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A Highly Sensitive and Simple Method of Genomic Subtraction

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ABSTRACT

We describe a method, named quantity compensated genomic subtraction, for isolating rare genomic DNA (gDNA) difference fragments between two complex eukaryotic genomes. Because PCR is used to compensate for quantity after each round of subtraction, up to a dozen iterations are possible with only a small initial amount of target materials (1 μg) after each round of subtraction. We successfully applied this technique to tilapia (*Orechromis niloticus*) genome (diploid >10^9 bp) mixed with a small amount of lambda DNA, which served as the "difference fragments" target. The magnitude of enrichment was 10^5 and the subtractive products were highly representative.

Key words: *Genomic subtraction, Lambda DNA, Orechromis niloticus.*

INTRODUCTION

Genomic subtraction is an essential tool for cloning the difference fragments between two different genome sets, but it can be used successfully only when the difference fragments are large, highly repetitive, or represent a large proportion of the total genome (Straus and Ausubel, 1990). Furthermore, even when subtractive steps are reiterated, difference fragments are enriched only 100 to 1000 times (Wieland *et al.*, 1990). Another technique, representational difference analysis (RDA) (Lisitsyn *et al.*, 1993), achieves 10^5-10^6 enrichment, but unfortunately, RDA procedures are both complicated and tedious, and when inappropriate restriction enzymes are chosen, the subtractive products will not be representative.

According to theoretical analysis (Ermolaeva and Wagner, 1995; Milner *et al.*, 1995; Sverdlov and Ermolaeva, 1993), rare or short difference fragments can be highly enriched from complex genomes if sufficient rounds of subtraction are available. This is almost impossible because after several rounds of subtraction, the starting materials remained are not sufficient to be PCR amplified and analyzed within acceptable amplifying cycles. Here we developed a simple method, which makes multiple rounds of subtraction available and requires only a little starting target DNA. Our method is outlined in Figure 1. The testers (including the "difference fragment" target) were partially digested with *Sau*3AI and ligated to adapters. The drivers were also partially digested with *Sau*3AI and then biotinylated. Testers and drivers in excess were reassociated during a period of incubation at a stepwise decreasing temperature. Biotinylated tester-driver hybrids were then removed by streptavidin-coated beads, while the unbound testers were collected and amplified by 3 cycles of PCR to compensate for the quantitative losses. The amplified testers were then brought to the next round of subtraction. Subtractive steps can be

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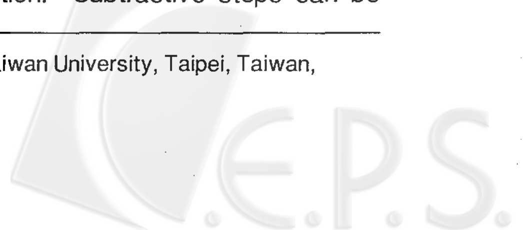

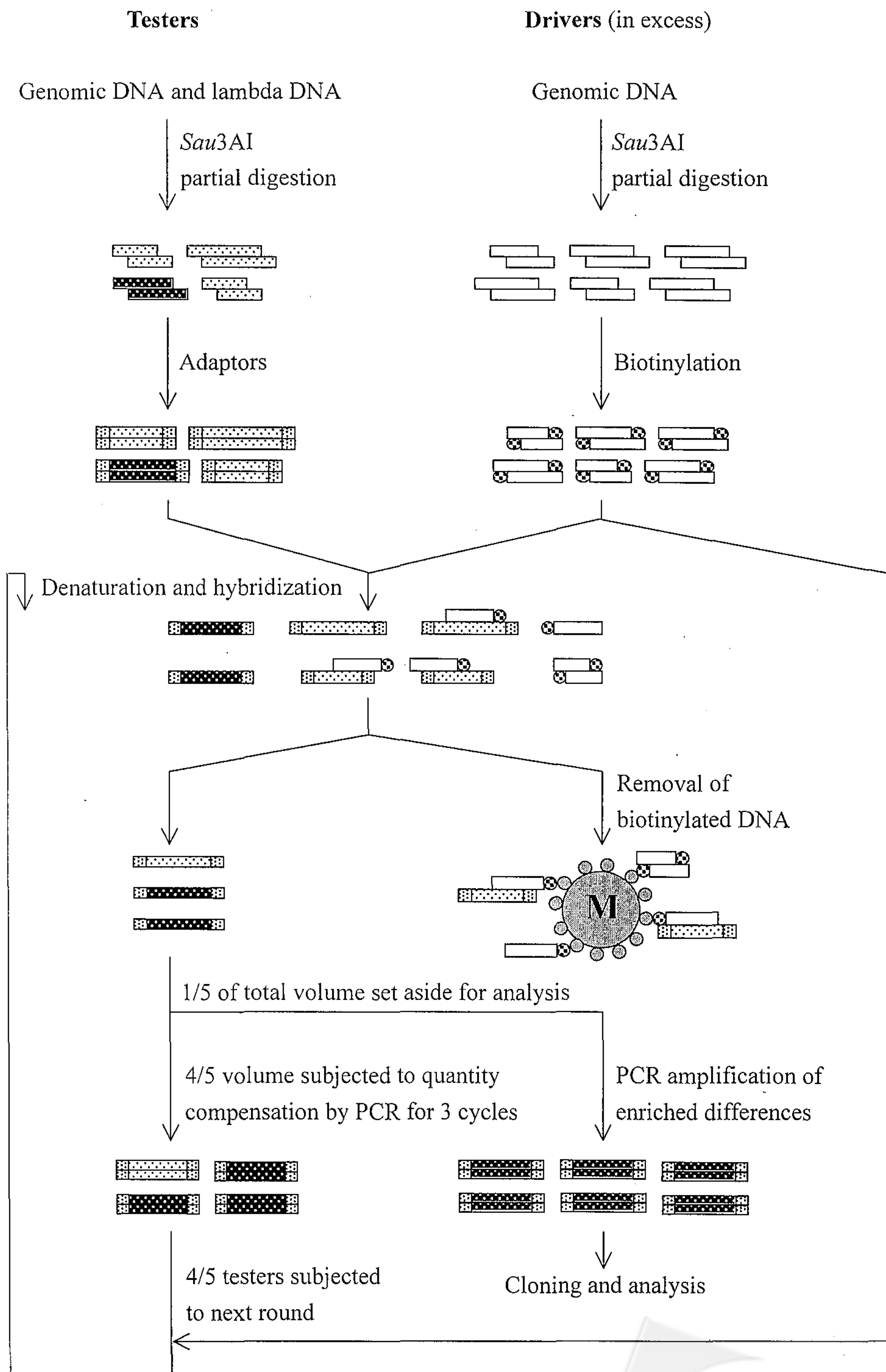

Fig. 1. Schematic of quantity compensated genomic subtraction. ▭ and ▭ represent tilapia gDNA for testers and drivers, respectively; ▬ , lamda phage DNA served as the difference fragments; □ , adaptors; ● , biotinylated dNTP. M, streptavidin-coated magnetic beads.

repeated until a satisfactory magnitude of enrichment is achieved.

MATERIALS AND METHODS

All gDNA was extracted from the blood cells of tilapia. Drivers (0.5-4.0 kb) were prepared by recovering *Sau*3AI partially digested gDNA from agarose gel. Recovered DNA was precipitated by ethanol with the addition of glycogen (Boehringer Mannheim), and then biotinylated in a 200 μl 3'-end fill-in reaction mixture consisting of 100 μg *Sau*3AI partially digested DNA, 1 × DNA polymerase buffer (Promega), 100 U Klenow fragment (Promega), 50 μM biotin-21-dUTP (Clontech), and 50 μM each of dATP, dGTP, and dCTP. The reaction was incubated at 37 ℃ for 2 hr and finally stopped at 70 ℃ for 10 min. The reaction mixture was divided into 10 equal parts, which were subsequently purified with the testers. Testers were prepared in a similar manner, except that 150 fg lambda DNA was added to the tilapia gDNA (1 μg) as the difference fragment before partial digestion with *Sau*3AI. DNA was recovered as described above. Recovered DNA was 3'-end filled-in and ligated to adapters in a 15 μl reaction mixture consisting of 1 μg blunt end DNA, 3 μg *Eco*RI (*Not*I) adapters (BRL), 1 × ligation buffer (Promega), 5% PEG8000 (Sigma) (Pfeiffer and Zimmerman, 1983; Hayashi *et al.*, 1986), 1.2 μM hexamminecobalt (III) chloride (Sigma) (Rusche and Howard, 1985), and 4.5 U T4 DNA ligase (Promega). The reaction was incubated at 16 ℃ overnight and finally stopped at 70 ℃ for 10 min. One of the driver aliquots was then added to the reaction mixture, and the free excess adapters and dNTPs were removed by Sephadex G-50 (Pharmacia) spun column (Sambrook *et al.*, 1989). The eluate was extracted once by phenol/CIA and precipitated by ethanol. The DNA pellet of the tester and driver mixture was resuspended in 15 μl hybridization buffer consisting of 0.1 M trisodium citrate, 1 M NaCl, and 5 mM EDTA (pH 7.2).

The hybridization mixture was overlaid with mineral oil (Perkin Elmer), denatured at 99 ℃ for 10 min, and then incubated at 80 ℃ for 30 min. Subsequently, the incubating temperature, which was controlled by a thermal reactor (Hybaid), was stepped down at a rate of 1 ℃ per 30 min until the temperature reached 65 ℃. The temperature was held at this point and incubation was allowed to proceed for a further 6 to 12 hr. After hybridization, the mixture was mixed with 100 μg ice-cold Streptavidin MagneSphere Paramagnetic Particles (Promega), which were pre-washed 3 times by ice-cold 0.5 × hybridization buffer, and then resuspended in a 85 μl of the same buffer. This was followed by incubation on ice for 40 min with periodic agitation to prevent magnetic particles precipitation. Still standing on ice, the biotinylated DNA hybrids were removed by magnetic separator (Sigma), and the DNA in the supernatant was precipitated by ethanol. One fifth of the subtracted testers were set aside for analysis, while the remaining 4/5 aliquot were subjected to PCR amplification to compensate for DNA losses during the above procedures.

For quantity compensation, the reaction mixture (50 μl) consisted of subtracted testers, 1 × DNA polymerase buffer, 1.5 mM $MgCl_2$ (Promega), 2 U DNA polymerase (ProZyme II, Protech), 1 μM primer (5'-AATTCGCG GCCGCGTCGAC-3'), and 200 μM of each dNTP. The PCR reaction was run 3 cycles of: 1.5 min at 94 ℃, 1.5 min at 56 ℃, and 3 min at 72 ℃. This was followed by a final extension at 72 ℃ for 15 min. The amplified testers were then pooled with another aliquot of the drivers, purified as before, and then subjected to another round of subtraction. The portion of the subtracted testers set aside for testing was also subjected to PCR under identical conditions except that (i) the temperature profile was 1.5 min at 94 ℃, 1 min at 56 ℃, and 1.5 min at 72 ℃ for 50 cycles with a final 15 min extension at 72 ℃, and (ii) 2 U DNA polymerase was added to the reaction at the beginning of the 26th cycle. After amplification, the PCR products were purified by Sephadex G-50 spun columns, phenol/CIA extraction, and ethanol precipitation with the addition

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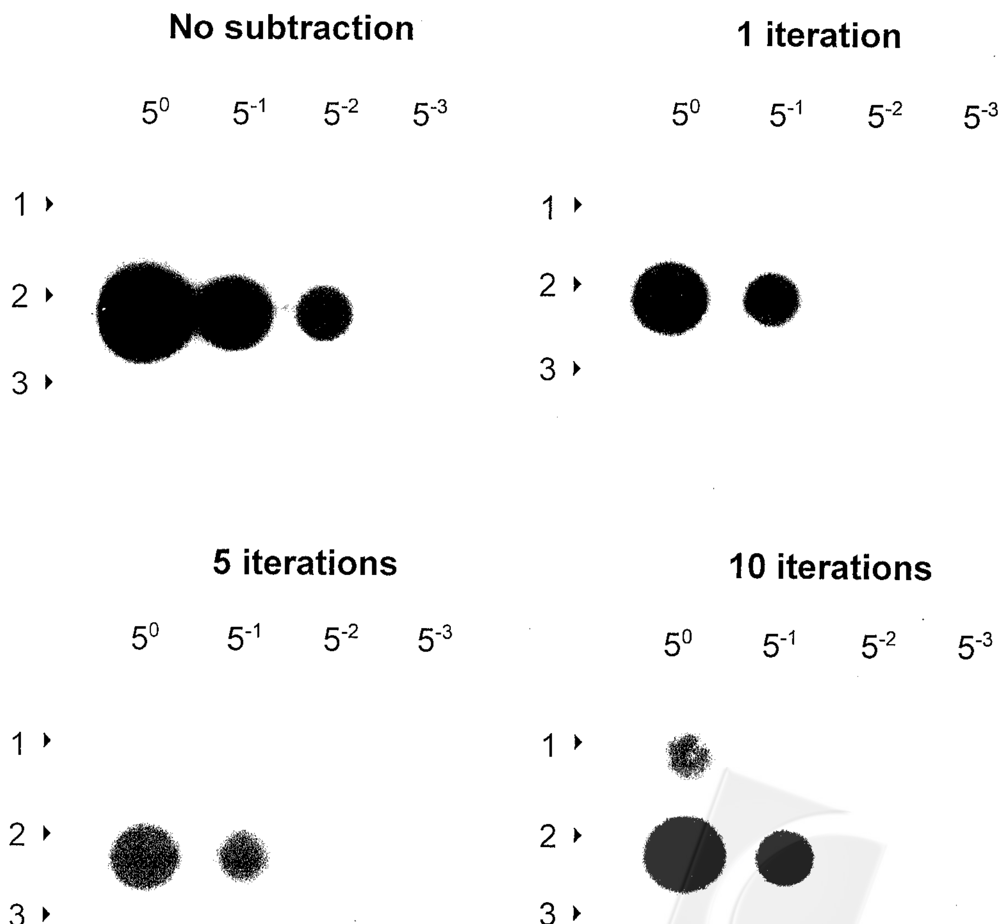
of glycogen. One fifth of the purified DNA was then subjected to second PCR following exactly the same protocol.

The second PCR products were isotope-labeled by either random priming or PCR. For random priming, 50 ng subtracted testers were labeled by the Prime-a-Gene Labeling System (Promega) according to the manufacturer's instructions. For PCR labeling, the reaction mixture (30 μl) consisted of 1/30 volume of the original second PCR products, 1 × DNA polymerase buffer, 1.5 mM $MgCl_2$, 2 U DNA polymerase, 1 μM primer, 50 μCi [α-^{32}P]dCTP (Amersham), and 50 μM of each dNTP. The temperature profile of the reaction was 1.5 min at 94 ℃ followed by 5 min at 56 ℃, and this cycle was repeated 12 times. After labeling, Southern blotting was used to assess how well the target lambda DNA fragments were represented by the subtracted products, while the magnitude of enrichment was analyzed by dot blotting (Brown, 1995). For Southern blotting, lambda DNA fragments digested by *Sau*3AI were transferred to a nylon membrane (Micron Separations Inc.) from 2.5% agarose gel. For dot blotting, denatured testing DNA was transferred to the membrane via Minifold (Schleicher &



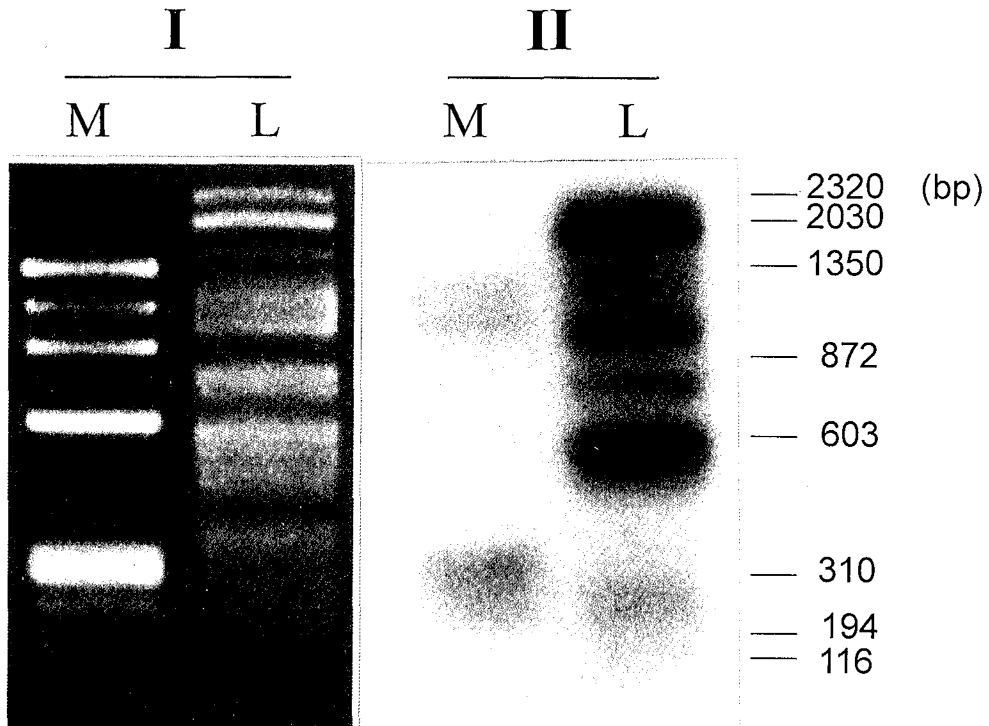


Fig. 2. Assessments of enrichment and representation of subtracted testers. (A) Dot blotting analysis was used to assess the magnitude of enrichment of lambda DNA served as the "difference fragments" in subtracted testers. Row 1: lambda DNA; row 2: tilapia DNA; and row 3: pUC19 DNA (control). The template DNA was serially diluted as shown above each lane. (B) Southern blotting analysis was used to assess how well the digested lambda DNA was represented in subtracted testers. After 10 iterations, subtracted testers were isotope-labeled and used as probes in Southern blotting (II) of the corresponding 2.5% agarose gel (I). M: ϕX174/*Hea*III marker. L: lambda DNA/*Sau*3AI.

Schuell, Inc.). In both cases, the membranes were probed with isotope-labeled subtracted testers in the presence of the denatured calf thymus DNA (Sigma). Non-specific DNA binding was eliminated from the membrane by sequentially washing with 4 × to 0.1 × SSC with 0.1% (w/v) SDS.

RESULTS AND DISCCUSSION

The enrichment and sensitivity of quantity compensation genomic subtraction

Ten rounds of subtraction were performed. After the 5th round, the magnitude of enrichment had reached about 10^5 (from 1.5×10^{-7} to 10^{-2}). Target enrichment was only slightly improved after another 5 rounds (Fig. 2A). Although the approach we describe here is thus slightly less sensitive than RDA (10^5-10^6 enrichment) (Lisitsyn *et al.*, 1993), it is still 100 to 1000 times more sensitive than traditional genomic subtraction methods. Furthermore, a magnitude of enrichment of 10^5 after 5 rounds of subtraction suggests that it should be possible to use this method to isolate a one-copy gene (less than 1 kb) from a highly complex genome.

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Representation of subtracted products

When isotope-labeled subtracted testers from the 10th round were hybridized to lambda DNA, the subtracted products were found to be highly representative, covering nearly all sizes of *Sau*3AI-digested lambda DNA fragments (ranging from 150 bp to 2.3 kb) but with different frequencies (Fig. 2B). This compares very favorably with RDA, in which the subtracted products represent only a small proportion (about 1/100) of the target lambda DNA and are also mostly confined to the smaller fragments (less than 700 bp) (Lisitsyn *et al.*, 1993). Furthermore, our procedures are much simpler.

Conclusions

The approach described here may be particularly useful in cloning small difference fragments between two complex genomes. This highly sensitive technique should also be useful for enriching rare differentially expressed cDNA, unique one copy genes or deletions in a complex eukaryotic genome, and even virus DNA, which often can be found in only a few cells in an infected cell population. The procedures are simple, the subtractive products are highly representative, and the multiple rounds of subtraction ensure that the sensitivity is high.

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複雜染色體的差減雜交：一個高靈敏度且簡易的新方法

廖希文[1]．蔡懷楨[2]

分離複雜染色體中少量的特有DNA片段，在分子生物的研究十分重要，然而，目前的方法不是靈敏度過低，無法分離出微量的特有DNA片段，不然就是步驟繁複，而且分離出的特有DNA片段並不具有高度代表性，所以，我們發展出一種更新、更有效的方法，具有高靈敏度，而且操作步驟簡易。在本研究中，以加在吳郭魚染色體中(雙套染色體大於10^9 bp)極微量的目標lambda DNA (總量僅占全部的1.5×10^{-7})爲模式進行研究，結果顯示只需極少量(1 μg)的目標群DNA (tester DNA)就可以進行十數次以上的差減雜交，使得原本存在於兩個染色體組中的相異DNA片段，所占總比例的增強倍數高達1.5×10^6倍，和已知的染色體差減雜交法相比，不僅具有最高的靈敏度，分離出的目標DNA也具有高度的代表性，而且操作步驟非常簡單，我們稱它爲總量補償染色體的差減雜交法(quantity compensation genomic subtraction)。

關鍵詞：染色體差減雜交，噬菌體DNA，吳郭魚。

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