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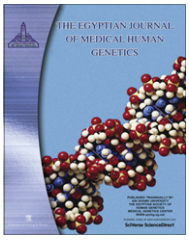

ORIGINAL ARTICLE

Use of denaturing gradient gel electrophoresis in screening unknown β-thalassemia mutations in Egyptian patients

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KEYWORDS

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Screening

Abstract The molecular defects resulting in a β-thalassemia phenotype, in the Egyptian population show a clear heterogenic pattern. Many studies have embarked on the molecular detection and characterization of these mutations, using a wide array of the available techniques with successful detection of both known and unknown mutations. PCR based techniques, as well as direct DNA sequencing are effective with some limitations as regards the time, effort and high cost to reach a final diagnosis. Intermediary screening techniques have proved to be effective tools to overcome these drawbacks. This study aims to assess the use of the denaturing gradient gel electrophoresis (DGGE)[1] to detect β-thalassemia mutations prior to the performance of direct sequencing to minimize the cost and workload involved in the process. In this study, forty-two previously genotyped patients in a study by El-Gawhary et al. in 2007, have been analyzed by DGGE for fragment 2 then 1. These are the β-globin gene fragments showing the majority of the β-thalassemia mutations. Sixty-eight alleles out of 79 mutant alleles in total were detected within these two fragments. The 11 undetected alleles comprise 9 alleles that require further examination using other DGGE fragments (0, 4 and 5) and correspond to -87(C > G), intervening sequence (IVS)II-1(G > A), IVS II-745, and IVS II-848(C > A). The remaining two that failed detection correspond to codon (CD) 37(G > A). Although, IVS-II 745(C > G) is undetectable within these two fragments, its 100% linkage polymorphism (+20 C > T) was detected in fragment 1 gel. DGGE is a sensitive

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[1] DGGE1: denaturing gradient gel electrophoresis.

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technique to screen for β-thalassemia mutations. For simultaneous analysis of multiple samples with unknown mutations, it is recommended that direct DNA sequencing be coupled with DGGE whenever available to reduce time, effort and cost.

1. Introduction

β-Thalassemia is the most common genetically inherited hemoglobin disorder in Egypt with a carrier rate varying from 5.3 to ⩾9% as reported by a recent study [1]. This is a group of disorders occurring almost entirely as a result of autosomal recessive inheritance of 2 β-globin gene point mutations. However small deletions of one to a few base pairs, as well as large deletions affecting the β-globin gene cluster occurring commonly in parts of the world, may cause this disorder. This genetic defect results in reduced or absent synthesis of the β-globin chain.

Increasing research is accumulating hundreds of mutations that are now known to cause this disease worldwide, with clustering of few numbers of mutations for every community [2]. The study of this genetic heterogeneity in every population has provided a new understanding of the disease phenotype, guided the management of the disease, and allowed successful prevention of the disease through prenatal diagnosis programs.

To date, the molecular characterization of the Egyptian population has employed variable techniques for direct mutation detection for known mutations and others for identification of unknown mutations. Polymerase chain reaction (PCR) based techniques such as the amplification refractory mutation system (ARMS), the synthetic oligonucleotide hybridization, the restriction enzyme digestion of the product or the use of denaturant high performance liquid chromatography (DHPLC) has greatly facilitated the screening for known mutations [3–6]. Furthermore, the use of PCR amplification and direct sequencing has permitted the accurate characterization of other unidentified alleles [6,7].

Although the automated fluorescent capillary based DNA sequencing is a very reliable method for the detection of unknown mutations and/or polymorphisms, it is tedious, time consuming as well as expensive. DGGE is considered one of the most sensitive and specific mutational scanning techniques [8]. Under appropriate conditions, all base pair substitutions, frameshifts, and deletions less than 10 bp can be resolved from the wild type sequence using DGGE [9].

The present study was performed to assess the usefulness of DGGE as a screening technique for the detection of unknown β-globin gene mutations in samples of known β-thalassemia patients before direct DNA sequencing is performed. Thus only a small area of the gene needs to be sequenced, therefore reducing costs, time and workload.

2. Subjects and methods

Forty-two subjects were included in this study. These comprised thirty-nine selected β-thalassemia patients (18 β-thalassemia intermedia and 21 β-thalassemia major), one obligate β-thalassemia carrier, and two healthy controls. Selection of the patients was done to include those having known genotypes previously published by an earlier study [7] to allow objective evaluation of DGGE when used to screen β-thalassemia mutations. The genotypes of the patients included the homozygous, the heterozygous and the double heterozygous patterns (Table 1).

The subjects were comprised of 15 females and 27 males. The patients' ages ranged between 2 and 24 years, those with β-thalassemia trait were 30 years old, while the two controls were 24 and 26 years old, respectively.

2.1. Molecular studies

DGGE involves the electrophoresis of double stranded DNA molecules through a polyacrylamide gel containing increasing concentrations of denaturing agents such as formamide and urea. The separation of the DNA fragments is based on the melting properties of the DNA molecule which in turn is based on the nucleotide sequence of the fragment under study. Double stranded molecules differing by single base changes can thus be separated, as they have different electrophoretic mobility as they undergo partial denaturation along the denaturant gradient.

The DNA fragments from an individual heterozygous for a mutation or polymorphism will show four bands on DGGE. The two faster migrating bands are the homoduplexes corresponding to the wild type and the mutant alleles. The additional bands that migrate at a slower rate in the gel consist of the two heteroduplexes formed by re-sorting of strands during PCR, which are less stable and melt out at a lower denaturing condition (Fig. 1).

For DGGE analysis the β-globin gene is divided into 5 fragments (Fig. 2). Analysis starts with fragments 1 and 2 where the majority of mutations are located (Table 7). If no abnormality is observed, analysis continues with fragments 0, 4 and 5 [4].

Genomic DNA was extracted from the peripheral blood leucocytes of EDTA anticoagulated fresh blood using QIAamp DNA Blood Mini KitT from QIAGEN©.

PCR amplification was done for 2 β-globin gene fragments using 2 sets of DGGE–PCR primers (Table 2) from Genosys© USA.

PCR reaction mix was done by adding 1.5 μl of extracted DNA to the PCR master mix [5 μl of 10X buffer from GE Healthcare, UK, 8 μl of 1.25 mM dNTPs from Invitrogen, USA, 1 μl of each primer (25 pmol), 35 μl H_2O, 0.3 μl AmpliTaq from GE Healthcare, UK (1.5 U) with a total of 51.8 μl in each reaction]. The 10X buffer preparation is 500 mM KCl, 15 mM Mg Cl_2, and 100 mM Tris–HCl (pH 9 at room temperature). An initial denaturation was held at 94 °C for 4 min. This was followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1.5 min and extension at 72 °C for 2 min. In the last cycle, extension was prolonged for 7 min. Amplified products of patients and controls were run on 2% ethidium bromide agarose gel. The thermal cycler used was an MJResearch© PTC-100T (USA).

DGGE was performed for all patients and controls first examining PCR products for fragment 2. Fragment 1 PCR

Table 1 List of genotypes of patients and controls included in the study.

Patient genotype	Number of patients (42)
-87(C > G)/-87(C > G)	3
IVS I-1(G > A)/IVS I-1(G > A)	4
IVS I-6(T > C)/IVS I-6(T > C)	15
IVS II-745(C > G)/IVS II-745(C > G)	4
CD 37(G > A)/CD 37(G > A)	1
CD 27(GCC > TCC)(Hb Knossos)/IVS I-1(G > A)	1
CD 27(GCC- > TCC)(Hb Knossos)/IVS I-6(T > C)	1
IVS I-1(G > A)/IVS I-6(T > C)	2
IVS I-110(G > A)/CD 27(GCC > TCC)(Hb Knossos)	1
IVS I-1(G > A)/IVS I-110(G > A)	1
CD 5(-CT)/IVS I-110(G > A)	1
IVS I-110(G > A)/IVS II-848(C > A)	1
IVS II-745(C > G)/IVS II-848(C > A)	1
IVS I-6(T > C)/IVS II-1(G > A)	1
IVS I-1(G > A)/IVS II-745(C > G)	1
IVS I-6(T > C)/IVS I-110(G > A)	1
IVS I-6(T > C)/N	1
N/N	2

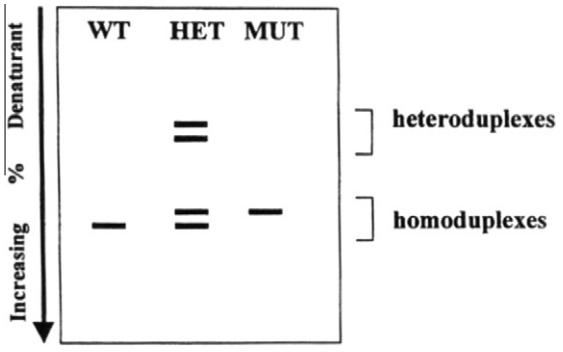

Figure 1 Schematic diagram to show the principle of the DGGE (provided by The Cyprus Institute of Neurology and Genetics Thalassemia Group). WT: Wild type homoduplex–HET: Heterozygous, the 2 faster migrating bands represent the wild and mutant homoduplexes and the 2 slower bands represent the heteroduplex bands of reassorted PCR strands–MUT: mutant homoduplex. The mutant homoduplex on the actual gel may migrate faster or slower than the wild type homoduplex and is determined by its melting temperature.

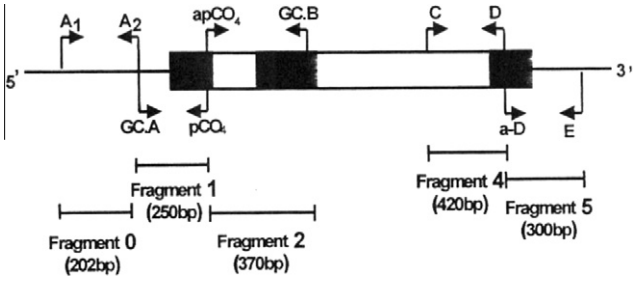

Figure 2 Showing the five DGGE fragments of the β-globin gene (provided by The Cyprus Institute of Neurology and Genetics Thalassemia Group). Fragment 1 covers the promotor region and first exon. Fragment 2 covers the first intron and the second exon.

product analysis was then done only for those samples whose mutations (either one or both) were not detected by the first fragment.

DGGE was performed using a Bio-Rad DCode systemT (Bio-Rad©, USA). The polyacrylamide gel was prepared using 6% acrylamide/bisacrylamide 37.5:1 with a denaturing gradient of 35–75% urea and formamide. The loading buffer used was ficoll-400 bromophenol blue/xylene cyanol.

3. Theory of the study

Establishing an optimum protocol for the analysis of β-thalassemia mutations must be population targeted and accommodated to the investigative facilities of different research centers. The appropriate use of the widely available techniques can improve the services, allowing better diagnosis and establishing successful disease prevention programs at an adequate cost. β-Globin gene mutations span the entire coding as well as non-coding regions. While whole genome sequencing is a breakthrough in the sequencing technologies which highly increases the through-put and reduces the cost of direct sequencing, yet automated fluorescent capillary-based DNA sequencing is still more widely available. The use of DGGE is a widely accepted intermediate mutational screening technique, to facilitate analysis of large genes, and shows additional advantages in prenatal and pre-implantation genetic diagnosis in β-thalassemia.

4. Results and discussion

The clinical groups of the patients in this study are mentioned in Table 3.

The patients' results were compared to a known normal as well as a known heterozygous control for the IVS-1-110 mutation which were run simultaneously with the patients' amplified products for fragment 2.

The results of the gel electrophoresis for fragment 2 PCR products revealed 19 mutant homoduplex bands representing the patients that carry homozygous mutations, seven double

Table 2 Primer sets used for PCR for DGGE analysis.

DGGE-Fragment	Primer- direction	Primer sequence
Fragment 1 (300 bp)	Forward primer	5' CGC CCG CCC CGC CCC CGT GCC CCC CGC GCC CGC CCC GCC CCC GTA CGG CTG TCA TCA CTT AGA CCT CA 3'
Fragment 1	Reverse primer	5' CAA CTT CAT CCA CGT TCA CC 3'
Fragment 2 (420 bp)	Forward primer	5' GGT GAA CGT GGA TGA AGT T 3'
Fragment 2	Reverse primer	5' GCG GGC GGG GCG GGG GCA CGG GGG GCG CGG CGG GCG GGG CGG GGG TGC AGC TTG TCA CAG TGC AGC TCA CT 3'

Table 3 Clinical groups of β-thalassemia patients included in the study.

Group	Clinical presentations	No of patients
A	Healthy controls	2
B	Obligatory carrier	1
C	β-Thalassemia intermedia	18
D	β-Thalassemia major	21

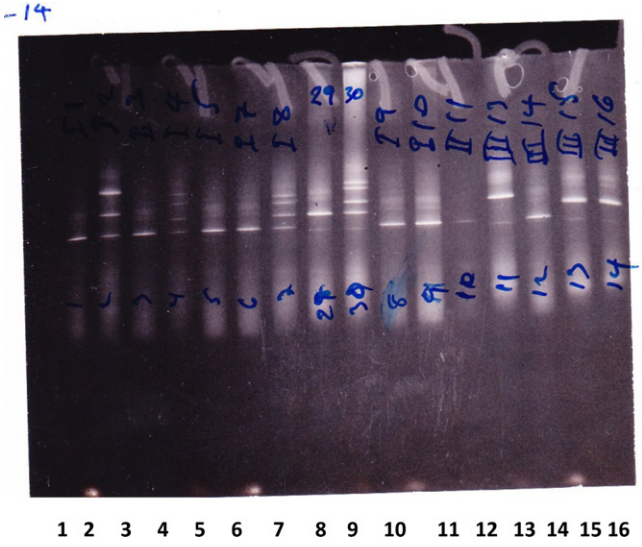

Figure 3 From left to right: Lane 8 (sample No. 29): normal control (previously genotyped). Lane 9 (sample No. 30): heterozygous IVS-1-110 control (previously genotyped). Lanes 1, 3, 5, 6, 10, 11, 12, 13, 14: mutant homoduplex bands. Lane 2: double heterozygous heteroduplex bands. Lanes 4 &7: heterozygous heteroduplex bands. Lanes 15 & 16: normal homoduplex bands.

mutant heteroduplex bands representing patients that carry double heterozygous β-thalassemia mutations, six normal/mutant heteroduplex bands representing the patients heterozygous for one β-thalassemia mutation detectable within this fragment (Fig. 3). The remaining ten samples showed normal homoduplex bands representing patients that have no mutations/polymorphisms detectable within the tested fragment (Table 4) .The latter 10 patients as well as the six heterozygous patients (16 in all) were subjected to fragment 1 DGGE analysis. Their DGGE–PCR products were compared to those of a known normal control as well as a known heterozygous control for silent CD2 polymorphism. Results showed four mutant homoduplex bands, three normal/mutant heteroduplex bands and nine normal homoduplex bands (Table 5).

Table 4 The results of DGGE fragment 2 analysis for all subjects included in the study.

DGGE analysis for PCR products	No of patients	%
Normal homoduplex	10	23.9
Double mutant heteroduplex	7	16.6
Normal homoduplex/mutant heteroduplex	6	14.3
Mutant homoduplex	19	45.2
Total subjects	42	100

Table 5 The results of DGGE fragment 1 analysis for normal and heterozygous samples by fragment.

DGGE analysis for PCR products	No of patients	%
Normal homoduplex	9	56.25
Double mutant heteroduplex	0	0
Normal homoduplex/mutant heteroduplex	3	18.75
Mutant homoduplex	4	25

Since DGGE is only a screening technique to detect the presence of the mutation in a specific DNA fragment, these results cannot be considered conclusive as to the type of the mutation until direct DNA sequencing is performed.

Therefore after analysis of eighty-four chromosomes of all patients, controls and one carrier, by fragments 2 and 1 of the β-globin gene, 68 mutant alleles out of seventy-nine (86.0759%) were detected, whereas 11 mutant alleles (13.924%) remained undetected within these 2 fragments and require further screening by other DGGE fragments (Fig. 4). Out of the 68 detected mutant alleles 46 belong to 23 patients, in whom both the mutations were detected within these 2 fragments and therefore need no further screening for their samples by other DGGE fragments (0, 4 and 5) (Fig. 5). The results of the DGGE were then compared with the genotypes of the patients (Table 6). The undetected alleles were found to correspond to the following mutations: -87, IVS II-1, IVS II-745, IVS II-848, CD 37. The 100% linked polymorphism (+20 C > T) to the IVS II-745, and not the mutation itself, was detected as it lies in the 5' untranslated region of the gene (an area covered by fragment 1).

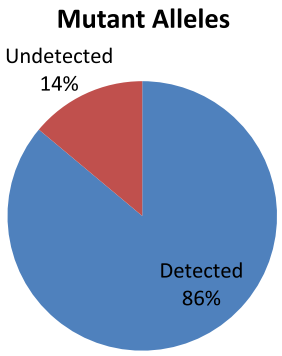

Figure 4 Results of chromosome analysis by fragments 1 &2. Undetected mutant alleles represent approximately 14% of the total.

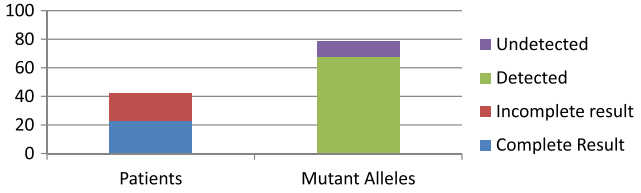

Figure 5 Results. After analysis by fragments 1 & 2, twenty-three patients showed complete results. Sixty-eight alleles were detected while eleven alleles were not detected from a total of seventy-nine.

β-Thalassemia is the commonest cause of chronic hemolytic anemia in Egypt. It remains a major public health problem. Studies on the Egyptian population have shown a few common mutations plus a wide range of less common mutations. In this study we aim to show the result of employing denaturing gradient gel electrophoresis technique to localize the β-thalassemia mutations in a group of Egyptian patients. Two β-globin gene fragments, denoted fragments 1 and 2, were amplified by DGGE–PCR primers and then analyzed by DGGE for samples of 39 patients, two healthy controls and 1 β-thalassemia trait individual, with a total of 42 subjects and 84 chromosomes of previously identified genotypes[7]. These 2 fragments are known to show the majority of β-thalassemia mutations (Table 7). Using these 2 fragments, 68 (68.0759%) mutant alleles were detected, while the remaining 11 (13.924%) mutant alleles were undetected. By comparing the latter alleles to their genotypes they were found to correspond to -87, IVS II-1, IVS II-745, IVS II-848, and CD 37. Theoretically, CD 37 should be detected by DGGE fragment 2. The other mutations require further analysis by the other DGGE fragments which are known to show the less commonly occurring β-thalassemia mutations in the Mediterranean region (refer to Fig. 2). Mutations within the second intron (fragment 3) are not possible to detect as it harbors a number of polymorphisms which distorts the results [4]. During each DGGE run, both a normal control known to carry the wild type gene as well as a known heterozygous control which is detectable within the chosen gene fragment are included. These controls assess the validity and performance of the technique [8], and for expert hands the heterozygous control may give a clue about similar mutations/polymorphisms when run simultaneously on the same gel. Although this is an obvious advantage, all samples must be sequenced to identify the type of mutation involved. Failure to detect mutations may also be due to Taq induced errors which may reduce the sensitivity of the DGGE system [9]. Experimental determination of optimal electrophoresis conditions allows maximum retrieval of results [8,10].

A study carried out for the molecular characterization of β-thalassemia in Egypt, using PCR amplification and restriction site analysis followed by DGGE and direct sequencing was able to detect twelve different mutations in the Suez Canal area [6].

A large study on 118 patients identified the largest spectrum of β-thalassemia mutations so far reported in Tunisia (18 distinct mutations), using a combination of several PCR based techniques, DGGE and direct nucleotide sequencing [11].

Another study was done for the molecular characterization of β-thalassemia in Syria, that used gene amplification with specific oligonucleotide primers, enzyme analysis, denaturing

Table 6 Comparison between patient genotypes and results of DGGE.

Patient genotype	Fragment 2	Fragment 1
-87/-87	Normal homoduplex	Normal homoduplex
IVS I-1/IVS I-1	Mutant homoduplex	Not done
IVS I-6/IVS I-6	Mutant homoduplex	Not done
IVS II-745/IVS II-745	Normal homoduplex	Mutant homoduplex
CD 37/CD 37	Normal homoduplex	Normal homoduplex
CD 27(Hb Knossoss)/IVS I-1	Double mutant heteroduplex	Not done
CD 27(Hb Knossoss)/IVS I-6	Double mutant heteroduplex	Not done
IVS I-1/IVS I-6	Double mutant heteroduplex	Not done
CD 27(Hb Knossoss)/IVS I-110	Double mutant heteroduplex	Not done
IVS I-1/IVS I-110	Double mutant heteroduplex	Not done
CD 5 –CT/IVS I-110	Normal/mutant heteroduplex	Normal/mutant heteroduplex
IVS I- 110/IVS II-848	Normal/mutant heteroduplex	Normal homoduplex
IVS II-745/IVS II-848	Normal homoduplex	Normal/mutant heteroduplex
IVS I-6/IVS II-1	Normal/mutant heteroduplex	Normal homoduplex
IVS I-1/IVS II-745	Normal/mutant heteroduplex	Normal/mutant heteroduplex
IVS I-6/IVS I-110	Double mutant homoduplex	Not done
IVS I-6/N	Normal/mutant heteroduplex	Normal homoduplex
N/N	Normal homoduplex	Normal homoduplex

Table 7 Some β-globin gene mutations and polymorphisms that can be detected by DGGE fragments 1 & 2.

Fragment 1	Fragment 2
−30(T > A/C)	IVS I-1(G > A)
+20* (C > T)polymorphism	IVS I-5(G > A/T)
+33(C > G)	IVS I-6(T > C)
CD 2(CAC > CAT)polymorphism	IVS I-110(G > A)
FSC-5(–CT)	IVS I-116(T > G)
Hb-S(GAG > GTG)	IVS I-130(G−C/A)
Frameshift(FSC)-6 –A	CD 25/26(+T)
FSC-8(–AA)	CD 27(GCC > TCC) Hb Knossos
FSC-8/9(+G)	CD 36/37(−T)
CD 15(TGG > TGA)	CD 37(TGG > TGA)
CD 22/23/24 del –AAGTTGG	CD 39(CAG > TAG)
	CD 37/38/39–GACCCAG
	CD 44(−C)

* 100% linkage polymorphism cis to the IVS II-745.

gradient gel electrophoresis and direct sequencing. By combining these three approaches, seventeen different mutations were identified, with successful mutation detection in almost 90% of the chromosomes studied [4].

A study was done including 95 Egyptian thalassemic patients, and used PCR–ARMS complemented by direct DNA sequencing for the detection of common and uncharacterized mutations. Combining these two techniques, mutations of all patients were fully characterized [7].

A recent study carried out on 172 patients from Western Saudi Arabia, which used PCR-ARMS and direct DNA sequencing, successfully identified 23 β-thalassemia mutations [12].

The discrepancy between the above studies, as regards the approaches used in the molecular characterization of β-thalassemia alleles in different populations, with or without applying DGGE as a screening technique for unknown mutations before DNA sequencing, may be attributed to the question of availability of this technique in different research laboratories.

5. Conclusion

Although it is equally reliable to detect β-thalassemia mutations using DNA sequencing, both common as well as rare ones, without prior DGGE analysis of samples, the cost can be significantly higher. In smaller or lower budget centers with more limited resources, cost and workload reduction is of essence. Therefore the application of a fast accurate and cheap technique can make a big difference and improve services. The use of DGGE as an intermediate technique is excellent for that purpose, particularly when multiple samples are simultaneously analyzed. The recommended approach is first to use one or more of the PCR-based techniques, to detect common mutations, followed by denaturing gradient gel electrophoresis [4,6,11], or other mutational screening techniques [13,14], and DNA sequencing for detection of rare ones. The use of DGGE has an added advantage in the prenatal diagnosis of β-thalassemia [15]. Once the mutations are characterized, testing other family members and prenatal tests are much simpler. DGGE also has a wide application in PGD[2] as it facilitates simultaneous analysis of more than one mutation in a single PCR fragment; it detects the presence of normal alleles and monitors the occurrence of allelic drop-out through the expectation that heterozygous samples have more than one electrophoretic band on analysis [16].

[2] PGD2: preimplantation genetic diagnosis

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