). Following assessment for second polar body extrusion, activated oocytes were individually cultured in time-lapse incubation (Embryoscope) for 5–6 days. Good-quality blastocysts were biopsied using Navilase-assisted techniques. Both biopsied

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cells and complementary blastocysts were stored at −80°C until processed at Progenesis (San Diego, California, USA).

Sample amplification and sequencing

DNA from cell lines, WGA embryo biopsies and haploid controls was subjected to whole-genome amplification using the Ion ReproSeq protocol (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each sample (250 pg) was barcoded and amplified. A 480-SNP panel was targeted via multiplex PCR, followed by linker incorporation and barcode tagging. Library quantification was performed using the Qubit High Sensitivity dsDNA Kit (Thermo Fisher Scientific, California, USA). Low DNA, inconclusive, or complex abnormal samples (three or more chromosomes abnormal) were excluded. SNP libraries were co-sequenced with corresponding PGT-A libraries using a combined ReproSeq workflow. We used comparable read number and coverage for PGT-A, typically 200 000–300 000 reads per sample, with an average read size of 130 bp.

Data processing and bioinformatic analysis

Sequencing outputs were processed via the open-source software 'BAM-read-count'. SNP reads were filtered to include only alleles with a minimum read depth of 10 and a minor allele frequency threshold of ≥20%. The resulting .tsv files were compiled into .csv matrices for allele frequency analysis, and SNP alleles were grouped into BAF categories of 0%, 33%, 50%, 66% and 100%, all performed using a proprietary algorithm. PGT-A data were analysed using Ion Reporter Software (V.5.18), while SNP-based ploidy assessments were carried out using a proprietary algorithm. Ploidy scores were calculated using defined formulas for the classification of haploidy, triploidy and diploidy.

Ploidy calling process

The ploidy calling process of the Previda Plus test first checks for the haploidy status, then for the triploidy and diploidy status. Haploid calling was determined based on BAF100: if BAF100 showed an overwhelming result, it was classified as haploid and if BAF100 was between 0.9 and 1, the sample was also considered haploid. It was

Table 1 Analysis of haploid embryos

Sample_ID	AF 0%	AF 33%	AF 50%	AF 66%	AF 100%	Haploidy score	Triploidy score	Final call
1a	235	2	0	2	235	59.75	1	Haploid
1b	1	0	0	0	1	0	0	Insufficient reads
2a	251	2	2	2	251	42.83	0.67	Haploid
2b	230	2	2	3	230	33.86	0.71	Haploid
3a	156	43	38	41	156	2.28	0.69	Inconclusive*
3b	175	27	19	26	175	3.43	0.74	Inconclusive*
4a	239	1	4	1	239	40.83	0.33	Haploid
4b	20	1	0	1	20	11	1	Insufficient reads
5a	257	1	4	1	257	43.83	0.33	Haploid
5b	234	1	0	2	234	79	1	Haploid
6a	257	1	0	1	257	129.5	1	Haploid
6b	235	0	0	1	235	236	1	Haploid
7a	226	1	0	2	226	76.33	1	Haploid
7b	187	1	0	4	187	38.4	1	Haploid
8a	220	0	0	2	220	111	1	Haploid
8b	188	0	2	2	188	48	0.5	Haploid
9a	293	0	2	1	293	98.67	0.33	Haploid
9b	211	1	1	3	211	43.2	0.8	Haploid
10a	245	1	0	2	245	82.67	1	Haploid
10b	241	1	0	3	241	61.25	1	Haploid
11a	259	0	0	2	259	130.5	1	Haploid
11b	202	1	0	5	202	34.67	1	Haploid
12a	237	0	2	0	237	119.5	0	Haploid
12b	233	2	0	3	233	47.6	1	Haploid

*Inconclusive based on criteria described in the 'Materials and methods' section.
AF, allele frequency.

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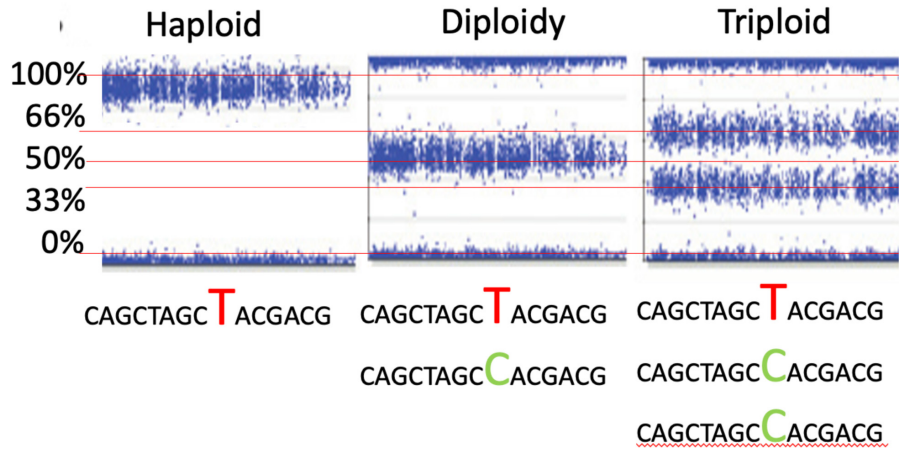

Figure 1 B-allele frequency patterns for haploid, diploid and triploid results.

calculated as haploidy score=log2((BAF33+BAF50+BAF66+BAF100)/(BAF33+BAF50+BAF66)). Online supplemental table 2 and table 1 show the haploidy results using both score methods. Online supplemental figure 1 shows the correlation of the two methods and the cut-off values of each method. Figure 1 shows BAF patterns for haploid, diploid and triploid embryos.

If haploidy status was >0.90 for method 1 or >3.0 for method 2, then the embryo was classified as a haploid, otherwise the analysis continued for triploid and diploid scoring using a cut-off derived from allele frequencies.

Online supplemental table 2 and table 1 show the number of SNPs that were not informative or undetected (BAF00), or were approximately 33% (BAF33), 50% (BAF50), 66% (BAF66) or 100% (BAF100) of the sample.

If the haploidy score was >0.90, the embryo was considered haploid, irrespective of the BAF33, BAF50 and BAF66 frequencies, which in haploid embryos are basically noise. An example is sample 1a in table 1, with 235 SNPs being BAF100, 235 being BAF0 but with two BAF66 and two BAF33. The origin of the sample controls was blinded to the PGT laboratory personnel processing the samples.

Reproducibility of triploidy detection

The reproducibility of this procedure was measured by running eight reference samples and 12 clinically confirmed samples. The results are illustrated in online supplemental table 1. Results show a high correlation between the two data sets obtained from the duplicated runs by two different operators and two different PCR instruments used. Two sets of repeatability results show a high correlation coefficient of R^2=0.9667 (figure 2).

Patient and public involvement statement

We did not include the opinion of patients or the public in the design of this research.

Data availability statement

All data generated in this study have been presented here or in the online supplemental file 1.

RESULTS

Characteristics of samples processed

We used 99 donor eggs to generate haploid embryos as haploid controls. We also used eight Coriell cell lines (four diploid and four triploid) as controls (online supplemental table 1) and 266 amplified DNA (WGA) de-identified samples obtained from embryo biopsies that we had previously analysed with our standard PGT-A (which did not include the use of SNPs) (figure 3).

Validation of diploidy and triploidy detection

To validate the Previda Plus SNP-based test for ploidy classification, we tested 20 control samples (online supplemental table 1), including four diploid and four triploid Coriell cell lines, five WGA-confirmed triploid embryo biopsies (all 69,XXY) and seven WGA-confirmed diploid embryos that resulted in live births. They were analysed in replicas with a total of 63 samples analysed (online supplemental table 1). All triploid samples were correctly identified based on the 3:2:1 autosome:X:Y chromosomal

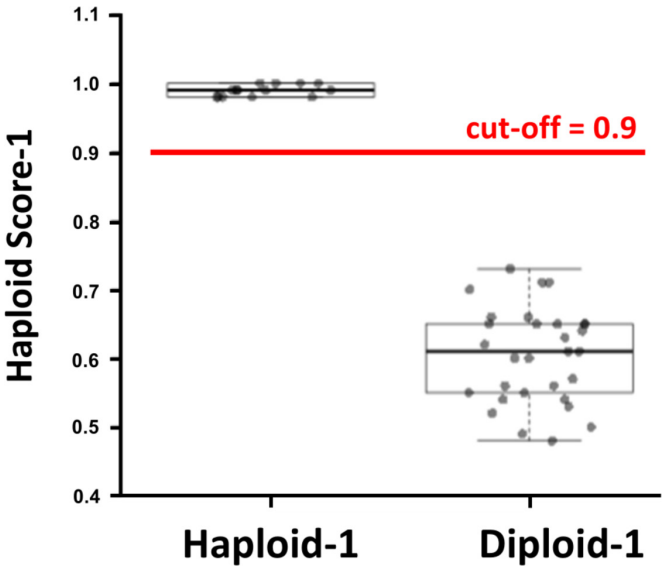

Figure 2 Box plots of the haploid score-1 and its cut-off values.

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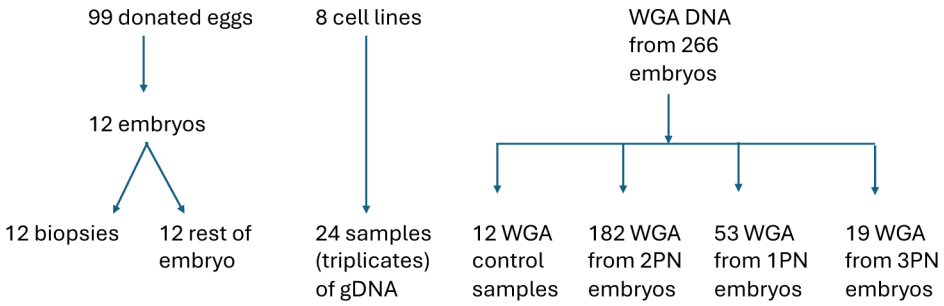

Figure 3 Flow of samples. 1PN, single pronucleus; 2PN, two pronuclei; 3PN, three pronuclei; gDNA, genomic DNA; WGA, whole-genome amplified.

ratio and BAF profiles. Similarly, all diploid embryo WGA samples were confirmed diploid. In total, 63 samples were 100% correctly identified (95% CI 0.943 to 1.0).

Haploid embryo creation and validation of haploidy

A total of 99 vitrified oocytes were warmed to validate haploidy detection, of which only 70 survived thawing, a substandard rate. After parthenogenetic activation, 28 (40%) of the eggs cleaved, and 12 developed into blastocysts (table 1). PGT analysis of the biopsy and the respective whole embryo showed that 11 samples had haploid results. Both the whole embryo and biopsy displayed BAF profiles dominated by homozygosity and X chromosomes, with none showing Y chromosomes. The remaining embryo (embryo 3) was inconclusive, with two biopsies of the same embryo scoring lower than expected for both haploidy and diploidy. This was considered a 'no call', neither a false positive nor a false negative.

Ploidy assessment of 1PN and 3PN embryos

A total of 254 embryos derived from zygotes with different PN numbers were analysed (table 2). Of those, 64% (34 out of 53) of 1PN embryos were diploid, while the remaining 36% were classified as haploid. All haploid 1PN embryos lacked a Y chromosome, confirming their classification as true haploid embryos. Among 3PN embryos (n=19), 42% (eight out of 19) were diploid, and the remainder were triploid, either 69,XXX or 69,XXY, indicating that polyspermy was unlikely and retention of the second polar body was the more likely mechanism.

Table 2 Ploidy of 1PN, 2PN and 3PN embryos analysed by PGT-A and SNP test

	1PN		2PN		3PN	
	Male	Female	Male	Female	Male	Female
Haploid	0	18	1*	0	0	0
Diploid	20	14	86	93	6	2
Triploid	0	1	1*	1	6†	5†

*Considered false positives (1%, 2/182). The triploid one was 46,XY by PGT-A and triploid by SNPs.
†Six were XXY and five were XXX.
PGT-A, preimplantation genetic test for aneuploidy; 1PN, single pronucleus; 2PN, two pronuclei; 3PN, three pronuclei; SNP, single-nucleotide polymorphism.

Among the 182 2PN embryos, 98.9% (179 out of 182) were confirmed diploid, one was confirmed triploid and two were considered false positives. Of the false positives, one was diploid (46,XY) by PGT-A and triploid by SNPs. The other false positive was male and haploid.

Parent of origin analysis

Parental origin and relationship analysis was performed on 900 parental WGA samples, demonstrating 100% accuracy (60/60 true positives, 840/840 true negatives). These were obtained with research consent through routine Preimplantation Genetic Diagnosis for Monogenic diseases (PGT-M) cases. These results contributed to an overall accuracy of 99.44%, 95% sensitivity and 99.76% specificity. Importantly, parental samples are not required for ploidy assessment (including triploidy and haploidy) or for analysis of 1PN/3PN embryos, unless parental origin determination is explicitly needed. The overall accuracy is considered 99.72% (1795/1800) across all samples. Figure 4 contains the similarity scores between every embryo and couple.

DISCUSSION

The SNP-based ploidy test here reported demonstrated high classification accuracy when applied to control samples of known genetic status, including the following: diploid and triploid cell lines previously classified as diploid, triploid embryo biopsies and haploid embryos generated via parthenogenetic activation. The addition of SNP genotyping data enables the differentiation between diploid, triploid and haploid embryos, a critical distinction that traditional NGS-based PGT-A methods cannot make. The ability of the SNP analysis to detect allelic balance and variation across hundreds of loci further enhances the test's capacity to differentiate diploidy from triploidy and haploidy. Although other techniques have been described to assess triploidy and haploidy based on SNP analysis,[12 13 18] the current approach has a novel element in that it allows adding the SNP data ad hoc after standard PGT-A using NGS. The scoring system here developed relies on well-characterised BAF patterns to distinguish between ploidy states. Haploid samples showed dominant homozygosity (BAF 0% or 100%), while triploid samples showed intermediate frequencies (BAF 33% and 66%). These scoring approaches proved

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	PARENTAL COUPLES														
EMBRYO ID	41T70p	3LVQFp	OCL33p	NNEEXp	PASITp	NG188p	9LV12p	9MSK6p	YUM8Ep	RM3D8p	VTP1Zp	Y8NOVp	Z5YKQp	9WO2Wp	K6332p
41T70e-1	0.98	0.78	0.82	0.75	0.74	0.76	0.76	0.75	0.79	0.73	0.75	0.82	0.76	0.79	0.79
41T70e-2	0.99	0.74	0.79	0.72	0.74	0.79	0.75	0.69	0.76	0.71	0.76	0.77	0.74	0.76	0.75
41T70e-3	0.98	0.69	0.79	0.69	0.69	0.72	0.7	0.67	0.69	0.7	0.72	0.71	0.75	0.73	0.69
41T70e-4	1	0.74	0.76	0.71	0.75	0.7	0.7	0.74	0.75	0.68	0.73	0.78	0.74	0.77	0.69
3LVQFe-1	0.76	0.98	0.75	0.75	0.78	0.73	0.73	0.77	0.72	0.77	0.72	0.79	0.73	0.72	0.8
3LVQFe-2	0.75	0.99	0.75	0.72	0.76	0.7	0.74	0.76	0.74	0.75	0.72	0.8	0.72	0.76	0.82
3LVQFe-3	0.73	0.96	0.71	0.74	0.75	0.71	0.68	0.75	0.7	0.7	0.68	0.76	0.72	0.73	0.76
3LVQFe-4	0.79	0.99	0.72	0.74	0.73	0.73	0.72	0.76	0.7	0.71	0.68	0.76	0.74	0.72	0.73
OCL33e-1	0.8	0.77	0.98	0.78	0.76	0.76	0.78	0.72	0.76	0.74	0.77	0.76	0.74	0.76	0.77
OCL33e-2	0.75	0.76	0.97	0.74	0.72	0.73	0.75	0.73	0.75	0.7	0.79	0.74	0.7	0.77	0.77
OCL33e-3	0.82	0.76	0.98	0.79	0.77	0.74	0.75	0.73	0.8	0.74	0.76	0.78	0.76	0.81	0.83
OCL33e-4	0.76	0.7	0.98	0.73	0.72	0.74	0.8	0.71	0.76	0.68	0.78	0.74	0.69	0.76	0.77
NNEEXe-1	0.73	0.76	0.77	0.98	0.76	0.71	0.75	0.73	0.77	0.73	0.77	0.74	0.79	0.74	0.77
NNEEXe-2	0.76	0.76	0.78	0.97	0.76	0.72	0.75	0.72	0.76	0.7	0.72	0.75	0.79	0.71	0.77
NNEEXe-3	0.74	0.77	0.81	0.99	0.76	0.76	0.81	0.78	0.75	0.75	0.77	0.78	0.77	0.79	0.76
NNEEXe-4	0.75	0.75	0.82	0.97	0.76	0.71	0.75	0.72	0.74	0.69	0.75	0.73	0.75	0.73	0.73
PASITe-1	0.79	0.77	0.75	0.74	0.99	0.74	0.74	0.76	0.7	0.73	0.76	0.72	0.73	0.76	0.75
PASITe-2	0.71	0.71	0.72	0.73	0.93	0.67	0.71	0.77	0.72	0.72	0.79	0.76	0.81	0.72	0.74
PASITe-3	0.75	0.75	0.71	0.68	0.91	0.65	0.66	0.72	0.65	0.67	0.72	0.67	0.7	0.75	0.71
PASITe-4	0.78	0.75	0.7	0.72	0.98	0.7	0.74	0.76	0.73	0.73	0.73	0.76	0.75	0.7	0.71
NG188e-1	0.71	0.71	0.76	0.75	0.76	0.96	0.75	0.71	0.78	0.68	0.73	0.71	0.72	0.75	0.74
NG188e-2	0.73	0.7	0.76	0.72	0.78	0.98	0.77	0.73	0.76	0.71	0.75	0.73	0.76	0.76	0.75
NG188e-3	0.73	0.68	0.76	0.7	0.74	0.96	0.74	0.73	0.74	0.72	0.74	0.75	0.7	0.78	0.73
NG188e-4	0.76	0.73	0.72	0.7	0.72	0.97	0.7	0.73	0.75	0.7	0.74	0.75	0.72	0.79	0.72
9LV12e-1	0.76	0.72	0.79	0.68	0.73	0.72	0.97	0.68	0.73	0.68	0.76	0.68	0.75	0.77	0.74
9LV12e-2	0.73	0.73	0.81	0.74	0.76	0.76	0.96	0.72	0.77	0.67	0.72	0.76	0.73	0.74	0.79
9LV12e-3	0.75	0.73	0.72	0.71	0.74	0.73	0.98	0.7	0.72	0.68	0.73	0.66	0.77	0.73	0.76
9LV12e-4	0.73	0.72	0.77	0.72	0.76	0.68	0.98	0.7	0.75	0.68	0.72	0.73	0.7	0.71	0.75
9MSK6e-1	0.8	0.71	0.74	0.72	0.75	0.75	0.68	0.97	0.74	0.71	0.73	0.76	0.74	0.77	0.73
9MSK6e-2	0.71	0.75	0.72	0.7	0.81	0.76	0.76	0.99	0.71	0.69	0.74	0.77	0.73	0.74	0.75
9MSK6e-3	0.76	0.73	0.74	0.72	0.79	0.74	0.7	0.98	0.73	0.71	0.74	0.73	0.74	0.74	0.75
9MSK6e-4	0.75	0.76	0.76	0.76	0.8	0.71	0.73	0.99	0.77	0.68	0.76	0.77	0.78	0.8	0.75
YUM8Ee-1	0.78	0.71	0.79	0.69	0.78	0.73	0.75	0.69	0.97	0.71	0.74	0.71	0.73	0.77	0.71
YUM8Ee-2	0.73	0.73	0.77	0.74	0.73	0.76	0.71	0.77	0.99	0.69	0.75	0.75	0.74	0.81	0.78
YUM8Ee-3	0.76	0.75	0.75	0.73	0.72	0.79	0.74	0.72	0.98	0.66	0.71	0.74	0.76	0.79	0.75
YUM8Ee-4	0.76	0.72	0.79	0.71	0.74	0.75	0.72	0.71	0.99	0.69	0.74	0.72	0.73	0.76	0.75
RM3D8e-1	0.74	0.7	0.75	0.73	0.76	0.71	0.7	0.73	0.74	0.94	0.77	0.71	0.76	0.78	0.76
RM3D8e-2	0.74	0.75	0.74	0.72	0.75	0.69	0.7	0.74	0.68	0.96	0.74	0.72	0.69	0.71	0.76
RM3D8e-3	0.74	0.71	0.72	0.72	0.74	0.72	0.68	0.69	0.68	0.96	0.75	0.74	0.72	0.75	0.75
RM3D8e-4	0.73	0.7	0.73	0.72	0.78	0.71	0.7	0.72	0.73	0.98	0.77	0.78	0.72	0.73	0.77
VTP1Ze-1	0.72	0.67	0.75	0.72	0.75	0.74	0.74	0.72	0.69	0.71	0.98	0.74	0.69	0.74	0.72
VTP1Ze-2	0.73	0.69	0.79	0.68	0.74	0.74	0.77	0.69	0.72	0.7	0.98	0.77	0.74	0.72	0.73
VTP1Ze-3	0.72	0.73	0.79	0.68	0.76	0.76	0.79	0.75	0.72	0.76	0.99	0.79	0.71	0.74	0.77
VTP1Ze-4	0.7	0.72	0.82	0.76	0.78	0.77	0.77	0.72	0.73	0.72	0.99	0.75	0.7	0.74	0.77
Y8NOVe-1	0.77	0.76	0.78	0.69	0.73	0.72	0.73	0.71	0.71	0.73	0.76	0.98	0.71	0.7	0.75
Y8NOVe-2	0.8	0.82	0.79	0.74	0.75	0.73	0.71	0.77	0.77	0.7	0.75	0.99	0.72	0.73	0.78
Y8NOVe-3	0.75	0.73	0.74	0.67	0.74	0.72	0.71	0.7	0.72	0.66	0.75	0.96	0.66	0.74	0.74
Y8NOVe-4	0.79	0.82	0.77	0.74	0.72	0.72	0.73	0.78	0.75	0.7	0.76	0.99	0.75	0.74	0.75
Z5YKQe-1	0.74	0.68	0.71	0.7	0.76	0.73	0.75	0.73	0.7	0.71	0.72	0.72	0.98	0.73	0.7
Z5YKQe-2	0.78	0.71	0.72	0.72	0.79	0.74	0.77	0.78	0.73	0.72	0.71	0.75	0.96	0.76	0.72
Z5YKQe-3	0.77	0.75	0.7	0.71	0.75	0.75	0.74	0.74	0.73	0.71	0.7	0.73	0.97	0.74	0.69
Z5YKQe-4	0.75	0.69	0.69	0.68	0.77	0.75	0.74	0.75	0.72	0.68	0.72	0.71	0.97	0.74	0.7
9WO2We-1	0.78	0.73	0.75	0.72	0.77	0.77	0.75	0.76	0.76	0.71	0.76	0.75	0.77	0.99	0.74
9WO2We-2	0.79	0.69	0.77	0.75	0.74	0.8	0.75	0.76	0.78	0.73	0.73	0.75	0.75	1	0.75
9WO2We-3	0.75	0.64	0.69	0.69	0.74	0.72	0.72	0.71	0.77	0.71	0.68	0.73	0.7	0.96	0.71
9WO2We-4	0.75	0.65	0.73	0.7	0.77	0.76	0.71	0.73	0.75	0.74	0.74	0.72	0.74	0.98	0.71
K6332e-1	0.74	0.73	0.75	0.69	0.77	0.76	0.71	0.79	0.79	0.76	0.76	0.78	0.72	0.77	0.99
K6332e-2	0.74	0.73	0.76	0.67	0.74	0.75	0.73	0.74	0.78	0.77	0.77	0.74	0.72	0.74	0.97
K6332e-3	0.74	0.72	0.78	0.7	0.78	0.76	0.72	0.76	0.72	0.72	0.74	0.76	0.69	0.77	0.98
K6332e-4	0.74	0.75	0.75	0.73	0.77	0.74	0.76	0.8	0.73	0.77	0.78	0.81	0.72	0.77	0.97

Figure 4 Paternity testing results.

robust, with accuracy, sensitivity and specificity rates nearing 100%.

A major strength of this study is the use of biologically derived haploid embryos as controls, generated through parthenogenetic activation of non-viable donor oocytes. To the best of our knowledge, this is the first study using validated haploid embryos to assess the performance of a ploidy-testing method. This step was necessary because haploid cell lines do not exist and 1PN embryos are often misclassified as haploid due to asynchronous PN formation or PN fusion.

Parental origin analysis further strengthens the utility of this method in clinical scenarios, especially for confirming embryo identity, detecting contamination and verifying paternity. Notably, our method does not require parental DNA for ploidy determination alone, reducing logistical burdens in embryo assessment workflows. Instead, since the SNPs can be

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added ad hoc, after NGS, this can be used in standard PGT-A in cases where the IVF centre suspects a possible mix-up of embryos or gametes before embryo transfer.

Importantly, these features are especially critical when assessing embryos with abnormal pronuclear formation, where standard morphology or copy number analysis alone may be insufficient for accurate diagnosis. Our data indicate that a large proportion of 1PN (64%) and 3PN (42%) embryos are diploid and thus potentially viable, challenging conventional morphological classifications that often lead to their disposal. These findings are consistent with prior reports[12 17] and emphasise the necessity for molecular validation in embryo assessment. Considering that 5%–11% of ICSI eggs are 1PN and over 60% are diploid, morphological assessment is discarding more diploid embryos than PGT-A misdiagnosis (0%–4%.[16 25 26]

A limitation of this method is its inability to differentiate between some forms of tetraploidy (eg, 92,XXYY vs 46,XY), which share similar copy number and SNP signals. The test would be able to flag uniparental disomy or maternal contamination if it detected more SNPs. Maternal contamination of a male embryo could be confused as triploidy, although this is unlikely with proper laboratory procedures. Contaminated samples would be expected to have BAFs at 25, 50 and 75. However, the error rate in SNP frequency using this panel, due to repeated amplification cycles, creates an error window that makes precise calculation of allele frequency challenging. For this reason, we use ranges to identify 66% and 33% allele frequencies. Maternal contamination detection can be addressed by comparing SNP panel of a maternal sample to the embryo. High similarity (ie, 90%) will indicate maternal contamination. In this study, maternal samples from the PGT-A study were not available.

Another limitation is the potential inability of this method to detect diploid/triploid mosaic embryos. These mosaics are very rare, usually caused by delayed polyspermy, where one sperm decondenses in an already segregated cell, resulting in some cells being diploid and others triploid.[27] Most probably, a larger SNP panel would be necessary to detect them.

In conclusion, this study provides strong support for the practice of integrating SNP genotyping into routine PGT-A pipelines, especially when embryos have ambiguous PN morphology or when additional parental origin analysis is necessary.

Contributors SM, the guarantor, wrote the manuscript and partially designed the experiments. NC-B produced the haploid embryos and together with MC helped develop the haploidisation experiments. HK and MP performed PGT and provided the samples for analysis. NA assisted with writing and study design. As both reviewers noted issues with grammar, we used ChatGPT in some paragraphs to improve wording. AI was NOT used to generate results or conclusions or for any purpose other than improving grammar.

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Competing interests NA is an owner of Progenesis. NC-B is an owner at Embryotools. MC is an owner of Herasight.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

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