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Identification of microsatellite markers <1 Mb from the *FMR1* CGG repeat and development of a single-tube tetradecaplex PCR panel of highly polymorphic markers for preimplantation genetic diagnosis of fragile X syndrome

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Purpose: To develop a single-tube polymerase chain reaction (PCR) panel of highly polymorphic markers for preimplantation genetic diagnosis (PGD) of fragile X syndrome (FXS).

Methods: An *in silico* search was performed to identify all markers within 1 Mb flanking the *FMR1* gene. Selected markers were optimized into a single-tube PCR panel and their polymorphism indices were determined from 272 female samples from three populations. The single-tube assay was also validated on 30 single cells to evaluate its applicability to FXS PGD.

Results: Thirteen markers with potentially high polymorphism information content (PIC) and heterozygosity values were selected and optimized into a single-tube PCR panel together with *AMELX/Y* for gender determination. Analysis of 272 female samples confirmed the high polymorphism (PIC > 0.5) of most markers, with expected and observed heterozygosities ranging from 0.31 to 0.87. More than 99% of individuals were heterozygous for at least three markers, with 95.8% of individuals heterozygous for at least two markers on either side of the *FMR1* CGG repeat.

Conclusion: The tetradecaplex marker assay can be performed directly on single cells or after whole-genome amplification, thus supporting its use in FXS PGD either as a standalone linkage-based assay or as a complement to *FMR1* mutation detection.

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Key Words: *FMR1*; fragile X syndrome; microsatellite markers; preimplantation genetic diagnosis

INTRODUCTION

Fragile X syndrome (FXS) is the leading known single-gene cause of inherited intellectual disabilities, with an estimated frequency of 1 in 5,000 males and 1 in 2,500–8,000 females.[1] It is most frequently caused by hyperexpansion and hypermethylation of a polymorphic CGG trinucleotide repeat in the 5′ untranslated region of *Fragile X Mental Retardation 1* (*FMR1*) gene on chromosome Xq27.3, resulting in transcriptional silencing of the gene.[2] Affected males typically have more severe phenotypes than females, including autism, intellectual and developmental disability, social anxiety, attention deficit hyperactivity, and other physical malformations.[3,4] Affected pregnancies can be avoided by preimplantation genetic diagnosis (PGD) or terminated after prenatal diagnosis.

FMR1 alleles can be categorized into four groups that are highly correlated with CGG repeat length: normal (NL; 5–44), intermediate/gray zone (IM/GZ; 45–54), premutation (PM; 55–200), and full mutation (FM; >200). Carriers of NL, IM, and PM alleles do not present any FXS symptoms. However, approximately 20% of female PM carriers are at risk for fragile X–associated primary ovarian insufficiency (FXPOI),[5,6] and approximately 40% of male PM carriers are at risk for fragile X–associated tremor/ataxia syndrome (FXTAS).[7,8] Furthermore, PM alleles are unstable and have a strong tendency to expand to FM alleles upon maternal transmission within one generation; therefore, women carrying these alleles have an increased risk of having pregnancies affected with FXS. The high population prevalence of PM alleles (1 in 130–250 females and 1 in 250–810 males[3]) further increases this risk. Consequently, female sex selection, which is commonly used to avoid X-linked disorders in general, is unsuitable for PGD of FXS because female carrier embryos may be affected. Therefore, it is necessary to exclude both male and female carriers of a mutant *FMR1* allele during PGD of FXS.

Southern blot analysis, the gold standard molecular diagnostic method for FXS, is incompatible with PGD due to its requirement for large quantities of DNA and the limiting genetic material from cleavage or blastocyst stage embryos. Polymerase chain reaction (PCR) amplification across the *FMR1* CGG repeat is commonly used for PGD of FXS. However, the technique is

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incapable of detecting all expanded *FMR1* alleles and therefore relies on detecting two different-sized normal *FMR1* alleles to exclude FXS in female embryos. This strategy will work only for the two-thirds of couples with informative normal *FMR1* alleles.[9,10] For the remaining one-third of couples with uninformative normal *FMR1* alleles, PGD of FXS relies entirely on linkage analysis of flanking microsatellite markers.[11–18] This usually involves prescreening each couple to identify informative markers[11,15,16] and PGD analysis involving multiplex PCR of a selected subset of markers, followed by nested secondary PCR of individual markers.[14–16] Thus far, at least 14 different makers have been used in PGD of FXS, with up to six markers successfully coamplified from a single cell.[18] Different combinations of informative markers have been used,[11,15,16] with each new marker combination requiring some customization and optimization to ensure successful coamplification of markers.

To increase the availability of closely linked informative markers and simplify their use in FXS PGD, an *in silico* search was performed to identify all markers within 1 Mb flanking the *FMR1* gene. Markers predicted to have low polymorphism or to be suboptimal for PCR amplification were filtered out, and 13 polymorphic microsatellite markers (*FXS146320*, *FXS146374*, *DXS998*, *FXS146706*, *FXS146782*, *DXS548*, *FXS147120*, *DXS731*, *FXS147174*, *FXS147197*, *FXS147217*, *DXS1215*, and *FXS147275*) were selected and optimized into a single-tube assay, together with *AMELX/Y* (*Amelogenin* gene on the X and Y chromosomes) for gender determination. Genotype analysis of genomic DNA samples from three populations confirmed the high polymorphism information content (PIC) and heterozygosity values of the panel markers, suggesting a high likelihood of assay informativeness of this tetradecaplex marker panel when applied to most, if not all, FXS PGD cases. This marker panel can be amplified from single cells, either directly or after whole-genome amplification, demonstrating its applicability for linkage-based FXS PGD either as a standalone assay or in conjunction with *FMR1* normal allele detection.

MATERIALS AND METHODS

Biological samples

Lymphoblastoid cell lines were purchased from the Coriell Cell Repository (Camden, NJ). Cord blood DNA of 272 anonymous, unrelated females (91 Malay, 87 Chinese, and 94 Indian) was used to evaluate the informativeness of each marker. No patients affected with FXS were involved in this study. DNAs or single cells were used for initial genotyping analysis of potential microsatellite markers, assay optimization, and determination of polymorphism and informativeness of selected markers. Single-cell isolation, lysis, and whole-genome amplification (WGA) processing have been described elsewhere.[19] This study was approved by the Institutional Review Board of the National University of Singapore (13-309E; 07-123E).

Identification and selection of microsatellite markers

DNA sequence within 1 Mb upstream and downstream relative to the *FMR1* CGG repeat was downloaded from the UCSC Genome Browser (ChXq27.3) (genome assembly GRCh37/hg19, February 2009 annotation). The strategy for identification and selection of markers has been described elsewhere.[19] Thirteen microsatellite markers with potentially high PIC and heterozygosity values were selected and optimized into a single-tube multiplex panel together with *AMELX/Y*.

PCR amplification

Single-marker PCR amplification was performed in a 50-µl reaction volume consisting of 10 ng genomic DNA, 2.5 U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 1× supplied PCR buffer containing 1.5 mmol/l $MgCl_2$ (Qiagen), 0.2 mmol/l deoxyribonucleotide triphosphates (Roche, Penzberg, Germany), and 0.2 µmol/l of each primer. Thermal cycling involved an initial 15-min enzyme activation at 95°C, 30 cycles of denaturation at 98°C for 45 s, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Multiplex PCR amplification was performed in a 20-µl reaction consisting of 10 ng of genomic DNA, 6 µl of lysed and neutralized single cell sample, or 2 µl of single cell WGA product as template, 1× Qiagen Multiplex PCR Master Mix (Qiagen), and 0.06–0.6 µmol/l of each primer. Thermal cycling involved an initial 15-min enzyme activation at 95°C, 30 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 1 min, ending with a final extension at 60°C for 30 min. Single-cell PCR conditions were identical to these except that 40 cycles were used.

Electrophoresis and analysis

A 1-µl aliquot of PCR product was mixed with 9 µl of Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.3 µl of GeneScan 500 ROX dye size standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min, cooled to 4°C, and resolved in a 3130xl Genetic Analyzer using a 36-cm capillary filled with either POP4 or POP7 polymer (Applied Biosystems). Samples analyzed using POP4 were electrokinetically injected at 1.2 kV for 18 s and electrophoresed for 25 min at 60 °C. Samples analyzed with POP7 were electrokinetically injected at 1.2 kV for 23 s and electrophoresed for 20 min at 60 °C. GeneScan analysis was performed using GeneMapper 4.0 software (Applied Biosystems).

Statistical analysis

Allele frequency, PIC,[20] expected heterozygosity (He),[21] and observed heterozygosity (Ho)[22] of each marker were calculated using Microsoft Excel.

RESULTS

One-hundred seventy potential microsatellite markers lying within 1 Mb upstream and downstream from the *FMR1* CGG repeat were identified by *in silico* mining, of which 146 satisfied the initial selection criteria (**Supplementary Table S1** online). Of these, seven markers were excluded due to their location within *Alu* repeats, and another seven markers were excluded because specific PCR primers could not be

satisfactorily designed. Following the preliminary screening, 13 microsatellite markers, including six established/published markers (*DXS998*, *FXS146706*, *FXS146782*, *DXS548*, *DXS731*, and *DXS1215*) and seven novel markers (*FXS146320*, *FXS146374*, *FXS147120*, *FXS147174*, *FXS147197*, *FXS147217*, and *FXS147275*), were selected after consideration of their polymorphism, informativeness, amplicon size, and location. Six of the 13 markers are located upstream of the *FMR1* CGG repeat and seven are located downstream. The distance of the furthest marker (*FXS146320*) from *FMR1* is <0.67 Mb. *DXS548* and *DXS731* are the closest established/published markers to *FMR1*. The thirteen markers were coamplified with *AMELX/Y* in a single-tube PCR reaction (**Figure 1**). Amplicons could be easily distinguished by their size ranges and peak colors.

To determine the allele frequency, PIC, He, and Ho values of the 13 microsatellite markers, we genotyped 272 DNAs from three populations (**Supplementary Table S2** online and **Table 1**). In total, 157 alleles were observed, with 6–22 alleles observed for each marker. Allele frequencies ranged from 0.0018 to 0.8235. Allele distributions of these markers varied in different populations and population-specific alleles were observed (**Supplementary Figure S1** online and **Supplementary Table S2** online). For example, alleles 116, 124, and 126 of *FXS146374* were observed only in the Indian population, whereas alleles 148, 150, and 152 were observed only in the Chinese population. Ten markers were highly polymorphic (PIC > 0.5), whereas the remaining three had 0.5 > PIC > 0.25 (**Table 1**). Markers *FXS147275* and *DXS548* were the most and least polymorphic, respectively, with He and Ho values ranging from a minimum of 0.31 (*DXS548*) to a maximum of 0.87 (*FXS147275*). Despite its low polymorphism, *DXS548* was included in the panel because it is one of the most well-characterized and recognized markers in FXS/*FMR1* analysis. The average informativeness of all markers in the panel was more than 0.65.

Population-specific differences in marker heterozygosity were also observed, to varying degrees (**Supplementary Figure S2** online and **Supplementary Table S3** online). In the Chinese and Malays, *FXS147275* was the most informative, with Ho of 0.89 and 0.90, respectively, whereas *DXS548* was the least informative (Ho = 0.23 and 0.31, respectively). In the Indian population, *FXS147120* and *FXS147275* were the most informative (Ho = 0.83), whereas *DXS548* had a Ho of 0.39. Of the 272 individuals genotyped, 271 (99.6%) were heterozygous for 3 or more of the 13 markers, whereas 263 (96.7%) were heterozygous for 6 or more markers (**Figure 2**). Importantly, 271 individuals (99.6%) were heterozygous for at least one marker on each side of the *FMR1* CGG repeat, and 261 (95.8%) were heterozygous for at least two markers on either side of *FMR1*

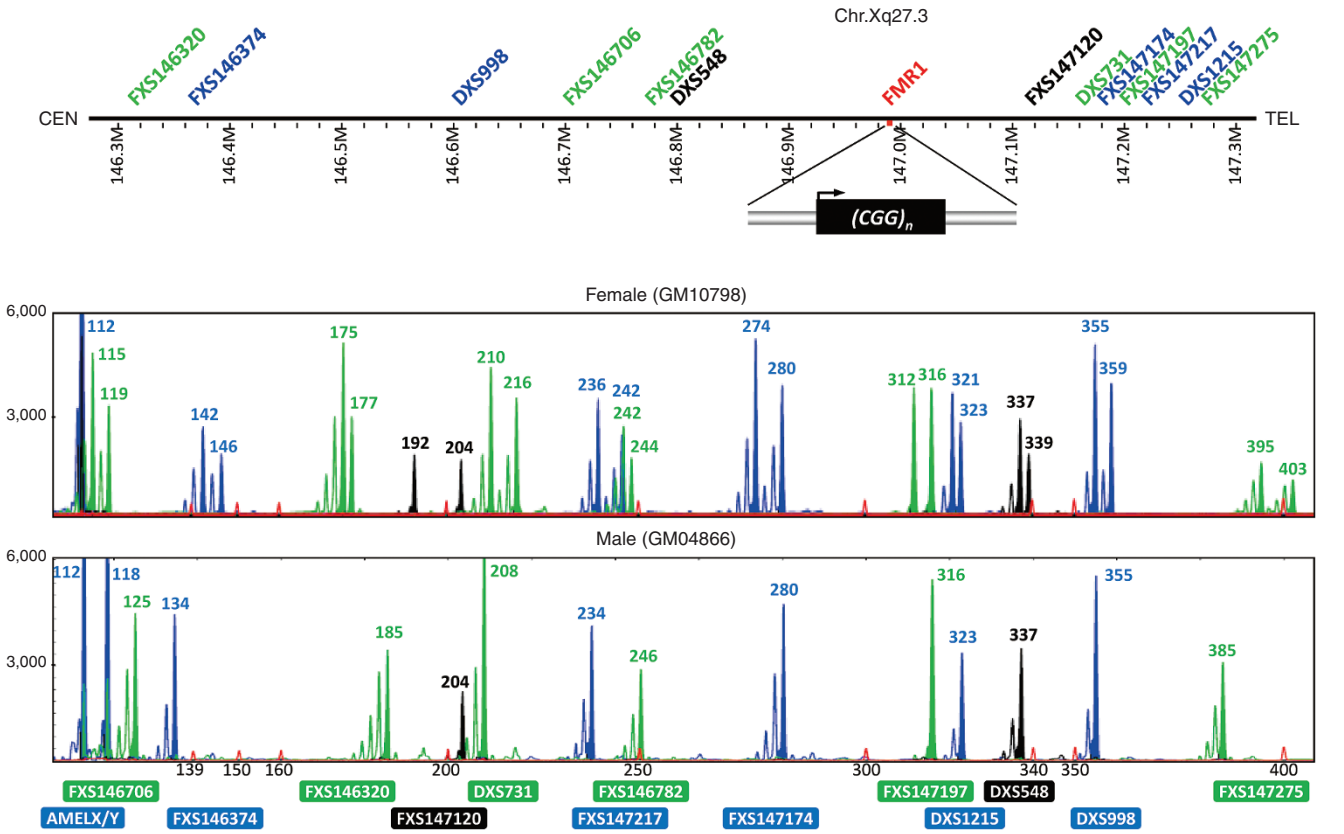

Figure 1 Single-tube multiplex PCR of 13 microsatellite markers flanking the *FMR1* CGG repeat on chromosome Xq27.3 and the *AMELX/Y* gene. Top, Schematic illustration of the chromosome Xq27.3 region showing relative positions of the *FMR1* CGG repeat and the 13 flanking microsatellite markers. Bottom, Representative electropherograms of multiplex PCR products including *AMELX/Y* after single-round amplification from 10 ng of female or male genomic DNA.

Table 1 Microsatellite markers flanking the *FMR1* CGG repeat used in the single-tube multiplex PCR assay

Marker name[a]	Repeat motif	Alleles[b]	Primer positions[c]	Concentration (μmol/l)	Amplicon size (bp)[c]	PIC[c]	He[c]	Ho[c]	AFR (%)[f]	ADO (%)[f]
FXS146320	(CT)n	17	F-HEX: 146320774-146320796 R: 146320929-146320946[d]	0.20	146–188	0.72	0.76	0.75	0.00	0.00
FXS146374	(AC)n	16	F-FAM: 146374676-146374700 R: 146374801-146374820	0.20	116–152	0.73	0.75	0.74	0.00	0.00
DXS998	(CA)n	6	F: 146607846-146607870 R-FAM: 146608177-146608198	0.15	340–358	0.50	0.54	0.49	0.00	0.00
FXS146706	(AC)n	10	F-HEX: 146706145-146706162 R: 146706249-146706273	0.40	109–127	0.61	0.64	0.66	0.00	3.33
FXS146782	(AC)n	14	F-HEX: 146782689-146782715 R: 146782912-146782933	0.30	223–255	0.76	0.79	0.76	0.00	0.00
DXS548	(GT)n	11	F-NED: 146803531-146803549 R: 146803840-146803862[d]	0.30	326–348	0.29	0.31	0.31	0.00	0.00
FXS147120	(AAAG)n	22	F-NED: 147120198-147120222 R: 147120366-147120389	0.06	164–324	0.85	0.87	0.84	0.00	10.00
DXS731	(TG)n	11	F-HEX: 147172458-147172481 R: 147172644-147172661	0.40	197–219	0.72	0.76	0.72	0.00	0.00
FXS147174	(GT)n	14	F-FAM: 147174729-147174747 R: 147174989-147175006[d]	0.10	258–288	0.58	0.61	0.57	0.00	0.00
FXS147197	(AAGG)n(A)n(GAAGG)n	6	F-HEX: 147197513-147197535[e] R: 147197812-147197832	0.12	309–325	0.47	0.56	0.57	0.00	3.33
FXS147217	(CA)n	10	F-FAM: 147217563-147217584 R: 147217782-147217805	0.20	222–248	0.71	0.75	0.73	0.00	0.00
DXS1215	(AC)n	6	F-FAM: 147268467-147268491[e] R: 147268764-147268785[d]	0.20	313–323	0.41	0.50	0.53	0.00	0.00
FXS147275	(AC)n	14	F-HEX: 147274920-147274938 R: 147275288-147275314	0.60	372–406	0.86	0.87	0.87	0.00	13.33

[a]Italicized markers are novel. [b]Alleles and genotypes were determined from 272 DNA samples. [c]Distance in base pairs (bp) from chromosome Xp-telomere based on genome assembly build GRCh37/hg19 (UCSC). [d]A GTTT tail sequence was added to the 5′ end of the primer. [e]Degenerate primer, S=C/G, V=A/C/G. [f]Values were determined from the analysis of 30 single lymphocytes from cell line GM10798.

ADO, allele drop-out rate; AFR, amplification failure rate; He, expected heterozygosity; Ho, observed heterozygosity; PIC, polymorphism information content.

(**Figure 3**). These results suggest that the multiplex panel contains sufficient marker redundancy to be informative for most FXS PGD cases, thereby avoiding the need for case-specific marker identification, selection, and panel customization.

To evaluate single-cell PGD suitability, the tetradecaplex PCR panel was validated on 30 single lymphoblasts isolated from the female lymphoblastoid cell line GM10798, which is heterozygous for all 13 marker loci (**Figure 1**). Successful coamplification of all 13 microsatellite marker loci and *AMELX/Y* was observed in all 30 cells (**Figure 4** and **Table 1**). Allele drop-out (ADO) was observed in only 4 of the 13 markers, with ADO rates ranging from 0 to 13.3%. The tetradecaplex PCR panel was also successfully amplified from WGA products of single cells, thus providing the flexibility to perform single-cell analysis either directly or after WGA (**Figure 4**).

DISCUSSION

Preimplantation genetic diagnosis of fragile X syndrome commonly relies on detection of the normal (nonexpanded) *FMR1* allele(s) by PCR amplification across the CGG repeat, with or without analysis of additional linked microsatellite markers to increase diagnostic confidence.[9,10,13–15,17,18] The larger mutant (PM and FM) *FMR1* allele is known to be highly refractory to PCR amplification and detection, especially when amplified from the limiting DNA of a single cell. This difficulty is further accentuated in the female heterozygote, where the normal allele is preferentially amplified and detected, at the expense of the expanded allele that fails to be detected. Therefore, to distinguish a normal female embryo from one carrying an expansion, the normal alleles of the male and female partner must differ in size, such that two normal alleles will be observed in a normal female embryo. This method is thus unsuitable for the one-third of PGD couples whose normal alleles are identical in size.[13,23]

Although the triplet-primed PCR (TP-PCR) method has been used successfully to detect *FMR1* CGG repeat expansions from genomic DNA,[24–27] and although PGD by TP-PCR has been described for myotonic dystrophy type 1 (ref. 10), PGD by direct TP-PCR has not been reported for FXS. This may be due to a combination of the extreme GC-rich sequence of the *FMR1* repeat and the limiting DNA available from a single cell. Therefore, for couples at risk for conceiving FXS-affected

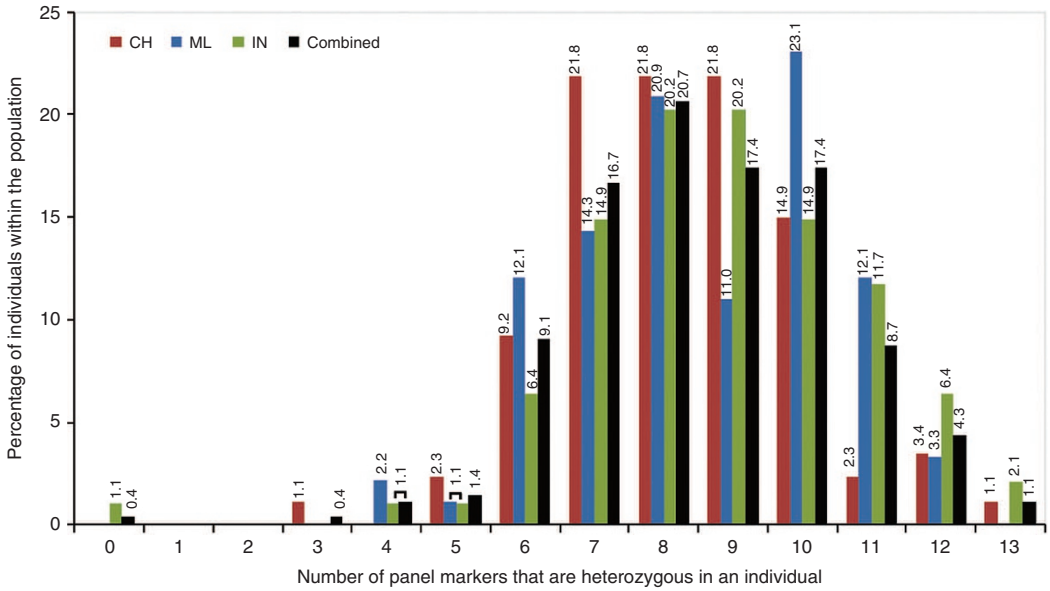

Figure 2 Percentage of individuals within the population who are heterozygous for different numbers of panel microsatellite markers. Colored bars indicate percentage of each population heterozygous for one or more panel markers. Black bars indicate the average percentage of the three populations. CH, Chinese; IN, Indian; ML, Malay.

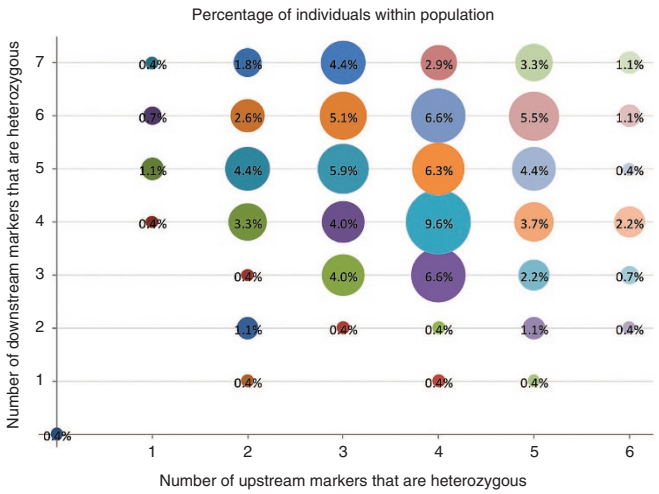

Figure 3 Percentage of individuals within the population heterozygous for different numbers of upstream and downstream flanking microsatellite markers. The x-axis represents the number of heterozygous upstream markers and the y-axis represents the number of heterozygous downstream markers.

children, PGD by linkage analysis of polymorphic markers is the only viable alternative, provided that a related index case is available to establish disease phase. Even when a couple's normal *FMR1* alleles are informative, linked marker analysis is performed in combination with *FMR1* CGG repeat analysis to minimize misdiagnosis caused by ADO and/or maternal/paternal DNA contamination.[13–18]

Several linkage-based assays have been published for the diagnosis of FXS.[11–18,28–34] Several of the reported markers are located more than 1 Mb away from the *FMR1* CGG repeat, which is not ideal due to the increased probability of recombination between marker and mutation. Also, the few closely linked markers (<1 Mb from *FMR1* CGG repeat) may not be informative for all or even the majority of FXS PGD couples. Prescreening of multiple markers is often required for each PGD case, with the subset of informative markers then selected for use either individually or in a nested or non-nested multiplex PCR.

Thus far, a maximum of six markers have been reported to be successfully coamplified from a single cell.[18] We have now more than doubled the number of markers that can be simultaneously amplified by combining 13 closely linked and polymorphic markers with *AMELX/Y* into a single-tube multiplex PCR panel. This panel is expected to have at least two informative markers on either side of the *FMR1* CGG repeat in a majority of at-risk couples, which will significantly reduce the need for assay customization. Also, with all panel markers <1 Mb away from the *FMR1* CGG repeat, the probability of an indeterminate diagnosis resulting from a recombinant haplotype is expected to be <1%.

Most existing linkage-based PGD assays use nested multiplex PCR followed by individual PCR reactions.[14–16] By multiplexing all 13 markers and *AMELX/Y* in a single round of PCR, the total number of reactions is significantly reduced, as is turnaround time, and the reduced number of steps translates into potentially fewer human errors. When the multiplex PCR assay was performed on 30 single cells, all cells amplified at all marker loci and ADO was observed in only four markers (**Table 1**). Marker *FXS147275* showed the highest ADO rate, likely due to its comparatively large amplicon size. However, the high marker redundancy of this panel lessens the adverse impact caused by individual marker ADO while simultaneously increasing power to detect exogenous DNA contamination,

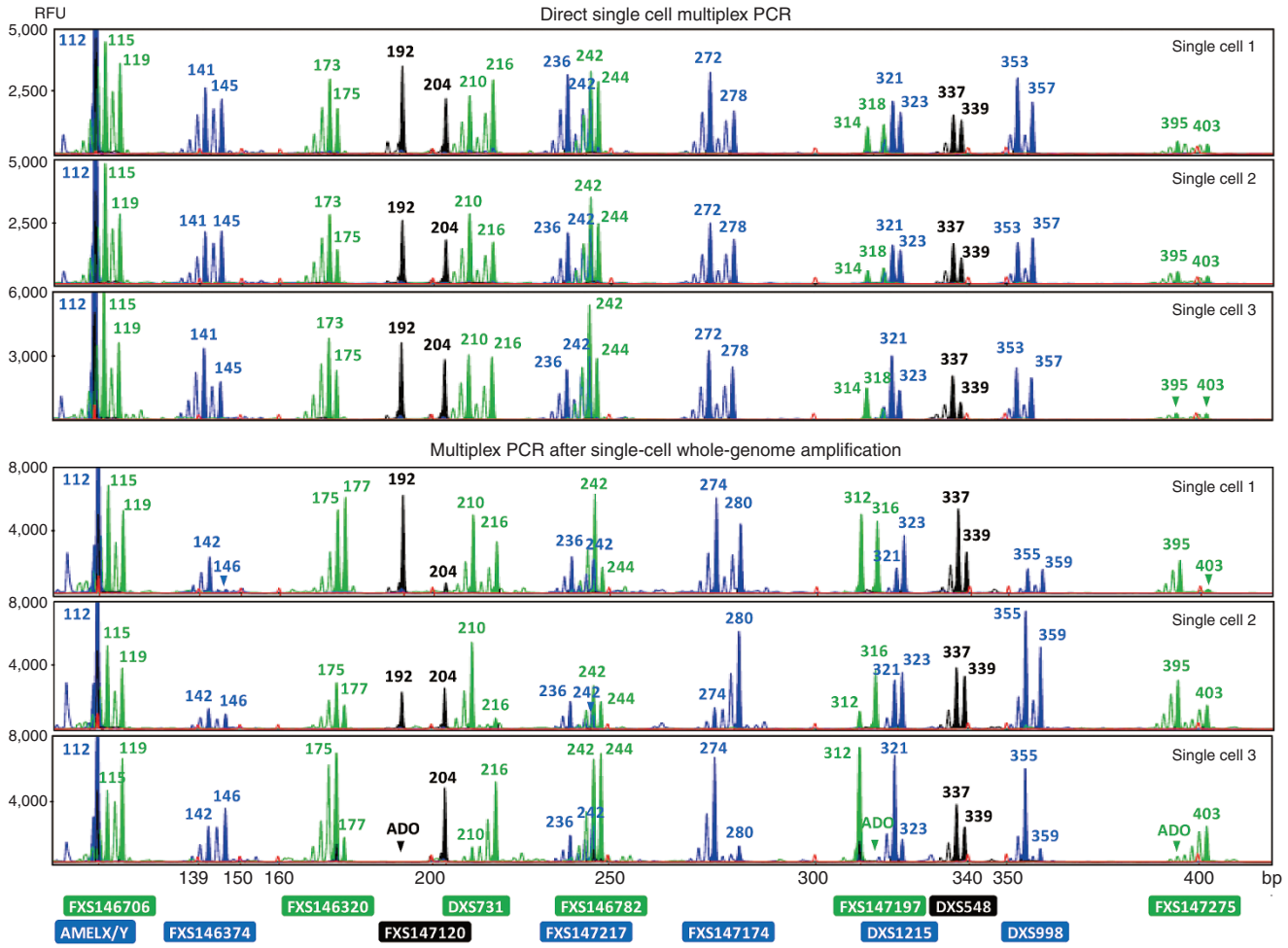

Figure 4 Representative electropherograms of amplification products after multiplex PCR directly from single cells or from single-cell whole-genome amplified material. Direct single-cell PCR products were analyzed using POP4, whereas PCR products generated from single-cell whole-genome amplification templates were analyzed using POP7.

the two major causes of misdiagnosis in PGD. Furthermore, successful amplification of the tetradecaplex PCR panel from single cells either directly or after WGA demonstrates its versatility for use in FXS PGD either as a standalone linkage analysis for couples who have uninformative normal *FMR1* alleles or as a complement to *FMR1* normal allele detection in the case of informative couples.

During the writing of this article, Kieffer *et al.*[18] reported an improved PGD assay for FXS that included five novel microsatellite markers. All five markers are among those that we also identified by *in silico* mining (**Supplementary Table S1** online), suggesting that our mining strategy was comprehensive. In this study, we determined the allele frequency distributions and informativeness of all 13 panel markers from three different populations for the first time (**Supplementary Table S2** online and **Table 1**). Of the markers in our panel, heterozygosity values of only three markers (*DXS998*, *DXS548*, and *DXS1215*) have been reported previously.[11,35,36] The reported heterozygosities for *DXS998* and *DXS1215* were marginally higher than in our populations, whereas that of *DXS548* was significantly higher. Nonetheless, we observed that 99.6% of individuals were heterozygous for three or more panel markers (**Figure 2**), with 95.8% of individuals heterozygous for at least two markers on either side of *FMR1* (**Figure 3**). These results strongly suggest that this panel will have sufficient marker redundancy even for WGA-based FXS PGD, where a minimum of two markers on either side of a mutation are recommended to be used,[37] thus minimizing the need for couple-specific marker panel customization. We also observed that individual marker informativeness varied among the three populations (**Supplementary Table S3** online). It is likely that these markers will also be informative in other populations, although interpopulation variation in informativeness of individual markers would be expected. Furthermore, a large number of markers will improve the likelihood of finding a subset of markers that will be informative, regardless of population or ethnicity. If the 13 panel markers are inadequate to meet the FXS PGD testing needs of any couple, then the other markers identified in this study (**Supplementary Table S1** online) should provide a useful resource for additional markers to be evaluated.

Conclusion

A single-tube tetradecaplex marker panel has been developed for use in FXS PGD. The multiplex PCR assay can be performed directly on single cells or after whole-genome amplification, thus supporting its use in standalone linkage-based analysis or as a complement to *FMR1* mutation detection.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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AUTHOR CONTRIBUTIONS

S.S.C. conceptualized and coordinated the project and experimental design, and revised the manuscript. M.C. participated in the design of the study, performed the experiments, data collection, analysis, and interpretation, and wrote the manuscript. M.Z. verified the experimental results and revised the manuscript. C.G.L. participated in the design of the study and revised the manuscript. All authors read and approved the final manuscript.

DISCLOSURE

S.S.C. is a founder of and an equity holder in TNR Diagnostics. All other authors declare no conflict of interest.

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