Genomic DNA amplification by the multiple displacement amplification (MDA) method

Roger S. Lasken[1]
J. Craig Venter Institute, 10355 Science Center Drive, La Jolla, CA 92121, U.S.A.

Abstract

Large amounts of DNA are frequently required for use in detection assays and genomic analysis. The limited availability of DNA can be a critical obstacle to meeting research and clinical needs. DNA amplification methods are often required to generate sufficient material from small specimens or environmental samples with low DNA content. The MDA (multiple displacement amplification) reaction is increasingly the method of choice for many applications because of its extensive coverage of the genome, the generation of extremely long DNA products compared with older whole genome amplification methods and the high DNA yields, even from exceedingly low amounts of starting material. Remarkably, MDA enables genomic sequencing even from single microbial cells. Some of the uses of MDA and its strengths and limitations will be discussed.

Requirement for large amounts of genomic DNA in laboratory procedures

Many genomic sequencing and detection methods require micrograms of DNA template [1]. Even highly sensitive analytical methods such as PCR are often constrained by a limited amount of DNA template when large numbers of different genetic loci are to be tested from a single sample. At the same time, the variety and difficulty of environmental and clinical samples under investigation have grown rapidly. Microbial ecology investigates genes and communities in many environments having low biomass, such as acid mine drainage and other extreme environments [2]. Detection of pathogens, either naturally occurring or present in biological weapons, can also be limited by low cell numbers. Biological specimens such as insect gut are another source of microbial DNA of interest. The newly announced Human Microbiome Project (http://nihroadmap.nih.gov/hmp/) seeks to investigate the microflora associated with the human body. This can include specimens with low bacterial numbers. Even acute blood infection may involve only a few bacterial cells per millilitre of blood. Other specimens such as stool have abundant microbial content but many rare species evade detection and analysis. Clinical diagnosis of chronic conditions may be difficult because of low numbers of bacteria. Genetic studies of human and other animal cells can also be constrained by availability of DNA, for example in genotyping of tumour biopsies for somatic mutations, and in the extreme case of genotyping individual blastomeres and sperm. Forensic applications also often require genotyping of DNA from limiting samples.

Key words: genomic DNA, multiple displacement amplification (MDA), quantitative PCR (qPCR).
Abbreviations used: CGH, comparative genome hybridization; FISH, fluorescence *in situ* hybridization; LCM, laser capture microdissection; MDA, multiple displacement amplification; STR, short tandem repeat; qPCR, quantitative PCR.
[1]email rlasken@jcvi.org

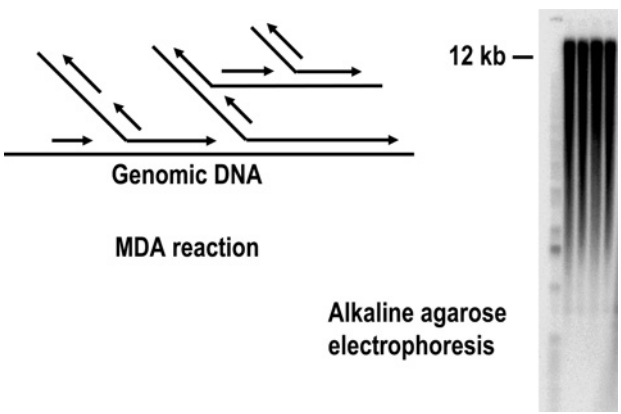
The MDA (multiple displacement amplification) reaction for amplifying genomic DNA

MDA [3,4] is a method to amplify DNA (Figure 1). Random primers are used to target the entire DNA template. The φ29 DNA polymerase [5], derived from the *Bacillus subtilis* bacteriophage φ29, is the preferred enzyme because of its extremely high processivity (the average number of nucleotides added to the 3′-terminus before the polymerase dissociates from the DNA) and its strong strand displacing activity (in which it displaces downstream complimentary strands as it copies the template strand). The polymerase adds an average of 70 000 nt each time it binds the primer template [6]. These characteristics allow the polymerase to copy over the same template multiple times, concurrently extending new primers while displacing the previously extended products. Exponential amplification results through a branched DNA intermediate structure. Another feature of the MDA reaction is that the random primers are protected by phosphorothioate linkages in the two 3′-terminal nucleotides [3]. This is necessary to prevent the DNA polymerase's associated 3′–5′ exonuclease proofreading activity from degrading the primers. The advantage of the proofreading activity is that it results in a low accumulation of mutations in MDA [7]. The base calling error rate in dideoxy DNA sequencing using DNA amplified by MDA was indistinguishable from that of unamplified template [8,9]. Some genomic sequence is lost during amplification by MDA; however, an estimated 99.8% of the genome is present [8].

MDA has the lowest amplification bias of any whole genome amplification method reported to date [10]. TaqMan qPCR (quantitative PCR) for 47 human genetic loci showed amplification bias over only a 6-fold range. These loci, which are present in single copies in the human genome, were represented after the amplification at ratios of between 0.5

Figure 1 | **The MDA reaction**

The DNA template is repeatedly copied by a branching mechanism in which φ29 DNA polymerase extends random hexamer primers as the strong 'strand displacement activity' concurrently displaces previously made copies. Denaturation and resolution of the amplified DNA on an alkaline agarose gel demonstrate that the synthesized strands have an average length of approx. 12 kb and range up to an estimated >100 kb. Reproduced from [32] with permission. © 2007 Elsevier.



and 3 copies per genome. The amplification bias increases with higher fold amplification and probably results from factors such as local sequence effects on the efficiency of the random primers. An important characteristic of MDA is that it tends to be a self-limiting reaction in which DNA is amplified to a fairly uniform concentration when the reaction is carried to completion (typically approx. 30–50 μg of DNA are produced from a 50 μl MDA reaction). Therefore the fold amplification that occurs largely depends on the amount of starting DNA template used. In the test of human DNA, 10 ng of DNA template (approx. 3000 genome copies) was amplified to a typical ~40 μg DNA yield, giving a 4000-fold amplification. The use of less template results in greater fold amplification and consequently greater amplification bias [2]. In the extreme case of amplification from the single genome copy in a bacterial cell (a few femtograms), a yield of 40 μg of DNA would be over a billion-fold amplification and bias will be far greater (see below). Amplification bias may complicate studies where the goal is to quantify precisely the relative amounts of DNA species present in a sample [12]; however, MDA-generated DNA is well suited for use in assays for detection or genotyping that are based on specific oligonucleotide probes or primers and for dideoxy DNA sequencing, the 454 method and other newer sequencing technologies.

Use of MDA in DNA sample preparation for genotyping and sequencing

MDA has been used in many laboratory procedures as a simple method for DNA sample preparation and amplification. MDA is available as a kit from commercial vendors (GenomiPhi and TempliPhi from GE Healthcare; Repli-g from Qiagen). The φ29 DNA polymerase and protected primers can be purchased separately from several vendors. MDA is widely used to restore depleted DNA samples that have been collected, for example, in population or epidemiological studies [1]. It is used to obtain amplified DNA from small specimens such as biopsies, minute amounts of blood and popular new sources of DNA such as buccal swabs [10]. MDA is used in genomic sequencing centres as a means to amplify plasmid DNA for use in high-throughput sequencing pipelines [7]. In addition to amplifying the DNA, it serves as a DNA sample preparation method that can replace older, more expensive methods to prepare plasmid DNA from cultures or colonies [13]. MDA is also useful for obtaining genomic DNA for genotyping. When high-quality DNA is available as a template, MDA accurately copies both alleles of a diploid locus [11], resulting in only approx. 1 SNP (single-nucleotide polymorphism) genotyping error per 1000 assays [14]. Reasonably accurate microsatellite genotyping has been obtained even from single human cells [15]. Out of 30 single-cell MDA reactions, 28 resulted in accurate genotyping, and equal representation occurred for both chromosomes for 14 different STRs (short tandem repeats). This was partly attributed to the lack of slippage artefacts for STRs using MDA [10,16–19] as apposed to profuse slippage for the PCR-based whole genome amplification methods [20]. Microsatellite testing of a few loci allowed haplotypes to be determined with 100% accuracy. One approach for improving genotyping accuracy where needed, as in use of poor-quality DNA template, is to prescreen MDA reactions by TaqMan qPCR [10,21]. qPCR of two diagnostic loci was highly indicative of the overall quality of the amplification. By carrying out genotyping studies only on those MDA reactions that pass this quality control, genotyping errors were nearly eliminated. For example, when MDA was intentionally carried out on highly degraded DNA template, genotyping errors occurred for 25% of assays but none occurred when only those MDAs passing the quality control were included in the study [21].

In an application to infectious disease, *Mycobacterium leprae* cells were isolated from skin of human leprosy patients and amplified by MDA for genomic analysis [22]. The fidelity of the amplified DNA was unaltered from that in the original specimens. The same procedure was also carried out on archived skin specimens from leprosy patients. Success of microsatellite analysis increased from 20 to 92% after MDA for these highly degraded archived samples and with no introduction of sequence errors. Genomic analysis with small specimens obtained by LCM (laser capture microdissection) can also require DNA amplification in order to obtain sufficient template for testing of more than a small number of genetic loci. MDA has been used with a variety of LCM sample types [23–25]. CGH (comparative genome hybridization) analysis of prostate cancer using MDA of LCM samples provided evidence that chromosomal rearrangements were non-random, consistent with a progression of events that promote tumour development, progression and survival [26]. However, genotyping errors have generally been higher

for studies where MDA was carried out from LCM samples compared with cultured cells or purified DNA template [23–25]. For example, LCM samples derived from at least 1000 cells generated errors in CGH analysis [24]. In contrast, extracted DNA from a similar number of cultured cells allowed highly accurate genotyping [11]. In another study, just ten cultured human cells were sufficient for MDA to produce amplified DNA that was indistinguishable from unamplified genomic DNA in genotyping assays [16]. This is consistent with data showing that only ten bacterial cells are sufficient for generating high-quality DNA by MDA [27]. Exciting work has even demonstrated the possibility of genotyping from single human cells using MDA to obtain DNA template. Single sperm cells were genotyped and haplotypes were determined [18]. Single blastomeres were genotyped as a means to carry out pre-implantation genetic diagnosis [16,17,28,29], although a high rate of genotyping errors noted in these papers must be taken into consideration when using the method for clinical diagnosis. The errors do not result from the fidelity of the φ29 DNA polymerase for nucleotide incorporation. Rather, it is likely that DNA damage and stochastic effects of amplifying from a single cell result in amplification bias between parental alleles of heterozygous loci leading to miscalling as homozygous.

Genomic sequencing from single bacterial cells

The U.S. Department of Energy funded the development of a single-cell sequencing method in 2001, and this was achieved [27] at Molecular Staging Inc. where MDA was developed. More than a billion-fold amplification by MDA generated micrograms of DNA from the few femtograms in one bacterial cell. Flow cytometry was used to obtain the single cells. MDA was also used with cells isolated by micromanipulation with glass capillaries to capture the cells with a microscope [30,31]. Unlike the low level of amplification bias obtained when starting from thousands of genome copies [10], MDA creates large bias and even loss of some sequence when starting from a single genome copy as a template [27]. Nevertheless, for uncultured bacteria, single-cell sequencing has enabled exciting progress in sequencing previously inaccessible microbes [30,32]. For example, sequences from a Crenarchaeota were obtained using FISH (fluorescence *in situ* hybridization) probes specific for both Archaea and Crenarchaeota to select the individual cells [33]. Even partial genomic drafts from amplified DNA can greatly advance biological research. A draft sequence estimated to cover 70% of the genome was obtained for *Beggiatoa* spp. [34], marine bacteria that had not been successfully cultured. Predicted genes were identified for a number of enzymes for sulfur oxidation, nitrate and oxygen respiration and CO_2 fixation, confirming a putative chemolithoautotrophic physiology. Two papers report genomic sequencing for TM7, a candidate phylum for which no sequenced members had existed. In one of them, FISH probes were used to isolate candidate cells by flow cytometry [35]. In the other [36], the cells were isolated on a microfluidic chip and MDA was carried out in a 60 nl chamber. DNA sequencing was highly accurate by the 454 method using MDA from single cells, whether the amplification was carried out on the microfluidic chip or by conventional MDA in 50 μl reactions [37]. As noted above, genotyping errors for DNA derived from a single human cell result from amplification bias rather than from the fidelity of the φ29 DNA polymerase for nucleotide incorporation. Similarly, amplification bias between different regions, starting with the single genomic template in a bacterium, results in loss of sequence; however, the sequences that are obtained are highly reliable due to fidelity of the polymerase. In several other studies, approx. 70–80% of novel bacterial genomes have been obtained starting with single cells. A recent review discusses the use of MDA for single-cell sequencing and the expected performance of the methods in generating draft genomic sequences [30]. MDA from single cells has also been used for PCR analysis of multiple genes by the MLST (multilocus sequence typing) method [38]. MDA was also carried out on 1–100 cells obtained by 'microcolony' methods [12], which require cell growth, but only for a low number of divisions. This approach has the advantage of amplifying from more than a single genome copy, thus reducing amplification bias and loss of sequence.

Future directions

MDA is enabling rapid progress in discovery of new environmental microbes. Single-cell genomics also promises to be useful in the study of the human microbiome and in infectious disease processes. It will be possible to discover uncultured bacteria living in the human body by sequencing directly from individual cells. Even for pathogens that can be cultured, it will be possible to analyse genetic diversity between individual cells without the selective loss of genotypes that results from growth in culture. Improvements to the MDA reaction reducing amplification bias will be needed to reduce genotyping errors when amplifying from single human cells and loss of genomic sequence when amplifying to sequence novel organisms. However, the future also promises to bring new advancements in the enzymology of DNA amplification methods. Perhaps even the ultimate goal in the biotechnology of DNA amplification, complete chromosomal replication *in vitro*, will be achieved. Efforts to improve the MDA reaction and optimize it for new applications are under way in many laboratories. Reduction of MDA reaction volume has improved the specificity of template amplification [39] and reduced bias [37]. Recent work has solved the reaction pathway by which certain chimaeric rearrangements can be generated by MDA and suggests approaches for reduction of chimaeras [40]. The growing demand for DNA substrate for use in genomic and detection applications underscores the continued need for innovation in the field of DNA amplification.

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