ORIGINAL ARTICLE

Direct assessment of hereditary hemochromatosis in preimplantation genetic testing

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Objective: Hereditary hemochromatosis (HH) is a common genetic disorder characterized by iron overload, which, if undiagnosed, can lead to severe organ damage. There are 4 types of HH. Type 1 HH, the most common form, is primarily caused by a common variant in Western Europe (p.Cys282Tyr, C282Y, or c.845 G>A). It is generally preventable during in vitro fertilization if proper genetic testing is performed before implantation. Here, we demonstrated a direct detection and cost-effective approach using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in preimplantation genetic testing (PGT) settings.
Design: We began by validating the assay with genomic deoxyribonucleic acid (DNA) from Coriell cell lines of known HFE C282Y genotypes, followed by testing patients' genomic DNA samples. After establishing the assay on genomic DNA, we extended the assay to whole-genome amplified DNA from embryo biopsies.
Subjects: The subjects include cell line samples and human specimens and human embryo biopsies.
Exposure: Patients and embryos either carried or did not carry the HFE C282Y variant in their genome. No intervention was applied.
Main Outcome Measures: The readout included the genotype of samples at the HFE C282Y locus and accuracy of PCR-RFLP results.
Results: An accuracy of >99% was achieved across 80 cell line samples, 38 patient samples, and 81 embryo biopsies.
Conclusion: In this study, we demonstrated the feasibility of using the PCR-RFLP approach to PGT. Specifically, we validated the assay for the HFE C282Y variant, the primary cause of type 1 hemochromatosis. The assay was tested on genomic DNA and DNA resulting from whole-genome amplification, achieving >99% accuracy, sensitivity, precision, and specificity. These results also suggest the possibility for extending the PCR-RFLP approach to cover a broader range of conditions, such as spinal muscular atrophy, to benefit more patients currently ineligible for testing at PGT laboratories. (F S Sci® 2025;■:■–■. ©2025 by American Society for Reproductive Medicine.)

Key Words: PGT, direct assessment, hereditary hemochromatosis, PCR-RFLP, C282Y

CLINICAL QUICK TAKE

- What clinical problem is addressed by these studies?
  - Many individuals carry the HFE C282Y variant without knowing it, which can increase genetic risk for their children.
  - We present a simple and cost-effective preimplantation genetic testing (PGT) screening approach specifically designed to detect this variant.
- What are the key findings?
  - The polymerase chain reaction-restriction fragment length polymorphism approach is accurate and reproducible for detecting HFE C282Y in PGT settings.
  - The assay performs direct detection, eliminating the need for haplotyping, prior testing for parental carrier status and PGT for monogenic disorders probe development.
- How do these findings apply to human fertility or the reproductive process?
  - This polymerase chain reaction-restriction fragment length polymorphism method can be directly applied to PGT for detecting genetic variants.
  - With modifications to primers and restriction enzymes, it also has the potential to screen for other conditions, such as spinal muscular atrophy.

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SQUIRE checklist was followed.
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Hereditary hemochromatosis (HH) is an autosomal recessive disorder characterized by excess iron absorption. This condition can lead to chronic liver diseases such as fibrosis, cirrhosis, and hepatocellular carcinoma if untreated (1). There are 4 types of HH. Type 1 HH, the most common form, is primarily caused by a common variant in Western Europe (p.Cys282Tyr, C282Y, or c.845 G>A) (2). Homozygosity for this variant in the HFE gene is what typically leads to the condition. In the general European population, approximately 0.4% of individuals are homozygous for this genotype, and this variant is responsible for up to 95% of HH cases (3). The American College of Medical Genetics Secondary Finding v3.1 list recommends reporting the HFE C282Y variant if detected (4). However, because of the high prevalence of this variant and its reduced penetrance, many carrier screening panels do not include it, meaning that families may be unaware of the risk of having an affected child (5). However, individuals who are homozygous—particularly males—can experience significant symptoms if the condition is undiagnosed (6).

Many genetic assays have been developed to directly probe this single nucleotide variant (SNV) in adults; however, only a few have been adapted or optimized for preimplantation genetic testing (PGT). Currently, many PGTs for monogenic disorders (PGT-M) rely on an indirect assessment method called haplotyping rather than direct SNV detection (Fig. 1A) (7). This approach requires deoxyribonucleic acid (DNA) samples from both parents and multiple embryos in a cycle. If only 1 embryo or only 1 parental DNA is available (e.g., in cases where donor DNA is unavailable), PGT-M cannot be performed because haplotyping is not feasible. Therefore, a cost-effective method for direct SNV detection is preferable.

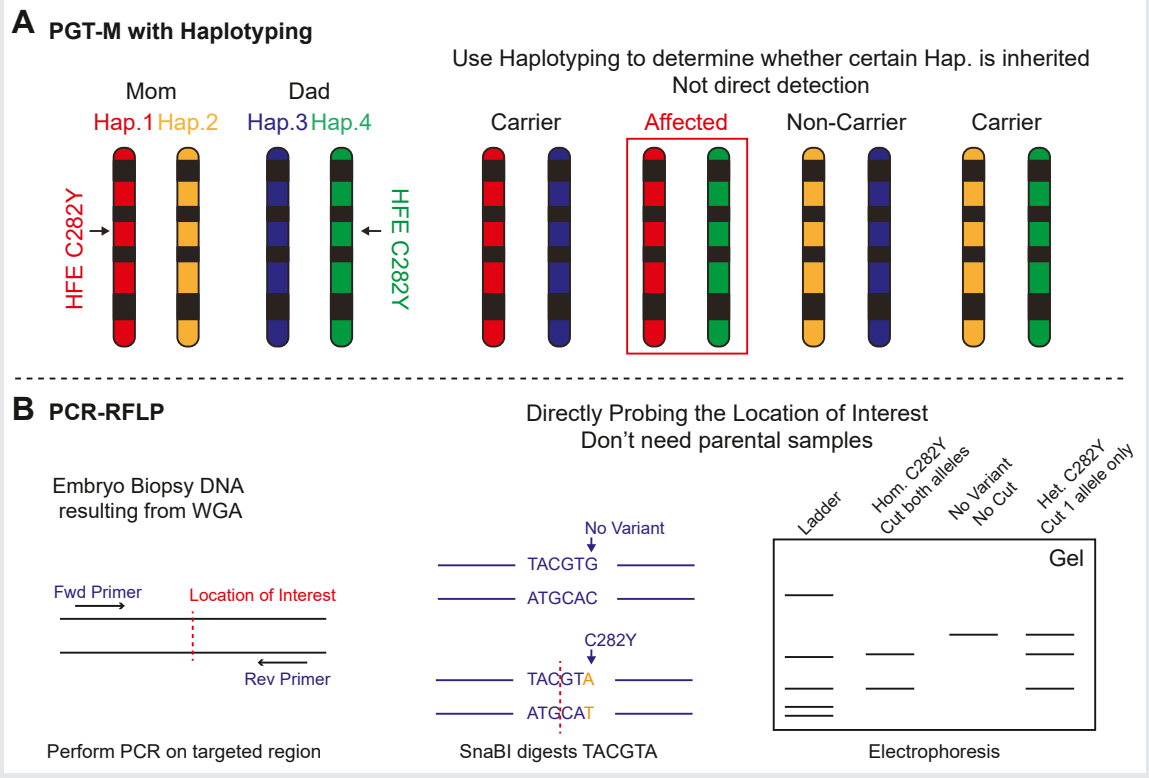
This study demonstrated the feasibility of adapting a direct detection assay for the HFE C282Y variant within a PGT setup using a cost-effective polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) approach. We began by validating the assay with genomic DNA from Coriell cell lines of known HFE C282Y genotypes, followed by testing patients' genomic DNA samples. After establishing the assay on genomic DNA, we extended the assay to embryo biopsies.

MATERIALS AND METHODS

Sample collection

Cell line DNA was purchased directly from Coriell (Camden, NJ, USA). The sample ID of each cell line is indicated in the text. The Coriell webpage provides a more detailed information on cell lines. This study did not require institutional review board

FIGURE 1



(A) Illustration of preimplantation genetic testing for monogenic disorders (PGT-M) haplotyping methodology for identifying affected embryos. (B) Schematic of the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) process, demonstrating the detection mechanism for specific variants.

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approval. The patient samples were submitted to laboratory for clinical testing, with consent provided for further research studies. All samples were deidentified before use.

DNA extraction

Saliva samples for this study were collected from patients using AccuGene's AccuSaliva Collection Kits (San Diego, CA). Deoxyribonucleic acid extraction was subsequently performed with the QIAamp DSP DNA Blood Mini Kit (Germantown, Maryland). Deoxyribonucleic acid concentration was measured using the Qubit Flex from Thermo Fisher Scientific (Waltham, MA). Deoxyribonucleic acid integrity was assessed with the Agilent TapeStation 4150 (Santa Clara, CA) using Genomic ScreenTapes. For quality assurance, samples needed a concentration of >3.5 ng/μL and a DNA integrity number of >5.5.

Embryo processing

Each embryo biopsy consisted of 3–5 trophectoderm biopsy cells with a maximum of 3-μL cell buffer in a 200-μL PCR tube. Whole-genome amplification (WGA) was performed directly in the PCR tube as described previously (8). Deoxyribonucleic acid concentration was measured using the Qubit Flex from Thermo Fisher Scientific.

Polymerase chain reaction-restriction fragment length polymorphism

For the positive and negative controls, cell line DNA samples NA14646 and NA12878 were prepared at a concentration of 20 ng/μL. Test samples were also prepared to a concentration of 20 ng/μL. The primers used were forward 5′-TGGCAAGGGTAAACAGATCC-3′ and reverse 5′-CTCAGGCACTCCTCTCAACC-3′, each at a concentration of 10 μM in Tris-ethylenediaminetetraacetic acid buffer from IDT (Coralville, IA).

The PCR mixture was prepared with 2 μL of DNA, 25 μL of DreamTaq PCR Master Mix (2×) from Thermo Fisher Scientific, 1 μL of each primer, and 21 μL of water, for a final volume of 50 μL. The thermocycler was programmed as follows: an initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 1 minute, 55 °C for 45 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes and a hold at 4 °C indefinitely.

After PCR, DNA purification was performed using 30 μL of Roche KAPA beads (Indianapolis, IN) according to the manufacturer's protocol, with elution in 30 μL of elution buffer from Qiagen (Germantown, MD). Next, 15 μL of purified DNA was combined with 5 μL of 10X NEB buffer, 1 μL of SnaBI enzyme (100 U/mL) from NEB (Ipswich, MA), and 29 μL of water, bringing the reaction volume to 50 μL. The mixture was incubated at 37 °C for 1 hour.

Finally, 1 μL of the resulting product was mixed with 3 μL of D1000 reagent and analyzed on the TapeStation 4150 using D1000 ScreenTape.

The output comma-separated values (CSV) files were exported for further analysis, with peaks below an intensity threshold of 0.2 filtered out as artifacts or interference.

A homozygous C282Y variant sample will display 2 bands: 1 at approximately 260 bp and another at 110 bp. A sample without the C282Y variant will show a single, uncut band at approximately 380 bp. A heterozygous C282Y variant sample will display 3 bands: 1 at the uncut size (380 bp) and 2 at the cut sizes (260 and 110 bp). If the resulting band pattern does not match these expected profiles, the results will be classified as indeterminate.

Sanger confirmation

Sanger sequencing and primer design were performed by Psomagen. Genotypes at the target sites were analyzed from chromatograms using in-house software.

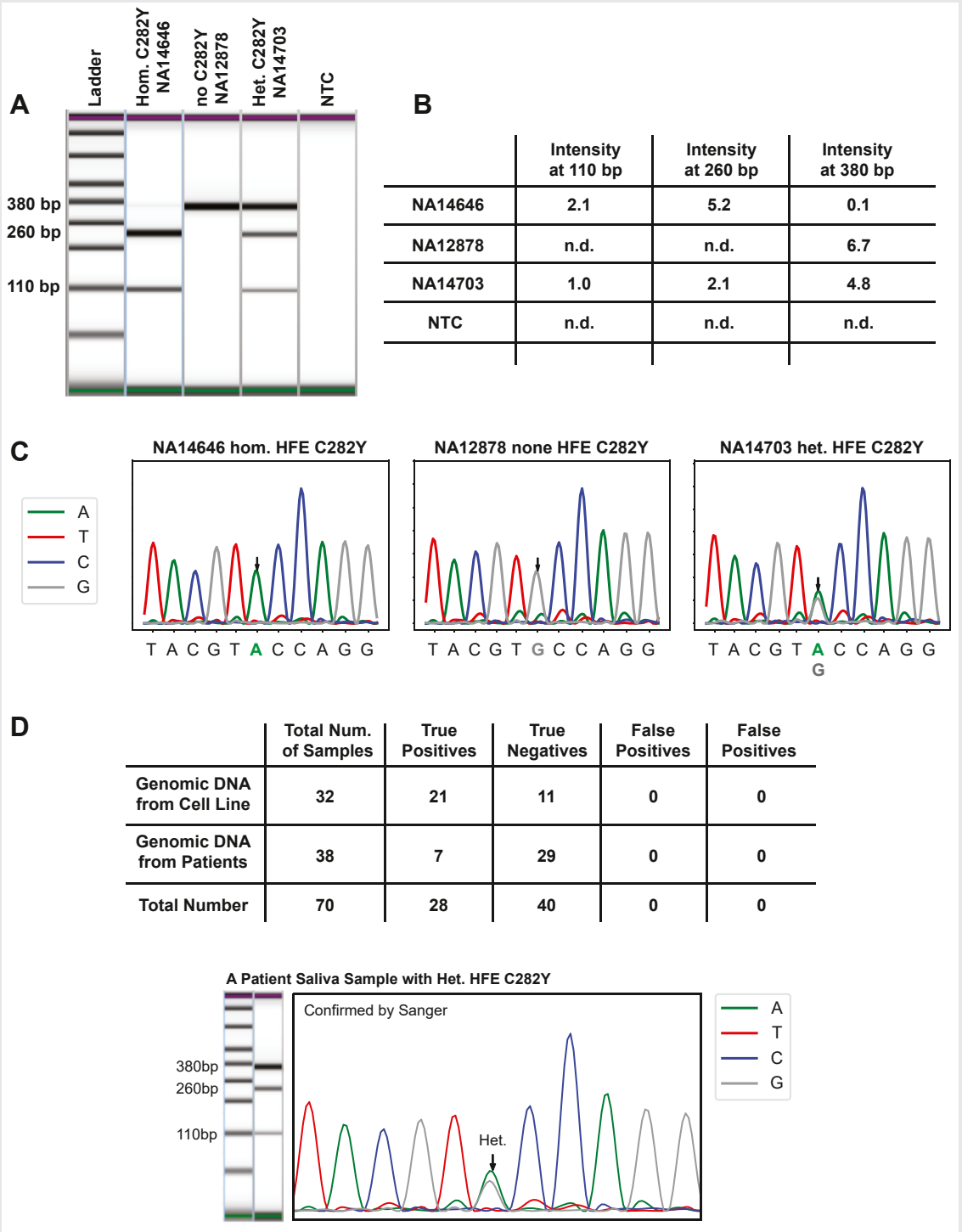
RESULTS

In the PCR-RFLP assay, we first used primers (5′-TGGCAAGGGTAAACAGATCC-3′ and 5′-CTCAGGCACTCCTCTCAACC-3′) to amplify a region of the HFE gene from Chr20:26,092,862 to Chr20:26,093,251 in the GRCh37 human genome, producing a fragment of approximately 380 base pairs (bp) (Fig. 1B, left) (9,10). If the HFE C282Y variant (c.845 G>A) is present, a unique TACGTA sequence exists within this fragment. The restriction enzyme SnaBI recognizes and cuts this sequence, producing 2 smaller fragments of approximately 260 and 110 bp (Fig. 1B, middle). Therefore, a homozygous C282Y variant sample will display 2 bands at approximately 260 and 110 bp. In contrast, a sample without the C282Y variant will show a single, uncut band at approximately 380 bp because it lacks the TACGTA sequence, preventing SnaBI digestion (11). A heterozygous C282Y variant sample will display 3 bands: 1 at the uncut size (380 bp) and 2 at the cut sizes (260 and 110 bp) (Fig. 1B, right).

This study used the Coriell cell line NA14646, derived from a patient with hemochromatosis and homozygous HFE C282Y as a positive control. In contrast, another Coriell cell line, NA12878, was used as a negative control because it does not carry any HFE C282Y variant, according to the published sequencing results from the National Institute of Standards and Technology (12).

As shown in Figure 2A, the targeted DNA segments were successfully amplified from genomic DNA using PCR and digested with SnaBI. The positive control NA14646 resulted in 2 bands: 1 at 110 bp and 1 at 260 bp, matching our prediction. The negative control NA12878 resulted in no digestion, showing an uncut band 380 bp due to the lack of the TACGTA sequence. Then, looking at another Coriell cell line NA14703, which carried the heterozygous HFE C282Y variant, it produced results with 3 bands: 110; 260; and 380 bp. This means that the allele with the C282Y variant was cut by SnaBI and the other allele without the C282Y variant remains uncut, consistent with the expected heterozygous genotype. Finally, the non-template control control exhibited no band, indicating no contamination occurred during the experiment. The 3 cell line samples were then confirmed by Sanger sequencing (Fig. 2C). Sanger chromatograms exhibited an A at the variant position in NA14646, consistent with the homozygous C282Y; an G at

FIGURE 2



(A) TapeStation ScreenTape results showed band patterns for homozygous C282Y, noncarrier, heterozygous C282Y, and nontemplate control samples. (B) Tabulated band intensities by size for each sample, demonstrating consistency with expected results. (C) Sanger sequencing confirmation for homozygous C282Y, noncarrier, and heterozygous C282Y samples. (D) Summary statistics of all samples tested using the polymerase chain reaction-restriction fragment length polymorphism assay and a Sanger illustration of a patient sample.

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FIGURE 3

A

Using DNA resulted from Whole Genome Amplification	Coriell Record on C282Y	PCR-RFLP on C282Y	Concordant?
NA14646	hom.	hom.	Yes
NA12878	n.d.	n.d.	Yes
NA14703	het.	het.	Yes
NA14180	hom.	hom.	Yes
NA14691	het.	het.	Yes
NA24695	n.d.	n.d.	Yes
NA6000	n.d.	n.d.	Yes
NA13591	n.d.	n.d.	Yes
NA24694	n.d.	n.d.	Yes
NA24631	n.d.	n.d.	Yes
NA14621	hom.	hom.	Yes
NA14651	hom.	hom.	Yes
NA14657	hom.	hom.	Yes
NA14652	het.	het.	Yes
NA14650	het.	het.	Yes
NA14641	het.	het.	Yes

B

	Total Num. of Samples	True Positives	True Negatives	False Positives	False Positives
Amplified DNA from Cell Line	48	30	18	0	0
Amplified DNA from Embryo Biopsies	81	41	40	0	0
Total Number	129	71	58	0	0

(A) Summary of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) results using deoxyribonucleic acid (DNA) obtained from whole-genome amplification, with all outcomes consistent with corresponding Coriell records. (B) Summary statistics for all whole-genome amplified samples tested using the PCR-RFLP assay, demonstrating high concordance and reliability of the method.

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the variant position in NA12878, indicating no variant; and a A/G in NA14703, confirming the heterozygosity.

Following the proof-of-concept experiments, we expanded the assay to a broader sample population, including additional Coriell cell lines and patient samples. For the patient samples, DNA was extracted from saliva and subsequently subjected to the PCR-RFLP assay (Fig. 2D, labeled as Genomic DNA from Patients). The results were validated by cross-referencing Coriell records or Sanger sequencing results, demonstrating an accuracy of >99%, sensitivity of >99%, precision of >99%, and specificity of >99%.

WGA is often necessary when the initial DNA quantity in a sample is insufficient for downstream genetic testing. For instance, in PGT, an embryo biopsy typically contains only 30–50 pg of DNA, far below the amount required for genetic analysis. Thus, WGA is generally performed before testing. Here, we performed the WGA as described previously (8) and obtained 3,000–5,000 ng of DNA, sufficient not only for ploidy screening such as PGT for aneuploidy (PGT-A; requires approximately 500 ng) but also for additional genetic testing. Consequently, PCR-RFLP was applied to whole-genome amplified DNA to evaluate its feasibility for directly assessing the HFE C282Y variant.

We performed WGA on 16 distinct Coriell cell lines, each containing 50 pg of DNA, yielding a mean of 3,000–5,000 ng of DNA per sample. Forty nanograms was taken for PCR-RFLP from each whole-genome amplified DNA sample. Initial tests on amplified DNA from these 16 Coriell cell lines showed over 99% concordance with Coriell records (Fig. 3A). We then expanded our testing to 32 additional cell line samples,

achieving results that were >99% consistent with Coriell records.

Next, we performed PCR-RFLP on DNA samples from embryo biopsies, processed as previously described (8). We obtained 3,000–5,000 ng of DNA per sample after WGA. From each sample, 40 ng of the resulting amplified DNA was used for PCR-RFLP. The 81 embryo biopsies tested showed >99% concordance with prior next-generation sequencing or haplotyping findings (Fig. 3B).

These findings demonstrate the feasibility and robustness of using a PCR-RFLP assay in PGT to directly detect the HFE C282Y variant without the need for parental genomes, which is required in traditional PGT-M.

DISCUSSION

The DNA used for PCR-RFLP is the same as that used for PGT-A. WGA from an embryo typically produces approximately 2,000 ng of DNA, with 500 ng allocated for PGT-A, leaving 1,500 ng available for additional testing. This surplus allows us to perform PCR-RFLP. Therefore, a single embryo biopsy provides enough DNA after WGA to support both PGT-A and PCR-RFLP testing. Additionally, because PCR-RFLP requires only 40 ng per test, this surplus allows laboratories to test many more variants, as long as primers and restriction enzymes can be properly designed.

Although this study focuses on the HFE C282Y variant, it opens the door for broader variant screening using PCR-RFLP methods. For example, PGT for spinal muscular atrophy has posed challenges for some patients because the cis or trans status of SMN1 cannot be concluded from carrier screening results (13). In many cases, these patients are rejected by PGT laboratories due to the absence of definitive genotypes necessary for haplotyping. Direct detection of embryo biopsies could benefit patients in such situations because it focuses on the embryo instead of the parents. The assay is now in the development phase.

Direct detection assay provides several advantages over indirect detection assay in PGT-M. In traditional PGT-M, where haplotyping is required, even with parental DNA available, testing is often not feasible when only a single embryo is present. In these cases, reliable haplotyping requires the inclusion of an additional family member, which complicates the process. However, direct detection eliminates the need for parental samples, simplifying the process and reducing costs.

From a cost perspective, PCR-RFLP has a reagent cost of under $10 per sample. At the same time, traditional PGT-M often requires several hundred dollars just to sequence the parental genomes before embryo testing.

However, PCR-RFLP has limitations. Designing and optimizing primers and restriction enzymes are necessary for testing new variants, making it more suitable for common pathogenic variants where primers and enzymes are readily available. Moreover, the method is not effective in PCR-challenging regions and is restricted to detecting single nucleotide polymorphisms and small indels. It cannot identify larger genetic variations, such as microduplications or microdeletions.

CONCLUSION

In this study, we demonstrated the feasibility of extending the adult PCR-RFLP approach to PGT. Specifically, we validated the assay for the HFE C282Y variant, the primary cause of hemochromatosis. The assay was tested on genomic DNA and DNA resulting from WGA, achieving >99% accuracy, sensitivity, precision, and specificity. We aim to expand the PCR-RFLP approach to cover a broader range of conditions, such as spinal muscular atrophy, to benefit more patients currently ineligible for testing at PGT laboratories.

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CRediT Authorship Contribution Statement

Qinnan Zhang: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Maria Katz:** Writing – original draft, Conceptualization. **Benjamin Podgursky:** Formal analysis, Conceptualization. **Nicholas Schuch:** Formal analysis, Data curation. **Shenglai Li:** Visualization, Formal analysis, Data curation. **Noor Siddiqui:** Resources, Funding acquisition. **Funda Suer:** Writing – original draft, Supervision, Methodology, Conceptualization. **Yuntao Xia:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Interests

The authors are employees and stakeholders of Orchid Health.

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