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Review

Whole genome amplification: Use of advanced isothermal method

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Laboratory method for amplifying genomic deoxyribonucleic acid (DNA) samples aiming to generate more amounts and sufficient quantity DNA for subsequent specific analysis is named whole genome amplification (WGA). This method is only way to increase input material from few cells and limited DNA contents. While PCR-based WGA methods have been under continuous development for over a decade, shortcomings of these methods enforced many researchers to switch to the use of non-PCR-based linear amplification techniques. Moreover, application of high fidelity and high possessive DNA polymerases enabled development of an isothermal WGA technique named multiple displacement amplification (MDA). MDA is not based on PCR and doses not require thermal cycling. It should be noted that, while MDA-based techniques proposed aiming to overcome the drawbacks of PCR-based methods however, MDA is still facing some challenges. It seems that PCR-based WGA methods also have some merits. One of the problems which encountered both MDA and PCR-based methods is in the amplification of degraded DNA templates. WGA methods such as T7-based linear amplification of DNA (TLAD), balanced-PCR amplification and restriction and circularization-aided rolling circle amplification (RCA-RCA) have been suggested to aim at amplification of such DNA templates.

Key words: Whole genome amplification, multiple displacement amplification (MDA), non PCR-based methods.

INTRODUCTION

WGA is a technique that is used for preamplification of the entire genome using random or degenerate primers. The technique is especially important in situations where amplification of entire template genome is the only way to overcome the problem of DNA source limitations. It is clear that this technique provides the possibility of genome-wide scans but the PCR-based approaches like the basic WGA methods encounters with shortcomings in some circumstances mostly due to the temperature changes during thermal cycling. In this paper other WGA methods that are proposed to reduce disadvantages of previous methods are described.

LINKER ADAPTOR TECHNIQUE- PCR (LA-PCR)

Ligation-mediated PCR (LMP) techniques involve ligating an adaptor sequence into a representation of DNA molecules, produced after enzymatic digestion, chemical

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cleavage or random shearing (Arneson et al., 2008c).

LAM-PCR method, initially described by Ludecke et al. (1989) and Saunders et al. (1989), involved restriction digestion of the DNA and ligation of adapters to serve as as priming sites for next PCRs. Subsequent PCR primers are complementary adapters. Ligation of short linker adaptors to the end provides a single annealing site for PCR priming and aiming to diminish some of the amplification bias associated with thermal cycling in which sequence has a large effect on both the denaturation of the template and the T_m of primers. Although linker adapters provide more uniform priming, due to requiring more steps in this method, only a limited subset of sequences is amplified (Lasken and Egholm, 2003). This method has high capability to produce PCR libraries for each chromosome that are used as probes for FISH (Peng et al., 2007). LAM-PCR was initially applied to the cloning of micro dissected chromosomes and detection of regions of DNA involved in DNA/protein interaction, not to the process of WGA (Hughes et al., 2005), however later optimized as a whole genome amplification technique.

Adaptor ligation mediated PCR of randomly sheared genomic DNA (PRSG)

A further adaptation of LMP type of whole genome amplification has been to ligate primer sequences on to randomly sheared genomic DNA in a procedure termed adaptor-ligation mediated PCR of randomly sheared genomic DNA (PRSG) (Tanabe et al., 2003). PRSG method is based on ligation-mediated PCR using fragmented DNA rather than enzymatically generated fragments for example, hydrodynamic shearing machine (Arneson et al., 2008b). For validation of whole genome amplification, Tanabe et al. (2003) have used exon amplification and genotyping of 307 microsatellites, in addition to array CGH. Analyzing of 307 microsatellites distributed throughout the genome revealed 84% were reproducibly amplified in PRSG DNA and 99% of this showed a consistency between the PRSG product and the original genomic DNA.

Single cell comparative genome hybridization (SCOMP)

Single cell comparative genome hybridization (SCOMP), initially described by Klein et al. (1999), is a form of LMP that was specifically designed for whole genome amplification from extremely limited sources of genomic DNA. SCOMP convert the genome to a high-complexity representation by digesting the restriction enzyme MseI to fragment less than 2 kb. This results in a smear, in the range of 100-1500 bp. After digestion with restriction enzyme, adaptors with specific primer sequences (specific to the restriction enzyme used) are ligated to the ends of the genomic DNA fragments and then amplified in a high-stringency PCR. In a pre-annealing step, two oligonucleotides are utilized to form an adaptor complex that can be ligated to the overhang on the genomic DNA fragments. (Arneson et al., 2008c; Hughes et al., 2005).

SCOMP product have been used for several downstream applications, including chromosomal comparative genomic hybridization (CGH), loss of heterozygosity (LOH) analysis and direct sequencing (Klein et al., 1999); however, there is inconsistency in the magnitude of amplification and elaborated protocol (Aviel-Ronen et al., 2006) in this method. For eliminating PCR-based introduction of bias, SCOMP is superior to DOP-PCR when using formalin-fixed paraffin embedded samples combined with laser capture microdissection (Stoecklein et al., 2002).

Since LMP technique has not yet been widely performed for WGA, associated problems have not been reported. Although initial results are promising, more investigations are required before determining relative advantages of LMP over other PCR-based methods (Hughes et al., 2005).

GenomePlex

OmniPlex technology, described by Sigma-Aldrich, converts non-enzymatic randomly fragmented genomic DNA into an *in vitro* molecular library of inherently amplifiable DNA fragments of defined size, followed by incubation at various temperatures to add adaptor sequences with specific PCR priming sites to both ends of each fragment.

By using a high-fidelity DNA polymerase, this library can be effectively amplified in several thousand fold, as well as it can be re-amplified to achieve a final amplification over a million fold without degradation of representation. The fragment library can generate milligram quantities of DNA starting with as little as 10-100 ng (Arneson et al., 2008a; Barker et al., 2004).

Rubicon Genomics commercializes different kits (Omniplex) that are used for the amplification of RNA, DNA and methylated DNA sequences. The GenomePlex tech-nique randomly cuts genomic DNA into a library of fragments, ranging from 200 to 1,500 base pairs in size, with an average of 400 bp. Universal priming sites (oligo with known 5' ends but degenerate 3'- ends) are added to the DNA fragments, which are then amplified using a high-fidelity DNA polymerase, without degradation of representation. In principle, GenomePlex is a hybrid WGA method that utilizes both LA-PCR idea and also OmniPlex technology. The advantages of this method are its capability to amplify degraded DNA, allowing for different variations and all steps can be performed in the same tube.

MULTIPLE-STRAND DISPLACEMENT AMPLIFICATION (MDA)

Multiple-strand displacement amplification (MDA) WGA which is an isothermal amplification method can be performed by application of one highly processive Phi 29

DNA polymerase (Bergen et al., 2005a), and this methods refer to hyperbranched strand displacement amplification (HSDA) (Aviel-Ronen et al., 2006). The base of strand displacement amplification (SDA) or multiple displacement amplification (MDA) is related to rolling circle amplification (RCA) type mechanism with circular DNA molecules. This method was initially used for the amplification of large circular DNA templates (Dean et al., 2001) and recently for the amplification of genomic DNA (Dean et al., 2002).

In MDA method, like other WGA approaches, there is no need to previous knowledge of the target template, however it is different from other WGA methods and is not based on PCR and repeated cycling (Lasken and Egholm, 2003).

In this method, at first the random hexamer primers anneal to the single stranded target molecule. As the DNA polymerase elongates the primer, the upstream DNA strands are displaced. Then the displaced DNA strands act as templates for new priming event and elongation in the opposite direction occur. This procedure continues and new DNA strands are displaced to produce new templates which result in a hyperbranched structure that generate high copies of the original DNA molecule (Lovmar and Syvanen, 2006).

SDA method uses either Phi 29 DNA polymerase or a combination of Bst polymerase and T4 gene 32 protein in conjunction with preferentially modified random primers to amplify the whole genome. Instead of repeated thermal cycles, a short initial (2-3 min) 94°C denaturation is followed by an enzyme specific incubation (Phi29: 30°C and Bst : 50°C) step of 6-18 h and a final 65°C step of 15 min to inactivate the enzyme (Dean et al., 2002; Lage et al., 2003; Silander and Saarela, 2008). It has shown that phosphorothioate modification of primers markedly stimulates the MDA reaction that result in amplifications of 104-106 folds (Dean et al., 2001). The phosphorothioate nucleotides protect primers from degradation by the 3'-5' exonuclease proofreading activity of the Phi29 DNA polymerase. The presence of an associated proofreading activity with the Phi 29 polymerase ensures high-fidelity amplification with an error rate of only 3×10^{-6} (in mutations/nucleotide) in the amplified DNA, compared with $\sim1\times10^{-3}$ generated by Taq DNA polymerase in a PCR reaction. Moreover, Phi29 DNA polymerase has extremely tight binding to the DNA template and a high rate of strand displacement synthesis through virtually any DNA sequence and secondary structure (Hosono et al., 2003). Phi29 DNA polymerase was used because of its ability to perform strand displacement DNA synthesis for more than 70,000 nt without dissociating from the template and its stability to synthetize DNA for many hours (Dean et al., 2001). This is one of the valuable advantages found for a DNA polymerase in the absence of cellular multi-subunits complexes (Panelli et al., 2006).

Average MDA product length is more than 10 kb. Using MDA method allows it to yield 20-30 µg products from as few as 1-10 copies of human genomic DNA (Dean et al., 2002). There are two commercial kits for MDA WGA techniques; Repli-G and GenomiPhi. It should be noted that there is a difference in yield between these MDA methods, probably due to using KOH alkali denaturation step prior to amplification in the Repli-G process which is more efficient at opening potential priming sites than the thermal denaturation used in the GenomiPhi protocol (Pinard et al., 2006). A main limitation of MDA method is its utilization for unusual templates such as DNA derived from fixed paraffin tissues, degraded DNA, cross linked DNA and short DNA fragments which are not well tolerated (Alsmadi et al., 2009).

COMPARISON OF MDA WITH PCR-BASED WGA METHODS

PCR-based WGA methods have been used for many applications; however their shortcomings have been well reported. Primer extension-based methods have susceptibility to bias and have a tendency to change the information of the original DNA (Kafshnochi et al., 2010). By the use of quantitative real time PCR, Dean et al. (2002) demonstrated an amplification bias of 10^3-10^6 between genomic loci in primer extension preamplification (PEP) amplified pro-ducts. In addition, PCR-based methods typically generate small products, usually 200 bp to 2 kb, which can limit some downstream applications. Furthermore, it has been well known that DOP-PCR does not provide complete coverage of all loci, or copy the target DNA in its entirety and can preferentially amplify shorter alleles, Alu sequen-ces and microsatellites. All of these factors can introduce experimental error regarding to loss of heterozygosity (LOH) studies and SNP analysis, hence many researchers use non-PCR-based linear amplification protocols (Hughes et al., 2005).

Compared to MDA, in PCR reactions, repetitive cycles of denaturation and annealing temperatures limit polymerase longevity and activity. The half-life for various Taq DNA polymerases ranges from 30 to 70 min at 95°C, resulting in a 50% or greater decrease in enzyme activity at the end of 40 cycles. By using isothermal reactions, the MDA methods can preserve enzyme functionality for a 16 h reaction, generating substantially more DNA in hyper branched mechanism of DNA amplification. It has been reported that PCR-related bias in products amplified, is caused by factors such as differential GC content and product reannealing. Moreover, not all amplicons are amenable to PCR amplification, resulting in missing sequence, the high-temperature denaturation required to render genomic DNA single-stranded for PCR-based WGA methods can cause cytosine deamination, and homoduplexes can form during the ramp from denaturation to annealing temperature.

All of these factors produce nonspecific amplification

artifacts up to 70% that lead to incomplete coverage. This is in accord with the high percentage of PEP and DOP generated sequences (60 and 80%, respectively as opposed to 40% for unamplified and MDA samples) that failed to map to the reference genomes (Pinard et al., 2006).

Despite of drawbacks well documented for PCR-based WGA methods, it should be noted that these methods also have some merits; however, their use should be restricted to DNA amplification for genotyping or marker identification purposes, not uniform genomic amplification for high accuracy whole genome sequencing. MDA-based techniques produced higher yields of the proper template (non-artifactual), however these approaches induced lower but still significant levels of bias (Lasken and Egholm, 2003; Pinard et al., 2006). comparison of several whole genome amplification methods such as DOP, PEP, Repli-g and GenomiPhi (as two procedures of MDA), show that all methods induced bias relative to the unamplified DNA, but MDA generated the least bias. Even so, MDA is still facing some challenges such as amplification yield, genome coverage, template independent DNA amplification (TIDA), and allele drop-out (ADO) (Alsmadi et al., 2009).

Handyside et al. (2004) proposed that, although MDA is an efficient method for whole genome amplification from single cells (efficiency rate of 92%), but as with all PCR-based methods, some preferential amplification of alleles, ADO and, in some cases, complete amplification failure was observed. This problem has decreased by optimizing of primer design, conditions of amplification and use of sensitive fluorescent PCR methods, but these cannot completely be eliminated, showing that they may be inherent to amplification from a single cell perhaps due to rapid degredation of the target DNA.

Sun et al. (2005) evaluated the relative efficiencies of some WGA methods with respect to their product yield, genome coverage, sensitivity and fidelity. This assessment is mostly based on analyzing a set of microsatellite markers by use of WGA products that was obtained from different protocols. They have reported similar sensitivity between I-PEP with MDA by using serial dilutions of starting gDNA that amplify 10 pg of genomic DNA (~6 pg/diploid cell). On the other hand, with this quantity of start material, allele dropouts frequently occur in both protocols. In both WGA methods, allelic dropouts were produced with 100 pg of gDNA. Though, by repeating PCRs on the same WGA yield, allelic dropout is not locus-specific, and data on all 13 determined loci could be obtained. Additionally, I-PEP products showed no allelic imbalance (one in 311 heterozygotes), however MDA products showed a large number of samples allelic imbalance (66 out of 311 heterozygotes). Conclusion of these findings show that the yield of MDA is higher than I-PEP but specificity of I-PEP products appears to be higher, particularly by analyzing microsatellite loci. Hence, analysis of SNPs shows that some markers might work better with products produced by specific protocol.

Primas-based whole genome amplification (pWGA)

Despite the advantages of the Phi29 DNA polymerase based MDA system, this system is still facing some limitations such as long time of amplification. In pWGA system, an initial heat denaturation step of the input DNA is often performed before the isothermal amplification to facilitate primer annealing. This step may result in mutations to the template and/or contaminations to the reaction. Unlike the PCR and MDA methods, in pWGA system, by utilizing bacteriophage T7 gp4 primase, there is no requirement of adding synthetic primers to the amplification reactions to synthesize primers on-template (Li et al., 2008).

This technology is based on the *in vitro* reconstitution of the naturally existing cellular DNA replication machinery of bacteriophage T7. Bacteriophage T7 has one of the simplest DNA replication systems. Only four proteins are needed to replicate the entire 40-kb linear genome of T7, that is, T7 gene 4 protein (T7gp4 with primas/helicase activity), T7 DNA polymerase holoenzyme (a heterodimer of two proteins, T7 gene 5 protein (T7gp5) and Escherichia coli thioredoxin, at 1:1 molar ratio, which T7gp5 encodes the 5'–3' DNA polymerase and the 3'–5' exonuclease and E. coli thioredoxin binds to T7 gp5 with an affinity of 5nM and improves the polymerase processivity) and a single-stranded DNA (ssDNA) binding protein encoded by T7 gene 2.5 (T7gp2.5). Amplification was carried out by incubating the reaction at 37°C for 30-120 minute and stopped by inactivation of T7 DNA polymerase at 65°C for 20 min.

Li et al. (2008) reported that the locus bias of method is less than 7-fold when input DNA was more than 10 ng. Even from as low as 1 ng of input (about 300 copies of the genome), the bias was only 11-fold which is similar to MDA method and much lower than PCR-based WGA. If the product is being used in following genome researches, such as comparative genomic hybridization (CGH), Low amplification bias is a prerequisite. It is proposed that from 10 ng of input DNA, all the 20 selected loci were amplified by pWGA in 173-1099-fold.

T7-based linear amplification of DNA (TLAD)

Liu et al. (2003) have devised a linear amplification protocol for genomic DNA, which originally was designed by Phillips and Eberwine (1996) to amplify mRNA for use on cDNA microarrays. T7-based linear amplification (TLAD) can generate microgram quantities of genomic DNA from as little as 2.5 ng of input DNA, whereas maintaining the variation in fragment size present in the starting material. In this method, the DNA is first digested with the restriction endonuclease, AluI. Terminal transferase is then used to

add polyT tails to the 3' ends of the digested DNA strands. An oligonucleotide primer, containing a 5' T7 promoter and a 3' polyA tract, is annealed to the genomic fragments, and Klenow is used for synthesis of the second strand. The reaction products of this synthesis are then used as templates for the *in vitro* transcription reaction. Particular use of this technique is amplification of the DNA extracted from FFPE tissue, as one of poor quality DNA. Unlike the PCR-based amplification, TLAD does not introduce the sequence and length-dependent biases, however disadvantages is the requirement for sample purification following each step that make prolonged protocol, cumbersome and result in sample loss. For these reasons, up till now, it has not been widely used (Hughes et al., 2005).

Balanced-PCR amplification

Amplification efficiency of MDA is reduced as the molecular weight of the starting material decreases, which is problematic for amplification of formalin-fixed archival DNA or low molecular weight DNA from deteriorated forensic samples. Klein et al. (1999) described that SCOMP utilizes DNA digestion and adaptor ligation to perform PCR-based whole genome amplification when starting from a single input cell. Because SCOMP uses digested and low molecular weight DNA as a starting material, which can amplify efficiently DNA from formalin-fixed samples. However, the issue of amplification bias in SCOMP method was not sufficiently addressed because this method was not validated at high resolution, that is, via array-CGH or on a gene-by-gene basis. Due to the PCR shortcomings, SCOMP is expected to cause substantial amplification bias. Based on these findings, Wang et al. (2004a) described a PCR-based technique to amplify genomic DNA that is called balanced-PCR amplification. This method does not require intact, long genomic DNA as starting material and allows removal of amplification bias caused by PCR saturation, in principle, like TLAD, this is a suitable method for low quantity DNA sources.

In the study performed by Wang et al. (2004a) based on this technique, genomic DNA (from two different origins, for example, one from cancer cells and another from normal cells) is first digested with a 4 bp cutting restriction nuclease (that is, NlaIII). Following ligation of composite linkers (AACTGTGCTATCCGAGGGAAAGGA-CATG and AACTGTGCTATCCGAGGGAAAGAGCAT G) to the two DNAs, the samples are mixed and PCR is performed in a single tube. The single tube amplification of the mixed samples is used to eliminate PCR biases associated with PCR saturation and impurities, while the polymerase has no capability to distinguish between alleles originated from normal or cancer genomes. In low yield of PCR, a nested (second PCR) is subsequently used to re-separate DNA fragments from the two original genomes on the basis of nucleotide `tags' incorporated in the composite linkers. The ability of balanced-PCR to overcome problems related with amplification of moderately degraded DNA may be associated with the initial digestion of DNA followed by adaptor ligation, which produces a significant number of DNA fragments lacking formalin-associated DNA damage that is able to amplify. It has been shown that performing of amplification in this method is not inhibited by formalin-induced DNA damage, it is suggested that to perform both balanced-PCR and MDA amplifications when DNA is isolated from fresh samples, because regards to gene amplification and deletion by the two methods can provide higher detection accuracy. It has been shown that MDA method can not amplify material from formalin-fixed sample of modestly degraded DNA, whereas balanced-PCR is capable of amplifying.

As balanced-PCR is not capable to amplify large (>2 kb) fragments which may potentially be present due to the location of successive NlaIII sites in a genome, this method is used to amplify a small fraction (Wang et al., 2004a).

Restriction and Circularization -aided rolling circle amplification (RCA-RCA)

Because of the inefficiency of thermostable DNA polymerases in amplifying DNA fragments with length more than 1 kb, balanced-PCR WGA method usually amplifies only a minor portion of the entire genome that is a genomic representation. This incomplete genome coverage may cause the loss of vital genetic information. Additionally, the amplification efficiency of MDA rapidly reduces as the molecular weight of the starting material decreases, therefore making it inappropriate for amplification of FFPE DNA or low molecular weight DNA from deteriorated forensic samples. For these reasons, Wang et al. (2004b) described RCA-RCA (restriction and circularization-aided rolling circle amplification), a new amplification methodology that overcomes problems associated with nucleic acid degradation and retains the allelic differences among amplified genomes while simultaneously achieving almost complete genome coverage. Thus, this is another suitable method for low quality DNA sources.

The principle of RCA-RCA method is the fragmentation of the genome with an appropriate restriction enzyme (for example, NlaIII) that cuts at least twice between successive DNA damage sites in FFPE samples, generates intact DNA fragments that can be circularized. After circularization and elimination of noncircular DNA via exonuclease, the circles are denatured to enable initiation of exponential, hyperbranched rolling circle amplification using random primers and Phi29 polymerase. Exponential amplification is enabled even if one of the two circularized DNA strands remains intact. Though, self-circularization of DNA fragments <250 bp has been recognized to be

inefficient, but during whole genome circularization, small DNA fragments (<200 bp) can cross-ligate to produce larger fragments and then circularize. During the subsequent amplification step, a considerably higher amplification could be expected for the small fragments relative to large fragments as the polymerase completes replication of a smaller circle faster, given a constant incorporation of nucleotides per second. However, this variability is smaller than one would expect (Wang et al., 2004b).

Wang et al. (2004b) reported that the concordance of RCA-RCA products to unamplified samples clearly exceeds that of all other available methods. In addition, the design of the RCA-RCA protocol allows any single enzyme or combination of enzymes to be substituted in place of NlaIII with no further modification. Therefore, by substituting the restriction enzyme in the first RCA-RCA step, it may be possible to recover samples of very high degradation.

CONCLUSION

With innovation of whole genome amplification technique, from this onwards, the amount of biological specimens and DNA quantity will not be a limiting factor in performing the advanced molecular genetic analysis. Although each of several proposed methods for whole genome amplification suffers from drawbacks and problems, however, strength of this technique cannot be ignored. Allele drop-out (ADO) is the main problem of all WGA approaches described to date. Therefore undoubted, the future attempts will focus on diminish and even elimination of this problem.

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