Whole-genome multiple displacement amplification from single cells

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Published online 30 November; corrected online 21 December 2006 (details online); doi:10.1038/nprot.2006.326

Multiple displacement amplification (MDA) is a recently described method of whole-genome amplification (WGA) that has proven efficient in the amplification of small amounts of DNA, including DNA from single cells. Compared with PCR-based WGA methods, MDA generates DNA with a higher molecular weight and shows better genome coverage. This protocol was developed for preimplantation genetic diagnosis, and details a method for performing single-cell MDA using the ϕ29 DNA polymerase. It can also be useful for the amplification of other minute quantities of DNA, such as from forensic material or microdissected tissue. The protocol includes the collection and lysis of single cells, and all materials and steps involved in the MDA reaction. The whole procedure takes 3 h and generates 1–2 μg of DNA from a single cell, which is suitable for multiple downstream applications, such as sequencing, short tandem repeat analysis or array comparative genomic hybridization.

INTRODUCTION

In some fields of current genetic research, such as forensic analysis, prenatal or preimplantation diagnosis or oncogenetics, DNA analysis has to be performed on small pools of cells or even single cells. The often minute amounts of genomic DNA are a limiting factor in these tests. Whole-genome amplification (WGA) is a method that has been developed to amplify a small amount of DNA template in such a way that the resulting sample has a higher DNA yield, but conserves the sequence representation of the template. PCR-based WGA methods include degenerate oligonucleotide-primed PCR[1], primer extension PCR[2] and ligation-mediated PCR[3]. The general applicability of these methods is hampered by non-specific amplification artefacts[4], incomplete coverage of loci[5] and the small size of the DNA products[1,2].

Multiple displacement amplification (MDA) has been described as a new method of WGA that leads to the synthesis of DNA with limited sequence representation bias[6,7]. This method is based on the annealing of random hexamers to denatured DNA, followed by strand-displacement synthesis at a constant temperature, resulting in DNA products of high molecular weight (up to 12 kb)[8]. As DNA is synthesized by strand displacement, a gradually increasing number of priming events occur, forming a network of hyperbranched DNA structures (see **Fig. 1**). The reaction can be catalyzed by the ϕ29 DNA polymerase or by the large fragment of the *Bst* DNA polymerase[9]. The first reports on single-cell MDA using the ϕ29 DNA polymerase were published in 2004 (refs. 10,11), and were soon followed by more exhaustive studies[12,13] and the first clinical applications in preimplantation genetic diagnosis[13–15]. MDA has also been used on single sperm cells[16] and on genomic DNA from just a few cells in forensic genetic analysis[17].

In our work, we compared the use of the *Bst* and the ϕ29 DNA polymerases in single-cell MDA. The *Bst* DNA polymerase produced low DNA yields (300–600 ng) when the input was 1 ng of genomic DNA or less. Moreover, the MDA products showed a strong representation bias and contained erroneous DNA sequences. On the other hand, the ϕ29 DNA polymerase generated 1–2 μg of DNA from single cells, with a high sequence fidelity and good genome coverage. We assayed several different amplification conditions and established an efficient, accurate, simple and fast protocol for single-cell MDA[12]. As the major risk of single-cell amplification is contamination, the different steps of the procedure were reduced to the strict minimum. The amplified DNA is suitable for multiple downstream applications, among them sequencing, short tandem repeat analysis (STR) and mutation analysis[12], or BAC array-comparative genomic hybridization (CGH)[18].

The main limitations of using this method on single cells are the high allele drop-out (ADO) and preferential amplification (PA)

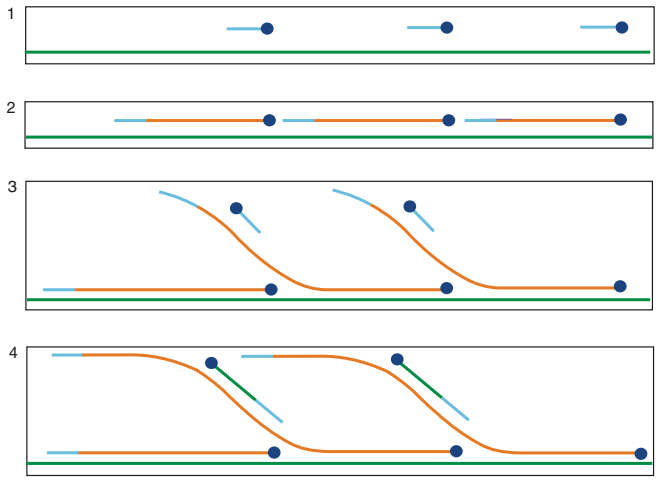

Figure 1 | Overview of the principle of multiple displacement amplification: (1) The random hexamers (represented by a blue line) bind to the denatured DNA (represented by a green line); (2) The ϕ29 DNA polymerase (represented by a blue circle) extends the primers until it reaches newly synthesized double-stranded DNA (represented by an orange line); (3) The enzyme proceeds to displace the strand and continues the polymerization, while primers bind to the newly synthesized DNA; (4) Polymerization starts on the new strands, forming a hyperbranched structure.

rates compared with direct PCR on DNA from a single cell (without previous WGA). ADO is defined as the random non-amplification of one of the alleles present in a heterozygous sample, and in our center was found, on average, in 25% of the loci amplified from single-cell MDA samples. The ADO rates ranged from 0–60%, which are comparable to those reported by other groups[13,15]. ADO decreases the accuracy of the genotyping of a sample and can lead to misdiagnosis in applications such as preimplantation and prenatal diagnosis. Two possible solutions to reduce the impact of ADO are to increase the number of loci that are studied and to analyze several replicates of the same sample. PA involves the relative overamplification of one of the alleles in comparison to the other. The amplification bias is random and noticeable at the locus level. When performing array-CGH, it can be observed that there is an overall equal amplification of all chromosomes and that the bias is generally restricted to a few random clones. When analyzing a set of STRs that are located close to each other and of which the linkage is known, it can be observed that PA is random and affects small stretches of genomic DNA. However, PA can be a serious hindrance when identifying STR alleles. Another consequence of PA is that, although MDA efficiently amplifies the genomic as well as the mitochondrial DNA, it should not be used for quantitative studies[12].

MATERIALS

REAGENTS

- Cell suspension (i.e., buccal cells, fibroblasts, lymphocytes or Epstein–Barr virus-transformed lymphoblasts) ▲ CRITICAL The cells should be freshly harvested. Samples that have been kept in phosphate-buffered saline (PBS) overnight at 4 °C or that have been frozen are less well amplified.
- Dithiothreitol (DTT; Sigma Aldrich, cat. no. D9779) ! CAUTION DTT is a harmful and irritating toxic product; use adequate protection when handling it, such as disposable gloves and mask. DTT is a powder that can be aliquoted and stored at 4 °C.
- KOH (Sigma Aldrich, cat. no. P-5958)
- PBS without calcium or magnesium (Sigma Aldrich, cat. no. P-4417-100TAB)
- NaCl (Sigma Aldrich, cat. no. S-5886)
- KCl (Sigma Aldrich, cat. no. P-5405)
- $NaH_2PO_4 \cdot 2H_2O$ (Sigma Aldrich, cat. no. S-5011)
- Glucose (Sigma Aldrich, cat. no. G-7021)
- $NaHCO_3$ (Sigma Aldrich, cat. no. S-5761)
- EDTA (Sigma Aldrich, cat. no. E-6511)
- Phenol red (Sigma Aldrich, cat. no. P-5530)
- Acetylated nuclease-free bovine serum albumin (BSA; Sigma Aldrich, cat. no. B4287-1G)
- GenomiPhi v2 DNA Amplification Kit (GE Healthcare, cat. no. 25-6600-30, 25-6600-31 and 25-6600-32) ▲ CRITICAL Optimization of the MDA technique for use on single cells was performed using the GenomiPhi Kit. Other MDA kits could, however, be used, and the Repli-g Kit (Qiagen, cat. no. 59043 and 59045) was also tested in our center. The results were similar to those obtained with the GenomiPhi Kit, but no exhaustive testing was performed. The Repli-g Kit produces a higher DNA yield due to a higher enzyme concentration, but is more expensive. The yield obtained with the GenomiPhi Kit is sufficient for PCR and array-CGH, but if a higher yield is necessary for other applications, it is probable that the adaptation of this protocol to the use of the Repli-g Kit will be straightforward. ▲ CRITICAL The enzyme mix of the GenomiPhi Kit must be stored at –70 °C (or –80 °C) and all other components may be stored at –20 °C. Thaw components on ice and maintain at 0–4 °C during handling.
- High Pure PCR Product Purification Kit (Roche, cat. no. 1732676) ▲ CRITICAL The use of other PCR product purification kits, such as the QIAquick PCR Purification Kit (Qiagen, cat. no. 28106 and 28104), have been shown to result in lower DNA yields (on samples generated simultaneously).
- Mineral oil (Sigma Aldrich, cat. no. M8410)

EQUIPMENT

- Stereomicroscope
- Mouth-controlled pipetting system (see **Fig. 2**)
- Fine hand-pulled glass capillary (Drummond microcaps 10 × 100 disposable micropipettes; Drummond Scientific) or flexible disposable tips (Stripper tips, available in different diameters; Mid-Atlantic Diagnostics)
- Drummond micropipette (Drummond microcaps 10 × 100 disposable micropipettes; Drummond Scientific)
- Silicone tubes, 6 × 10 mm and 3 × 5 mm, lengths of choice (~20 cm)
- Filter, 0.2 µm (Sartorius Minisart single-use sterile filter; Sartorius)
- Mouthpiece, i.e., mouthpiece of a Thoma pipette (Hirschmann Laborgeräte); an alternative is to use a 100 µl pipette tip ! CAUTION The use of mouth-controlled pipetting in a clinical setting is discouraged or prohibited in many laboratories. The main risk is the contamination of the sample or the operator with viruses or viral DNA. In a research setting, mouth pipetting can still be used as long as the possible contamination with viruses or viral DNA does not invalidate the procedure and/or does not present a risk for the operator. An alternative to mouth-controlled pipetting is the use of denuding and manipulation pipettes, as used in laboratories working in the field of reproductive medicine, i.e., the Flexipet pipette (Cook), the Stripper pipette (Mid-Atlantic Diagnostics) or the Swemed Pipette Holder (Swemed). Nevertheless, these systems do not allow manipulation to be controlled as accurately and finely as a mouth-controlled system.
- Laminar airflow
- 200 µl PCR tubes
- Sterile Petri dish (i.e., Falcon Petri dish; BD Biosciences, cat. no. 351016)
- Thermocycler
- Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies)

▲ CRITICAL Other methods of DNA quantification can be used, such as the Quantifiler Human DNA Quantification Kit (Applied Biosystems, cat. no. 4344790) or the PicoGreen dsDNA Quantification Kit (Molecular Probes, cat. no. P-7581).

REAGENT SETUP

Alkaline lysis buffer (ALB) 50 mM dithiothreitol (DTT) and 200 mM KOH. ▲ CRITICAL The KOH solution should be autoclaved prior to use, and the ALB should be stored at 4 °C for no longer than a week. In addition, do not use the ALB described by the manufacturer of the MDA kit, as the higher concentrations of DTT and KOH may damage the DNA.

Ca^{2+}- and Mg^{2+}-free medium 14 mM NaCl, 0.2 mM KCl, 0.04 mM $NaH_2PO_4 \cdot 2H_2O$, 5.5 mM glucose, 1.2 mM $NaHCO_3$, 0.02 mM EDTA, 0.01% (w/v) phenol red, supplemented with 15 mg ml^{-1} BSA. ▲ CRITICAL The medium should be autoclaved and can be stored for a year at 4 °C, in aliquots of 2 ml in eppendorf tubes. The BSA should be added immediately prior to its use. This can be done without calculating the exact amount, by adding a small amount to the aliquot using the tip of a spatula. A small amount is sufficient to avoid adhesion of the cells to the surface of the culture dish. Use a sterilized spatula and work in a laminar airflow to avoid contamination.

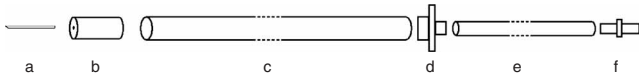

Figure 2 | Schematic representation of a mouth-controlled pipetting system. a: for small cells (i.e., lymphocytes), use fine hand-pulled glass capillaries. Larger cells (i.e., blastomeres or fibroblasts) can be picked up using flexible disposable tips. b: Drummond micropipette. c: silicone tube, 6 × 10 mm. d: filter, 0.2 µm. e: silicone tube, 3 × 5 mm. f: mouthpiece.

BOX 1 | WASHING CELLS IN PBS

1. Add 500 µl PBS to ~100 µl cell suspension and centrifuge at 4,000*g* for 5 min.
2. Discard the supernatant and resuspend in 500 µl of fresh PBS.
3. Centrifuge at 1,400*g* for 5 min.
4. Repeat Steps 2 and 3.
5. Discard the supernatant and resuspend the pellet in 100 µl fresh PBS.

PROCEDURE

Collection of single cells ● TIMING depends on the number of samples and the expertise of the operator (an experienced operator can tube 20 single cells in 30 min to 1 h)

1| Prepare a 200 µl PCR tube containing 1.5 µl ALB for each cell to be collected and keep the tubes on ice.

▲ CRITICAL STEP The single-cell collection and the set-up of the MDA reaction should be performed taking all necessary precautions to avoid contamination by extraneous DNA. During the single-cell collection, operators should use clean laboratory overcoats, masks, gloves and hairnets, and the stereomicroscope should be located in a laminar airflow in a separate area. All further handlings should be performed using laboratory overcoats and gloves, in dedicated areas, in laminar airflows and with sterile materials.

▲ CRITICAL STEP We have found that the use of 1.5 µl ALB leads to better results than using larger volumes. Nevertheless, 2.5 µl ALB can be used if 1.5 µl is too small a volume to correctly introduce the cells.

▲ CRITICAL STEP Ensure that the ALB droplet is at the bottom of the tube.

2| Prepare culture dishes with rows of 3 droplets (~3 µl) of Ca^{2+}- and Mg^{2+}-free medium and one large droplet (~20 µl). Cover with mineral oil.

3| For cells that are obtained from cell suspensions or trypsinized cell cultures (e.g., fibroblasts), wash cells three times in PBS (see **Box 1**).

▲ CRITICAL STEP This step is not necessary for polar bodies, single blastomeres or trophectoderm samples obtained from oocyte and embryo biopsy, or for samples such as laser-captured cells.

4| Use the mouth-controlled pipetting system (see **Fig. 1**) to transfer 1–2 µl of the cell suspension to the large droplet of Ca^{2+}- and Mg^{2+}-free medium in the culture dish.

▲ CRITICAL STEP The stereomicroscope can be used to visualize this step if desired, to ensure that the pipette enters the droplet and not the oil.

5| Use the mouth-controlled pipetting system to transfer 2–3 cells to each first droplet of the rows of three, visualizing this procedure under the stereomicroscope.

6| Wash a single cell by transferring it from droplet to droplet using the mouth-controlled pipetting system, visualizing this procedure with the stereomicroscope.

7| Transfer the cell into the 1.5 µl ALB contained in the 200 µl PCR tubes using the mouth-controlled pipetting system and keep the tube on ice. This step can be performed using either option A (for lymphocytes or lymphoblasts) or B (for larger cells, e.g., buccal cells, fibroblasts or blastomeres).

(A) Blind transfer (of lymphocytes or lymphoblasts)

(i) Insert the tip of the capillary into the ALB and blow a small amount of medium into it (~1 µl).

(B) Observed transfer (of larger cells, e.g., buccal cells, fibroblasts or blastomeres)

(i) Lay the tube on the surface of the microscope.
(ii) Focus on the ALB droplet.
(iii) Insert the capillary parallel to the surface of the microscope into the tube, avoiding touching the walls.
(iv) Insert the tip of the capillary into the ALB.
(v) Focus on the tip and blow gently until the cell is seen entering the ALB.

▲ CRITICAL STEP Avoid introducing more than 1–2 µl of medium into the ALB when transferring the cell. It will dilute the ALB, reducing its effectiveness, and may introduce inhibitors.

8| Add 1 µl of the last washing droplet to an additional PCR tube for use as a negative control.

9| Store the samples at –20 °C or –80 °C for at least 30 min.

■ PAUSE POINT Samples can be stored at –20 °C or –80 °C for up to 1 week.

▲ CRITICAL STEP Samples should not be kept (frozen) for longer than 1 week. Experiments performed at our center showed a decrease in efficiency after 1 week of storage.

Cell lysis ● TIMING 10 min

10| Incubate the samples in a thermocycler for 10 min at 65 °C.

▲ CRITICAL STEP The use of Neutralization Buffer following cell lysis, as suggested by the manufacturer of the MDA kit, is unnecessary when using the here-described ALB. Experiments performed at our center showed that the use of Neutralization Buffer results in higher background levels when performing array-CGH.

▲ CRITICAL STEP From here on, keep the samples constantly on ice.

MDA ● TIMING 2 h and 15 min

11| Add 9 µl of the Sample Buffer (containing random hexamers) from the GenomiPhi v2 DNA Amplification Kit to each sample and leave them on ice for the time it takes to prepare the master-mix of Reaction Buffer and the Enzyme mix (i.e., 5 min; the exact time is not critical).

▲ CRITICAL STEP A denaturation step at 95 °C, as recommended by the manufacturers, is not required prior to the MDA. As this high temperature may damage the DNA, we recommend avoiding this step.

▲ CRITICAL STEP In addition to the series of single-cell samples, at least one negative control should be prepared by including 1 µl of medium from the last washing droplet (see Step 8) instead of sample. Furthermore, it is useful to prepare a negative control that does not contain any of the products used during the single-cell collection (by using autoclaved MilliQ water instead of sample). In case of contamination, these negative control samples may be useful for helping to trace the source. A positive control can also be prepared by adding 1 ng of genomic DNA instead of sample. The GenomiPhi v2 DNA Amplification Kit contains a source of positive control DNA at 10 ng μl^{-1}, but DNA obtained from other sources can be equally used; at our center, genomic DNA extracted from the patients' blood is routinely used. A positive control may be useful to control for amplification failure in the single-cell samples. Successful MDA of the positive control sample indicates correct handling of the samples and products.

12| Prepare a master-mix that contains 9 µl of Reaction Buffer (containing nucleotides and additional random hexamers) and 1 µl of the Enzyme Mix, for each sample to amplify.

13| Pipette 10 µl of this mix into each sample and vortex gently.

14| Incubate the samples at 30 °C for 2 h, then at 65 °C for 10 min (to inactivate the enzyme).

▲ CRITICAL STEP The enzyme is very sensitive to temperature changes. It should not be frozen and thawed several times. It is best to aliquot the enzyme on arrival and avoid, as much as possible, any source of heat.

▲ CRITICAL STEP It is important to respect the incubation temperature of 30 °C. We unsuccessfully assayed other incubation temperatures.

▲ CRITICAL STEP Use a thermocycler for the incubation, as it ensures a constant temperature.

▲ CRITICAL STEP Incubating for more than 2 h (as suggested by the manufacturers) does not increase the final specific DNA yield.

■ PAUSE POINT The samples can be kept overnight at 4 °C.

Purification ● TIMING depends on the number of samples. Approximately 30 min for 24 samples.

15| Purify the MDA products following the protocol provided by the manufacturer of the DNA purification kit.

▲ CRITICAL STEP The final elution volume can be chosen, depending on the needs (50 µl or 100 µl of Elution Buffer).

Concentration measurement and storage ● TIMING 1 min per sample

16| Use the Nanodrop ND-1000 spectrophotometer to measure the concentration of MDA products to check if the MDA was successful.

■ PAUSE POINT The samples can be stored for more than 1 year at 4 °C or at −20 °C (if the samples are kept frozen, avoid repeated freezing and thawing to prevent DNA fragmentation).

● TIMING

Steps 1–9, 30 min to 1 h to collect 20 single cells (depending on the expertise of the operator)
Steps 10–14, approximately 2 h and 30 min
Step 15, 30 min for 24 samples (depending on the purification kit used)
Step 16, approximately 1 min per sample

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Low yield of DNA	Poor-quality cell	Use freshly harvested and washed cells.
		Use a different cell line/type (high levels of apoptosis/necrosis in the culture).
	No cell in the tube	Restart procedure. Use a positive control (tube containing 1 ng of genomic DNA), along with the single-cell samples to ensure that the MDA reaction did not fail.
	Poor lysis	Ensure that ALB is fresh.
		Avoid transferring more than 2 μl of Ca^{2+}- and Mg^{2+}-free medium in the ALB when collecting the single cells.
	Cell sample contains inhibitors (i.e., polyvinylpyrrolidone (PVP) in sperm samples, saliva in epithelial cells obtained from a mouthwash)	Give special attention to the washing procedure. Discard all supernatant in each step. Add one more washing step.
	Excess of BSA in cell sample	Use less BSA in the Ca^{2+}- and Mg^{2+}-free medium.
	Poor enzyme conservation (i.e., due to incorrect transport)	Use a fresh aliquot of enzyme.
		Use a new kit.
		Always keep the enzyme frozen (at –80 °C); aliquot the enzyme after delivery.
		Avoid sources of heat (i.e., fingers).
High yield of DNA in negative control samples	DNA contamination	Replace solutions and use autoclaved products.
		Use dedicated materials.
		Clean working area.
		Use adequate protection (gloves, masks, overcoat).
		Change gloves frequently.
	Background synthesis (*de novo* random DNA synthesis and/or contaminating host DNA)	Always keep the enzyme frozen and use the mix of Reaction Buffer and Enzyme mix immediately after preparation.

ANTICIPATED RESULTS

You may expect a DNA yield of approximately 1–2 μg. Negative controls (no template samples) show a low DNA concentration (up to 10 ng μl^{-1}). This DNA does not originate from contamination with human DNA from the laboratory environment, but possibly from *de novo* random DNA synthesis and/or contaminating host DNA that is present in the enzyme preparation. This method amplifies both the genomic and the mitochondrial DNA. The genome coverage of this method, when starting with 10 ng of genomic DNA, has been reported to be 99.82%[19]. The genome coverage of MDA from single cells has not yet been conclusively determined; however, array-CGH carried out on MDA-amplified DNA from single cells in our laboratory has not yet revealed any region of the genome that is refractory to MDA. Performing high-density single nucleotide polymorphism arrays may provide a more accurate method with which to estimate the coverage, and DNA fingerprinting can also be used to confirm that the correct DNA has been amplified.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com

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Erratum: Whole-genome multiple displacement amplification from single cells

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Nat. Protocols doi:10.1038/nprot.2006.326; published online: 30 November; corrected online 21 December 2006.

In the version of this article initially published online, the third and fourth panels of Figure 1 (p. 1965) were incorrectly drawn. Figure 1 has been corrected in all versions of the article.