ORIGINAL ARTICLE

Use of multiple displacement amplification to amplify genomic DNA before sequencing of the α and β haemoglobin genes

M Mai, J D Hoyer, R F McClure

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See end of article for authors' affiliations

Correspondence to:
Dr R F McClure,
Department of Laboratory
Medicine and Pathology,
Mayo Clinic, 200 First
Street SW, Rochester, MN,
55905, USA; mcclure.
rebecca@mayo.edu

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Aims: To evaluate the technique of multiple displacement amplification (MDA) for whole genome amplification from small volume blood samples before sequencing in a clinical test to identify haemoglobin gene mutations.

Methods: Phage ϕ 29 DNA polymerase was used to perform MDA, starting with either 1 μ l of blood or 1 ng of previously isolated blood DNA from 23 patients. The amplified products were then evaluated using a clinical test that involves sequencing the haemoglobin genes to detect mutations. The results were compared with the current clinical test method that uses genomic DNA isolated using column based technology.

Results: The MDA technique produced large quantities (theoretically approximately 2 mg) of DNA. The amplification procedure was extremely easy and took about four hours (less than one hour of hands on technician time and three hours for amplification). When MDA products were used in the same clinical test protocol as genomic DNA isolated using column technology, there was 100% concordance for detection of a variety of point mutations in the α 1, α 2, and β globin genes.

Conclusions: The MDA technique is useful for overcoming the problem of insufficient genomic DNA in clinical specimens requiring haemoglobin gene sequencing and could be useful for other clinical applications.

Obtaining a sufficient amount of high quality DNA from clinical samples to perform molecular tests is frequently a challenge. In our practice, DNA sequencing of globin genes is often indicated, but not possible because of insufficient blood remaining after the initial haemoglobin protein studies. This is particularly true for samples taken from infants, where obtaining the recommended volume of blood for testing is difficult. Our standard procedure includes genomic DNA extraction using column based technology, followed by globin gene specific polymerase chain reaction (PCR) amplification to provide a template for sequencing. In our experience with clinical specimens, genomic DNA from small numbers of cells is lost during column isolation and it is difficult to perform gene specific PCR directly from genomic DNA released from very small numbers of nucleated cells. Thus, it would be useful to have a reliable method for genomic DNA amplification, so that testing could be performed on a small number of nucleated cells.

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PCR based whole genome amplification methods have been described,^{1,2} although they have not been widely accepted as useful for amplifying clinical samples because of their complexity and lack of replication accuracy. Recently, phage ϕ 29 DNA polymerase has been used in a technique called multiple displacement amplification (MDA) to perform quick and accurate amplification of total genomic DNA from small numbers of cells.³ Because of the high processivity and high fidelity of the polymerase, many copies of double stranded linear DNA, each greater than 10 kb in length, can

be generated in the presence of random primers under isothermal conditions. Using the MDA technique, we easily produced large quantities of DNA from either 1 μ l of peripheral blood (approximately 5000 nucleated cells) or 1 ng of previously isolated genomic DNA. We then used the amplified DNA to obtain reliable results in our clinical test for α and β globin gene sequencing.

METHODS

Samples

Twenty three peripheral blood specimens sent to the Mayo Clinic laboratory for haemoglobin testing were used in a non-blinded, retrospective analysis. The specimens had been stored at room temperature for variable amounts of time (range, 2–7 days) and 11 of the samples had been frozen (–20°C; range, 2–18 months) before use.

Whole genome amplification using MDA

The kit (REPLI-g 625s) from Molecular Staging Inc (New Haven, Connecticut, USA) was used according to the manufacturer's instructions. Briefly, 1 μ l of blood (approximately 5000 nucleated cells) was diluted in 34 μ l of phosphate buffered saline, lysed by the addition of 35 μ l of Fast Lysis solution, and neutralised by the addition of 35 μ l of Lysis Stop buffer. A 2.5 μ l aliquot of the lysis product was then mixed with 40 units (0.5 μ l) of ϕ 29 DNA polymerase, 12.5 μ l of random primers and dNTPs, and 34.5 μ l of distilled H₂O to a final volume of 50 μ l and incubated at 30°C for 16 hours. In some cases, MDA was performed starting with genomic DNA isolated using column technology (see below). In these cases, the procedure was modified such that 1 ng of

Abbreviations: MDA, multiple displacement amplification; PCR, polymerase chain reaction

Table 1 PCR and sequencing primers for α and β haemoglobin genes		
Gene	Primer	Primer sequence
α Globin genes	PCR primers	AG-F 5' ccc gcg ccc caa gca taa ac
		AG1-R 5' ctg gca cgt ttg ctg agg gaa aa
		AG2-R 5' tgg cac att ccg gga tag aga
	Sequencing primers	AGS1-F 5' caa gca taa acc ctg gc
		AGS1-R 5' gag ccg tgg ctg agg tgg
		AGS2-F 5' caa ccg tcc tgg ccc cgg
		ASG2-R 5' gtg aac tgg gcg ggg agg t
		ASG3-F 5' cgg gtt gcg gga ggt gla g
		AG1S3-R 5' tgc tga ggg aaa aaa ctc agg
		AG2S3-R 5' tcc ggg ata gag aga acc c
β Globin gene	PCR primers	Exon 1-F 5' gta cgg ctg tca tca ctt aga c
		Exon 1-R 5' aaa ccc aag agt ctt ctc tgt c
		Exon 2-F 5' act ggg cat gfg gag aca gag a
		Exon 2-R 5' tgt ttc cca ttc taa act gta c
		Exon 3-F 5' cat att gct aat agc agc tac a
		Exon 3-R 5' ctc cca cat tcc ctt ttg agt
	Sequencing primers	Exon 1-F 5' aca ccc tag ggt tgg cca atc tac tc
		Exon 1-R 5' atg ccc agt ttc tat tgg tct cct taa
		Exon 2-F 5' ttg ggt ttc tga tag gca ctg act ct
		Exon 2-R 5' ttc tcc cct tcc tat gac atg aac tta
		Exon 3-F 5' taa ggc tgg att att ctg agt c
		Exon 3-R 5' gac tta ggg aac aaa gga acc ttg aat a

F, forward; PCR, polymerase chain reaction; R, reverse.

DNA was mixed directly with the enzyme and primers and incubated as described above.

Quantitative and qualitative analysis of the MDA products

The MDA products were quantitated using a fluorescence based method that is specific for double stranded DNA (PicoGreen®; Molecular Probes, Eugene, Oregon, USA). Aliquots (5 µl) of the MDA product were also analysed using agarose gel electrophoresis (1.0% agarose, 100 V, 60 minutes, stained with ethidium bromide) and the products visualised using the Alpha Imager gel documentation system (San Leandro, California, USA).

DNA isolation using column technology

The Wizard genomic DNA purification kit (Promega, Madison, Wisconsin, USA) was used to isolate genomic DNA from 0.3 to 3 ml of whole, never frozen blood using the manufacturer's instructions.

Table 2 DNA yield after MDA				
Patient	Specimen	Blood volume or DNA amount	Yield/ reaction* (µg)	Theoretical total yield† (µg)
1	Never frozen WB	1 µl	45	1875
2	Never frozen WB	1 µl	48	2000
3	Never frozen WB	1 µl	44	1833
4	Never frozen WB	1 µl	53	2208
5	Frozen WB	1 µl	53	2208
6	Frozen WB	1 µl	50	2083
7	Frozen WB	1 µl	50	2083
8	Frozen WB	1 µl	52	2167
9	Frozen WB	1 µl	50	2083
10	Frozen WB	1 µl	55	2292
11	Frozen WB	1 µl	56	2333
12	Frozen WB	1 µl	57	2375
13	Frozen WB	1 µl	57	2375
14	Frozen WB	1 µl	48	2000
15	Frozen WB	1 µl	48	2000
16	DNA	1 ng	51	
17	DNA	1 ng	50	
18	DNA	1 ng	50	
19	DNA	1 ng	59	
20	DNA	1 ng	57	
21	DNA	1 ng	46	
22	DNA	1 ng	49	
23	DNA	1 ng	56	

*Each reaction contained only 2.5 µl, equal to 2.4% of the 1 µl WB lysis product (see methods); †the theoretical total yield is the calculated yield that would have been obtained if the entire 1 µl of WB had been amplified.
MDA, multiple displacement amplification; WB, whole blood.

Sequencing of α and β haemoglobin genes

Table 1 shows the primers used for PCR amplification and subsequent sequencing of the α and β haemoglobin genes. PCR was performed in a 25 µl reaction volume containing 50 mmol/litre KCl, 10 mmol/litre Tris/HCl (pH 8.3), 200 µmol/litre each dNTP, 1.5 mmol/litre MgCl₂, 0.2 µmol/litre each primer, 1.25 units of hot start Taq DNA polymerase, and 5 µl of Q-solution (US-Qiagen, Valencia, California, USA). The reactions were amplified in an MBS Satellite thermal cycler (Thermal Hybaid, Waltham, Massachusetts, USA) with an initial incubation at 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds and 61°C for one minute, with a single final incubation of 72°C for one minute. Sequencing was then performed using the dye terminator method with fragment analysis by capillary electrophoresis on the ABI 377 (Applied Biosystems, Foster City, California,

Figure 1 Agarose gel electrophoresis of multiple displacement amplification using MDA products. The arrow designates a marker fragment of 23 kb. (A) Lanes 1–9 contain MDA products with the starting sample type shown above the lanes. Lanes 10 and 11 contain non-amplified genomic DNA (1.25 µg each) extracted using the column technique (see methods). (B) Lanes 1–8 contain MDA products and show the effect of decreasing amplification incubation time (see methods). Lanes 10 and 11 contain non-amplified genomic DNA (1.25 µg each) extracted using the column technique (see methods). WB, whole blood.

Figure 2 Representative sequencing traces showing concordant point mutation detection in two patients using DNA extracted with the column method and DNA amplified by multiple displacement amplification (MDA) as templates.

USA) using Chromas 2.0 sequence analysis software (Technelysium Pty Ltd, Tewontia, Australia). Both strands of DNA in each specimen were sequenced.

RESULTS

Quantitation using the fluorescence based method revealed that each reaction, starting with either never frozen or previously frozen whole blood, produced approximately 50 µg of double stranded DNA (range, 44–59 µg; table 2). Because only approximately 2.4% of the DNA released during the cell lysis step was amplified (see methods), the theoretical total yield from the amplification of 1 µl of whole blood was estimated to be approximately 2 mg (table 2). Similar yields were obtained when the starting template was 1 ng of genomic DNA isolated using column technology. Specimen age before processing did not appear to have a significant affect on the yield. Qualitative analysis of the MDA products using agarose gel electrophoresis (fig 1) revealed that the amplified fragments were mostly greater than 20 kb and showed smaller average fragment size than the genomic DNA extracted using column technology (fig 1A). In addition, although the manufacturer's recommendation for MDA incubation was 16 hours, as little as three hours of incubation

was sufficient to produce a substantial amount of DNA (fig 1B).

In each sample, the DNA sequence from both strands showed a normal haemoglobin gene sequence in the entire region expected to be normal, and the expected mutations were correctly identified (table 3; fig 2). There was 100% concordance between the sequences obtained using MDA products as a template and those obtained using DNA extracted by the column method as a template.

DISCUSSION

Our results show that MDA is a useful method for amplifying very small amounts of genomic DNA before haemoglobin gene sequencing. Reliable results were obtained using whole blood specimens subjected to a variety of pretest conditions, including variable lengths of room temperature incubation, freezing, or previous genomic DNA extraction using a column based method. As little as 1 µl of whole blood or 1 ng of previously extracted DNA were adequate amounts of starting material, making the procedure useful for even the smallest clinical samples available.

Although no DNA purification was performed when whole blood was amplified, the small amount of residual cell debris

Table 3 Sequencing results of extracted DNA and MDA products

Patient	Mutation name	Gene	Nucleotide position	Codon number	Normal sequence	Mutation identified*
5	Hb Buffalo	α1	424	89	CAC	CAG
7	Hb G-Pest	α1	377	74	GAC	AA [~] C
8	Hb Fuchu-I	α1	371	72	CAC	TAC
19	Hb Columbia Missouri	α1	420	88	GCG	GTG
22	Hb Bassett	α1	438	94	GAC	G [~] CC
20	Hb Buffalo	α1	424	89	CAC	C [~] AG
3	Hb Manitoba	α2	605	102	AGC	CG [~] C
6	Poly A signal mutation	α2	819		AATAAA	AATAAG
11	Hb Chicago	α2	705	136	CTG	ATG
12	Constant Spring	α2	723	142	TAA	CA [~] A
9	Intron mutation	α2	509		G	T
14	Hb Coen	α2	694	132	GTG	GGG
15	Hb Beth Israel	β	488	102	AAC	A [~] GC
1	Hb E	β	129	26	GAG	A [~] AG
2 and 4	Hb S	β	70	6	GAG	GTG
10	Hb Austin	β	304	40	AGG	A [~] GC
13	Intron mutation	β	148		G	C
16	Hb Atlanta	β	407	75	CTG	CCG
17	Hb Athens GA	β	303	40	AGG	A [~] AG
18	Hb β ⁰ Thal. (stop codon)	β	299	39	CAG	TAG
21	Promoter mutation (position -29)	β	-29		A	G
23	Promoter mutation (position -29)	β	-29		A	G

*In each sample, the same mutation was identified whether the sequencing template was MDA product or DNA isolated using the column method (see methods). Hb, haemoglobin; MDA, multiple displacement amplification.

Take home messages

- Multiple displacement amplification (MDA) produced large quantities of DNA in clinical specimens requiring haemoglobin gene sequencing
- The amplification procedure was extremely easy and took just four hours
- There was 100% concordance for detection of a variety of point mutations in the $\alpha 1$, $\alpha 2$, and β globin genes when compared with genomic DNA isolated using column technology
- The MDA technique is useful for overcoming the problem of insufficient genomic DNA in clinical specimens requiring haemoglobin gene sequencing and could be useful for other clinical applications

did not hinder the sequencing application being tested and additional purification steps were not felt to be necessary. A previous report of MDA indicated that the method results in an average product length of 10 kb, with a range from 2 to 100 kb.³ Agarose gel analysis of the MDA products in our study indicated that the average DNA length obtained was probably longer (> 20 kb), but otherwise appeared consistent with predicted results. We also found that as little as three hours of amplification was sufficient to produce ample DNA for our purposes, which shortened the procedure time considerably, so that the entire amplification procedure required only approximately four hours (less than one hour of hands on technician time and three additional hours for incubation).

"We found that as little as three hours of amplification was sufficient to produce ample DNA for our purposes, which shortened the procedure time considerably"

We feel that our sequencing results were 100% specific because the mutations identified are all previously reported haemoglobin abnormalities, the same mutation was detected using sequencing of both DNA strands, the remainder of the sequence in each case showed a normal haemoglobin gene sequence, and there was 100% concordance with the results obtained using genomic DNA isolated by a standard column method as the sequencing template. Thus, our results indicate that the MDA technique is sufficiently reliable for

amplification of peripheral blood samples before haemoglobin sequencing. Previous reports provide evidence to suggest that MDA is a robust and accurate technique, superior to PCR based genomic amplification methods.³⁻⁵ The error rate of $\phi 29$ polymerase is estimated to be only $1/10^6$ – 10^7 , compared with approximately $3/10^4$ for Taq polymerase,⁶ and $\phi 29$ shows less amplification bias than Taq.³ Although our clinical test for globin gene sequencing uses PCR, errors caused by Taq polymerase do not appear to be frequent enough to confuse test interpretation. The main goal of our study was to verify the previous claims of $\phi 29$ enzyme fidelity in the context of our specific clinical needs.

Although we have only tested the MDA method in the specific clinical application of haemoglobin gene sequence analysis, we feel that MDA should be equally useful in a wide variety of clinical applications using small numbers of nucleated cells from essentially any source. However, our search of the English language literature revealed no previous reports of MDA applications for routine clinical testing.

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Authors' affiliations

M Mai, J D Hoyer, R F McClure, Division of Hematopathology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester 55905, USA

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