Journal of Biochemistry and Molecular Biology, Vol. 34, No. 1, January 2001, pp. 59-65

JOURNAL OF
Biochemistry and Molecular Biology

A Simple and Efficient Subtractive Cloning Method

Hyun-Jin Min, Sang-Soo Park and Tae-Ju Cho*
Division of Life Sciences and Research Institute of Genetic Engineering,
Chungbuk National University, Cheongju 361-763, Korea

Received 30 October 2000, Accepted 4 December 2000

In subtractive hybridization, target sequences in the tester are enriched by hybridizing with an excess amount of driver, followed by removing the tester hybridized with the driver. All of existing subtractive cloning methods are designed to remove the tester/driver hybrid. The removal of hybrid, however, is often unsatisfactory for various reasons. In this study we developed a subtractive enrichment protocol in which the tester/driver can be completely removed by selecting only the tester/tester after hybridization. In this protocol both the tester and driver DNAs are ligated with same linker DNAs and amplified by polymerase chain reaction (PCR). The tester DNA is then digested with two different enzymes and used in subsequent hybridization with an excess driver. After hybridization, the DNA is ligated with the adaptor that is only compatible with the tester/tester. Since only the tester/tester can have the new adaptor, no tester/driver can be amplified by PCR in this protocol. Unlike other methods, a 100% subtraction efficiency can be achieved even though the enzymatic treatments used in the enrichment procedure are incomplete. Furthermore, only the hybridized tester DNA can have the new adaptor and be amplified by PCR, resulting in 100% denaturation in effect. The efficacy of this novel method was verified with the model system in which a known amount of the target sequence is included.

Keywords: Adaptor ligation, Subtractive cloning.

Introduction

Subtractive hybridization was successfully applied to clone mRNA sequences that are more abundant in one mRNA population than in another, or to clone a DNA that is deleted in a mutant genome (Sagerstroem *et al.*, 1997). In subtractive hybridization, the target sequence in one nucleic acid population ("tester") is enriched by hybridizing with a excess amount of another population ("driver") containing either less or no target sequence, followed by removing the common sequences from the tester. Removal of the common sequence is achieved by various strategies. The commonly used protocol employs a biotinylated driver DNA. In this protocol, the tester/driver hybrids formed during hybridization are removed using streptavidin or avidin (Sive and St. John, 1988; Barr and Emmanuel, 1990). The hybrids are also removed by various other means, including hydroxyapatite chromatography (Wieland *et al.*, 1993; Barila *et al.*, 1994) and chemical cross-linking (Hampson *et al.*, 1992). In some cDNA subtractions, cDNA covalently linked to a latex particle is used as a driver, and the hybrid is removed by simple centrifugation (Hara *et al.*, 1993).

All of the enrichment strategies are designed to remove the tester hybridized with a driver. However, the removal is rarely complete. Barr and Emanuel (1990) reported that, when samples of ^{3}H-labeled DNA were biotinylated by two cycles of photobiotinylation, approximately 1% of the ^{3}H label was not removed by even three cycles of a streptavidin binding and organic extraction. This might indicate that 1% of the DNA was unbiotinylated. Or, the result might reflect an incomplete separation of biotinylated DNA. Whatever the cause is, the incomplete removal of the tester/driver would result in the lowering of the efficiency of target enrichment. Also, in other protocols employing different physical separation methods, a complete separation of the tester/driver hybrid is seldom achieved.

Protocols of subtractive enrichment without using physical separation methods have also been developed. One such a method is the EDS method developed by Zeng *et al.* (1994). They incorporated phosphorothioate nucleotides into the 3' end of tester DNA. After hybridization, the DNA sample is treated with ExoIII and ExoVII exonucleases. These enzymatic treatments degrade the driver, hybrid tester and unhybridized tester DNA. In contrast, the tester/tester DNA is protected due to the thionucleotide incorporation. Yang and Sytkowski (1996) developed a different method employing enzyme treatment. They prepared the tester and driver DNAs by PCR amplification after ligating the same linkers. In this

*To whom correspondence should be addressed.
Tel: 82-043-261-2309; Fax: 82-043-267-2306
E-mail: tjcho@cbucc.chungbuk.ac.kr

protocol, the driver DNA was cut with restriction endonuclease to remove the linker, and then used in hybridization. After hybridization, the linker of the tester DNA in the tester/driver hybrid was removed by mungbean nuclease treatment, making the tester/driver hybrid unamplifiable. Lin and Ying (1999) devised yet another protocol that uses uracil-containing DNA as a driver. In this case, the tester/driver hybrid is removed by digestion with uracil-DNA glycosylase and single-strand-specific nuclease.

In the subtractive enrichment protocols, where enzymatic treatments are employed to remove the tester/driver hybrid, the efficiency of the enzymatic treatments is a key factor to the success of target enrichment. Lisitsyn *et al.* (1993) devised a novel procedure to remove the tester/driver hybrid without enzymatic treatments. In this strategy, they ligated the tester DNA to a dephosphorylated adaptor to make only one strand of the adaptor covalently linked to the tester. After hybridization, the DNA was subjected to fill-in reaction. By this procedure, only the self-annealed tester can have the adaptor sequence at both ends and be exponentially amplified by subsequent PCR. This leads to the efficient removal of the tester that is hybridized with the driver.

In this study, we developed a subtractive enrichment protocol that removes the tester/driver hybrid by selecting only the tester/tester after hybridization. This is done by selective adaptor ligation of the tester/tester DNA. The method developed in this study, therefore, is like the method of Lisitsyn *et al.* (1993) in that it does not rely on enzymatic treatments to remove the tester/driver hybrid. Yet, our method is simpler and more efficient than the method developed by Lisitsyn *et al.* (1993).

Materials and Methods

DNA samples The λ phages used in this study were derived from a soybean cDNA library constructed with λgt11 as a cloning vector. Hundreds of phages were plated in the presence of X-gal, and then white and blue plaques were isolated with toothpicks. The DNA prepared from a phage producing a blue plaque was used as a driver. The DNA prepared from a phage yielding a white plaque was used as a tester. The insert in the recombinant phage DNA was examined by PCR amplification using the flanking sequence of the insert as a primer. The recombinant phage DNA has an insert of about 1.3 kb, which produced 150, 200, 400, and 500 bp fragments when digested with *RsaI*. The *E. coli* genomic DNA was prepared from XL1-blue. The target DNA used with the *E. coli* DNA was the pGSS8 plasmid clone containing a rbcS cDNA insert (Cho *et al.*, 1992). The cDNA was synthesized with a cDNA synthesis kit from Promega using mRNAs from leaves of Chinese cabbage (*Brassica campestris*). In the cDNA synthesis, a set of oligo(dT) anchor primers were used in order to make cDNA synthesis start where the poly(A) tail begins. The oligo(dT) primers used were as follows: 5'CGGGGTAC$(T)_{15}$A 3' (RsaTA), 5'CGGGGTAC$(T)_{15}$C 3' (RsaTC) and 5'CGGGGTAC$(T)_{15}$G 3' (RsaTG). The target DNA that was used with the model cDNA was λgt11 DNA.

Preparation of the tester and driver DNA Genomic DNA, or cDNA to be used in subtractive hybridization, was digested with *RsaI*, and purified using a Qiagen tip-20 mini column. The *RsaI*-digested DNA was then ligated with an Am linker (Table 1), and 1 μl of the ligation mixture was directly used for amplification by PCR. 45 μl of the PCR mixture contains as follows: 1 μl of ligation mixture, 5 μl of 10X PCR buffer containing 15 mM $MgCl_2$, 5 μl of dNTP mixture, 0.5 μl of *Taq* DNA polymerase (Bioneer), and enough sterile distilled water to make a total of 45 μl. The condition of thermal cycling for PCR was as follows: 72°C for 15 min, 94°C for 3 min, and 25-30 cycles of 94°C for 1 min, 55°C for 10 sec, and 72°C for 2 min, and finally 72°C for 10 min. After the initial 10 min incubation at 72°C, 5 μl of primer (10 pmol/μl) was added, and mixed thoroughly by pipetting several times. The PCR product was purified using a Qiagen tip-20 column. In the case of the DNA to be used as a driver, the purified DNA was used in subsequent hybridization without further treatment. In the case of the DNA to be used for the tester, the DNA sample was divided into two aliquots. One aliquot was digested with *RsaI*, and the other was digested with *KpnI*. The two aliquots were then combined and purified again using a Qiagen tip-20 column.

Subtractive hybridization The tester DNA was mixed with the excess driver DNA, denatured, and hybridized. The hybridization mixture was as follows: 5.5 μl of driver DNA (2-5 μg), 1 μl of tester DNA (20-100 ng), 1 μl of a 100 mM sodium phosphate buffer (pH 6.8) containing 10 mM EDTA. After adding two to three drops of mineral oil, the mixture was denatured for 10 min in boiling water. 2.5 μl of the solution containing 4 M NaCl, 100 mM sodium phosphate (pH 6.8), 10 mM EDTA was then added, and mixed thoroughly by pipetting. The mixture was treated again at 99°C for 3 min, and then incubated at 70°C for 2-15 hr in either a heating block or thermocycler. After hybridization, the mixture was purified using a Qiagen spin column (silica membrane type). The DNA was eluted from the column with 35 μl of 10 mM Tris, pH 8.5, and 5 μl of the purified DNA was used for adaptor ligation. In the ligation reaction after the first round of subtractive hybridization, the reaction mixture contains as follows: 5 μl of DNA, 1.3 μl of 10X ligation buffer, 1 μl of K1AC adaptor, 1 μl of K1GT adaptor, 5.5 μl of sterile distilled water and 1 μl of T4 DNA ligase. After the second and third round of subtractive hybridization, K2AC/K2GT and K3AC/K3GT adaptors, respectively, were used. The sequences of the adaptors are shown in Table 1. After incubation at room temperature for 3 hr or overnight, 2 μl of the ligation mixture was used directly for PCR amplification using an appropriate primer, for instance a MA18 after the first round of subtractive hybridization. The condition of PCR amplification was the same as described above. When two or more rounds of subtractive hybridization were performed, the amplified DNA was purified using a Qiagen tip-20 column, and then used as a new tester after adjusting the DNA concentration to the initial condition.

Southern blot hybridization The tester, driver, target DNA and the DNAs obtained after subtractive hybridization (PCR products) were electrophoresed on 1.5% agarose gel, transferred to a Hybond N^+ nylon membrane (Amersham), and probed with the

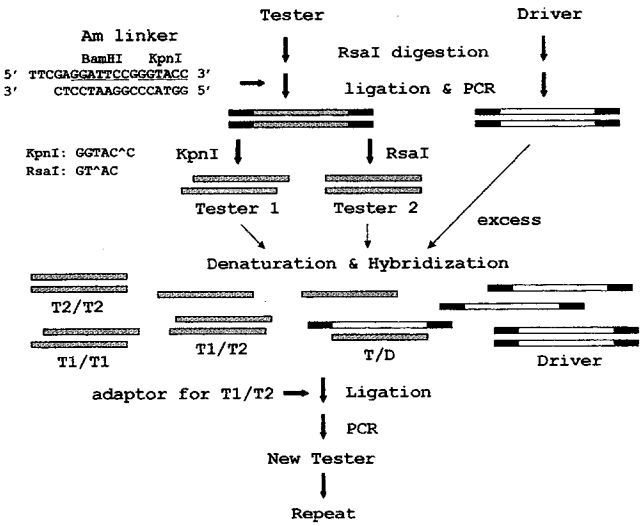

Fig. 1. Subtractive enrichment strategy employing selective adaptor ligation after hybridization. Tester and driver DNAs are digested with *RsaI*, ligated with Am linker, and amplified by PCR. The tester DNAs digested with either *RsaI* or *KpnI* are mixed, and then hybridized with an excess driver. After hybridization and selective adaptor ligation, only the hybridized tester/tester DNA can be amplified by PCR.

target DNA conjugated with thermostable alkaline phosphatase. DNA labeling, hybridization and detection were performed using AlkaPhos Direct kit from Amersham.

Results

In this study, we developed a highly efficient, but simple subtraction method. The experimental procedure of this method is depicted in Fig. 1. The primers and adaptors used in this study are shown in Table 1. In this method, the tester DNAs that are digested with two different restriction enzymes are hybridized with the excess driver, ligated with adaptors, and amplified by polymerase chain reaction (PCR). The adaptors used after hybridization were designed to be compatible only with the hybridized tester/tester DNA. In the protocol developed by Lisitsyn *et al.* (1993), a fraction of the tester DNA in the tester/driver hybrid and single-stranded tester can pick up the missing adaptor sequence when annealed with other tester DNA molecules. This might happen during the repeated cycles of denaturation, annealing and extension of PCR. Once they have adaptor sequences at both ends, they can also be amplified exponentially by PCR, leading to a decrease in subtraction efficiency. In our protocol, the selective amplification of the tester/tester by using the adaptors that are specific for the tester/tester eliminates the possibility of amplification by other forms of hybridization products. Thus, it is possible to achieve a 100% subtraction efficiency.

Table 1. Adaptors and PCR primers

PCR primers	
	KpnI
Am18	5' TTCGAGGATCCGGGTACC 3'
Kp1 (MA18)	5' AAGCTCCTAGGCGGTACC 3'
Kp2	5' CCTGGTAGGCATGGTACC 3'
Adaptors	
Am	5' TTCGAGGATCCGGGTACC 3' 3' CTCCTAGGCCCATGG 5'
K1AC	5' AAGCTCCTAGGCGGT**AC** 3' 3' GAGGATCCGCCA 5'
K1GT	5' CGCCTAGGAGC 3' 3' **TG**GCGGATCCTCGAA 5'
K2AC	5' CCTGGTAGGCATGGT**AC** 3' 3' CCATCCGTACCA 5'
K2GT	5' CATGCCTACCAG 3' 3' **TG**GTACGGATGGTCC 5'

Although the method developed in this study also employs enzyme treatments, the subtraction efficiency is unaffected by the enzyme reaction, unlike other methods. In the protocols employing enzymatic treatment to degrade the tester/driver hybrid, the efficiency of the enzyme reaction directly influences the efficiency of subtraction, since the undigested tester DNA in the tester/driver hybrid is amplified in PCR. However, in our method, the efficiency of the enzymatic treatment does not affect the subtraction efficiency, since undigested DNA is not amplified in PCR. In our protocol, selective adaptor ligation rather than enzymatic treatments provides a means to remove the tester/driver hybrid.

Another advantage of our protocol is that the undenatured tester DNA is not amplified by PCR, resulting in 100% denaturation in effect. In most subtractive enrichment experiments, the denaturation process is not seriously considered, although it may have a profound effect on the enrichment efficiency of target sequences. If, for example, denaturation is only 90% complete, then 10% of the common sequences cannot be removed. When this happens, the subtraction efficiency would at most be 90%, even if the rest of the procedure is very efficient. Incomplete denaturation can be envisioned, since the salt concentration in most hybridization buffers is usually high. Certain DNAs may also resist denaturation due to some sequence characteristics (e.g., high GC content).

When this newly devised protocol was employed to enrich targets in model systems, it turned out that the method was indeed highly efficient and reliable. To examine the efficacy of the method, a simple model system was first tested. The model system uses λgt11 DNA without insert as a driver, and the λgt11 DNA containing about a 1.3 kb cDNA insert was used as a tester. In this model system, therefore, the insert DNA serves as a target DNA. The insert DNA produced 150, 200, 400, 500 bp fragments when digested with *RsaI*. The phage DNAs were digested with *RsaI*, ligated with Am linker,

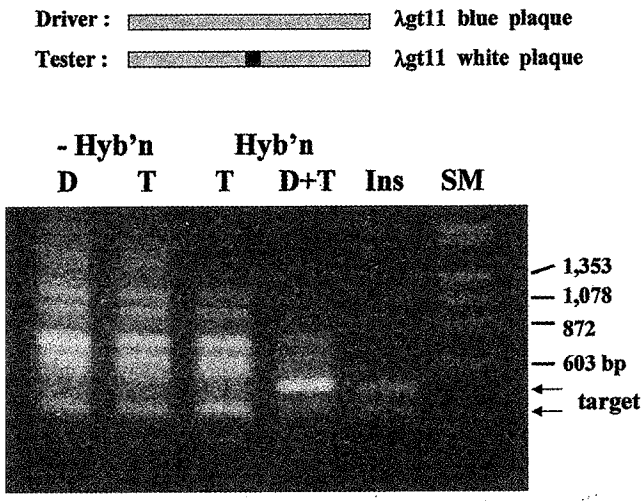

Fig. 2. Enrichment of a target in λ DNA. In this subtractive hybridization, the DNA from a white plaque was used as a tester, and the DNA from a blue plaque was used as a driver. 1 μg/ml of the tester DNA, previously digested with either *KpnI* or *RsaI*, was hybridized for two hours with 100 μg/ml of driver DNA. The hybridized DNA was then ligated with K1AC and K1GT adaptors and amplified by PCR. D, Driver; T, Tester; Ins, insert DNA that was amplified by PCR and digested with *RsaI*; SM, size marker.

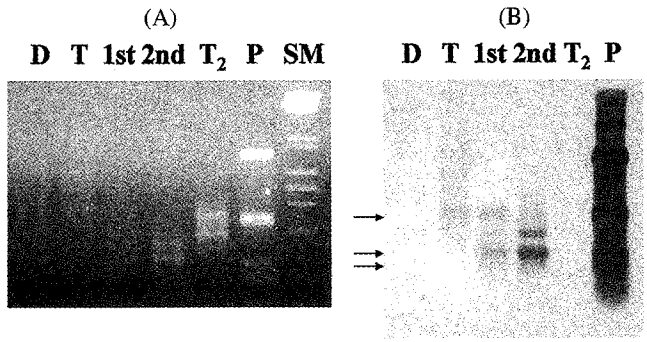

Fig. 3. Enrichment of a target in *E. coli* genomic DNA. In this model system, the pGSS8 plasmid DNA was used as a target. 5 μg/ml of the *E. coli* genomic DNA containing 0.1% pGSS8 DNA was hybridized for five hours with 500 μg/ml of the *E. coli* genomic DNA containing no pGSS8 DNA. The hybridized DNA was then ligated with AC and GT adaptors and amplified by PCR, as described in Materials and Methods. The PCR-amplified DNA was then used as a new tester in the next round of subtractive hybridization. The DNAs obtained in this experiment were size-fractionated by a 1.5% agarose gel electrophoresis (A), and analyzed by Southern blot hybridization (B). Arrows indicate target DNA fragments. D, Driver; T, Tester; 1st, DNA after the first round of subtractive hybridization; 2nd, DNA after the second round; T_2, DNA after the second round of self-hybridization; P, plasmid target DNA digested with *RsaI*; SM, size marker.

and amplified by PCR. The tester DNA was further treated with either *RsaI* or *KpnI*, and hybridized with the excess driver as described in Materials and Methods. The result of the subtractive hybridization using this model system is shown in Fig. 2. The strong bands that are present in the tester disappeared after subtractive hybridization. Instead, DNA bands corresponding to 400 and 500 bp fragments of the target DNA appeared. This indicates that the target DNA was successfully enriched.

In the next experiment, the *E. coli* genomic DNA containing a plasmid target DNA at a single copy level was used as a tester. The plasmid used was a pGSS8 clone that contains a rbcS cDNA insert (Cho *et al.*, 1992). The plasmid produces 150, 270, 350, 750, and 1800 bp fragments when digested with the *RsaI*. *E. coli* DNA without the plasmid DNA was used as a driver. The result of subtractive hybridization is shown in Fig. 3. As shown in Fig. 3A, both the tester and driver DNAs were seen as a smear before the subtractive hybridization. After subtractive hybridization, however, several bands showed up. When the tester DNA sample was subjected to the same procedure without the added driver DNA, several bands were also observed. However, the banding pattern was different. To examine whether or not the DNA bands appeared after the subtractive hybridization corresponded to the target sequences, Southern blot hybridization was performed using the target DNA as a probe. The result showed that the DNA sample obtained after two rounds of subtractive hybridization contained a considerable amount of the target sequences (Fig. 3B)). Among the targets, 270, 350, and 750 bp fragments were clearly seen. However, the 1.8 kb target DNA was not observed. The absence of the 1.8 kb target in the enriched DNA could be explained by the fact that the PCR process was inefficient in amplifying >1 kb DNA. The 170 bp target DNA was also not present in the enriched DNA sample. This might be due to removal of the small DNA during purification steps using Qiagen columns. In the case of the tester DNA sample subjected to the same procedure, the target sequence was not enriched.

Then, the method was applied to cDNA subtraction. The cDNA sample used in this experiment was derived from leaf mRNA of Chinese cabbage (*Brassica campestris*). The target used was λgt11 DNA. Data based on reassociation kinetics have shown that there are typically three abundance classes (slow, intermediate and fast reassociating components) in eukaryote mRNA (Lewin, 1975; Goldberg *et al.*, 1978). Also, the complexity of the mRNA in various tissues of higher organisms is 20,000~40,000 kb (Lewin, 1980). We assumed that the low abundance class comprised 50% of the total leaf mRNA, and that the complexity of the class was 25,000 kb. We assumed also that the intermediate and fast components comprised 25% and 25% of the total cDNA, respectively, with the sequence complexity of 500 kb and 25 kb, respectively. The total complexity would then be 25,525 kb. In another words, there are 25,525 different mRNA species, assuming that the average size of mRNA is 1 kb. Under these circumstances, the mRNA species in the intermediate and fast components are 25 times and 500 times more abundant respectively, compared to the slow reassociating component.

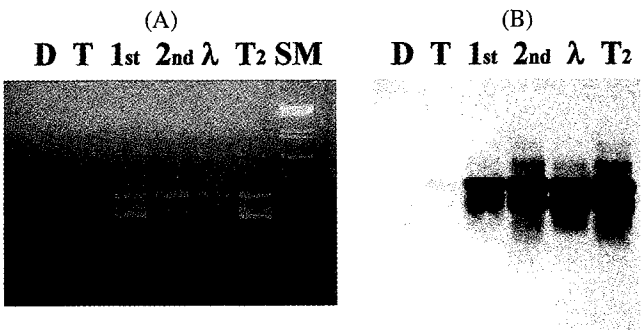

Fig. 4. Enrichment of a target in Brassica leaf cDNA. In this model system, λ DNA was used as a target. 10 μg/ml of the *Brassica* leaf cDNA containing 1.0% λ DNA was hybridized for 15 hours with 500 μg/ml of the cDNA containing 0.01% DNA. The hybridized DNA was then ligated with AC and GT adaptors and amplified by PCR, as described in Figure 3. The driver, tester and PCR products obtained from this experiment were size-fractionated by 1.2% agarose gel electrophoresis (A), and analyzed by Southern blot hybridization using λ DNA as a probe (B). D, Driver; T, Tester; 1st, DNA after the first round of subtractive hybridization; 2nd, DNA after the second round; T_2, DNA after two rounds of self-hybridization; λ, target; SM, size marker.

We used as a driver the cDNA sample containing the target at 0.01% of total DNA. At this level of target DNA, the abundance of the target in a driver roughly equals to 1/10 of that of the low abundance class. As a tester, we used the cDNA sample containing a 1% target DNA. In this model system, therefore, the target represents the gene whose expression is increased by 100-fold in the tester.

The result of the subtractive hybridization using this model system is represented in Fig. 4. As shown in Fig. 4A, some sequences were enriched as distinct bands even after just one round of subtractive hybridization. To examine if the DNA bands present in the enriched DNA sample corresponded to the target DNA fragments, Southern blot hybridization was carried out using the target DNA as a probe. The result shows that the bands of the enriched DNA sample indeed represent the target sequence (Fig. 4B)). In this experiment, the DNA sample containing only a tester was also subjected to the same procedure. Unlike the result obtained with the model system using *E. coli* DNA, the target DNA was also enriched by self-hybridization. This result reflects the nature of this subtractive enrichment method. In the subtractive hybridization employing the strategy selecting the tester/tester DNA after hybridization, kinetic enrichment also occurs. Under the circumstances, the DNA sequence of high abundance can be easily self-annealed to form a tester/tester. Once target sequences are enriched, the sequence would have a better chance to form a tester/tester in subsequent rounds. That is, target DNA can be enriched without subtraction with a driver. This result also implies that the abundance of most cDNA sequences in the tester is lower than that of the target DNA.

Discussion

The target enrichment with this novel method turned out to be very efficient, based on the comparison between the experimental results and the predicted output of the simulation. The simulation was performed as described by Cho and Park (1998) with the assumption that the subtraction efficiency was 99%. In the case of the model experiment using λ DNA, simulation shows that the target is enriched to 58% of total DNA after one round of subtractive hybridization. Fig. 2 shows that the level of target enrichment is comparable to that of the prediction. Simulation also shows that the single copy target in the *E. coli* genome is enriched to 1.4% and 38%, respectively, after the first and second round of subtractive hybridization. The experimental result also shows that the target DNA fragments were enriched as major DNA bands after two rounds of subtractive hybridization (Fig. 3). In the case of the simulation of the cDNA subtraction, the result of simulation shows that the target is enriched to 15% and 86%, respectively, after the first and second round of subtractive hybridization. The experimental result shows that the target showed up as major bands, even after the first round of subtractive hybridization (Fig. 4). Overall, the result of simulation indicates that the enrichment levels obtained in the model subtractive enrichment experiments are achievable only if the efficiency of subtractive enrichment is 99% or more.

The method we developed does not use biotinylated DNA or phosphorothioate nucleotide. It does not employ streptavidin/avidin, or various separation columns such as a hydroxyapatite column. Thus, it is simple. Nevertheless, the method is highly efficient. The high subtraction efficiency can be ascribed to the feature of the method that the tester/driver hybrid cannot be amplified in the PCR done after hybridization. Thus, virtually a 100% subtraction can be achieved. The strategy selecting only the tester/tester after hybridization also accounts for the high enrichment efficiency. In the case that the driver DNA is biotin-labeled, and the hybrid tester is removed using streptavidin or avidin, then both the single-stranded tester (ssT) and tester/tester (TT) are selected. By contrast, the protocol developed by Lisitsyn *et al.* (1993), and the protocols employing enzyme treatments (Zeng *et al.*, 1994; Yang and Sytkowski, 1996; Lin and Ying, 1999), select only the tester/tester after hybridization. As mentioned earlier, the strategy selecting ssT plus TT is subtractive in nature, whereas the strategy selecting only TT has a kinetic enrichment component as well. Since the kinetic component strongly favors the target DNA, the TT selection strategy is more efficient than the ssT+TT selection strategy. Computer simulation also showed that the enrichment strategy employing the TT selection is more efficient than the strategy employing the ssT+TT selection (Milner *et al.*, 1995; Cho and Park, 1998). The kinetic component can be enhanced by incorporating the phenol-emulsion reassociation technique (Kohne *et al.*, 1977; Travis and Sutcliffe, 1988), or by

including dextran sulfate in the hybridization solution (Barr and Emmanuel, 1990).

Another key feature of our method is that same linkers are ligated to both the tester and driver. In the protocols where the DNA sample after hybridization is amplified PCR, only the tester DNA should be amplified. To this end, the linker ligated to driver DNA is usually different than that of the tester. The addition of different linkers, however, may result in a differential amplification of the same sequence due to the different primers used in PCR. If the driver has the same linker as the tester, the linker in the driver should be removed before using in hybridization. Should the removal of the linker from the driver be incomplete, then the driver DNA behaves as a tester. This reduces the proportion of target in the tester and driver:tester ratio. The impact can be great, considering that the excess driver is usually used in subtractive hybridization. In the protocol developed in this study, by contrast, the linker DNA of the tester rather than the driver is removed. In this case, the tester retaining the linker behaves as a driver. However, the impact is negligible since again an excess driver is usually used in hybridization.

Generally, in PCR amplification, small DNAs are preferentially amplified. This biased amplification could also be observed in our hands. Although only a fragment of the target gene is good enough to identify and clone, it is probable that some target genes escape the subtractive enrichment procedure. Considering this possibility, it might be wise to perform another subtractive hybridization using the DNA digested with the restriction enzyme that is different from *RsaI*. One choice could be *AluI* as it also produces a blunt-ended DNA. In this case, the linker DNA having a *HindIII* site can then be used. Since the *HindIII* site is cleaved by *AluI*, the DNA digested with *AluI*, and ligated with a linker containing the *HindIII* site, can be similarly used as in this study.

The differential display method developed by Liang and Pardee (1992) has also been used to clone differentially expressed genes (Park *et al.*, 2000a, b). The technique is a simple and powerful method to detect both up-regulated and down-regulated gene expression in the same experiment. However, the method is not sensitive enough to detect rarely-expressed genes. For the same reason, the method is seldom used in cloning of a single copy target in a large genome. Hakvoort *et al.* (1994) reported that low-abundance genes were successfully detected by combining the two procedures; the target sequences were enriched by subtractive hybridization prior to differential display. Pardinas *et al.* (1998) included the subtractive hybridization step for a different purpose. One difficulty in the differential display is that numerous false clones are found after cloning the sequence showing the differential expression. They reported that contaminating DNA species could be successfully removed by subtractive hybridization.

Acknowledgments This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF 981-0502-012-2).

References

Barila, D., Murgia, C., Nobili, F., Gaetani, S. and Perozzi, G. (1994) Subtractive hybridization cloning of novel genes differentially expressed during intestinal development. *Eur. J. Biochem.* **223**, 701-709.

Barr, F. G. and Emmanuel, B. S. (1990) Application of a subtractive hybridization technique involving photoactivatable biotin and organic extraction to solution hybridization analysis of genomic DNA. *Anal. Biochem.* **186**, 369-373.

Cho, T.-J., Chung, K.-A., Lee, K.-J., Chung, U.-S., Cho, N.-J. and Chae, Q. (1992) Molecular cloning of a cDNA encoding a ribulose-1,5-bisphosphate carboxylase small subunit in soybean. *Kor. J. Biochem* (presently *J. Biochem. Mol. Biol.*) **25**, 658-663.

Cho, T. J. and Park, S. S. (1998) A simulation of subtractive hybridization. *Nucl. Acids Res.* **26**, 1440-1448.

Goldberg, R. B., Hoschek, G. and Kamalay, J. C. (1978) Sequence complexity of nuclear and polysomal RNA in leaves of the tobacco plant. *Cell* **14**, 123-131.

Hakvoort, T. B. M., Leegwater, A. C. J., Michiels, F. A. M. and Chamuleau, A. F. M. (1994) Identification of enriched sequences from a cDNA subtraction-hybridization procedure. *Nucl. Acids Res.* **22**, 878-879.

Hampson, I. N., Pope, L., Cowling, G. J. and Dexter, T. M. (1992) Chemical cross linking subtraction (CCLS): a new method for the generation of subtractive hybridization probes. *Nucl. Acids Res.* **20**, 2899.

Hara, E., Yamaguchi, T., Tahara, H., Tsuyama, N., Tsurui, H., Ide, T. and Oda, K. (1993) DNA-DNA subtractive cDNA cloning using oligo(dT)30-latex and PCR: Identification of cellular genes which are overexpressed in senescent human diploid fibroblasts. *Anal. Biochem.* **214**, 58-64.

Kohne, D. E., Levison, S. A. and Byers, M. J. (1977) Room temperature method for increasing the rate of DNA reassociation by many thousandfold: the phenol emulsion reassociation technique. *Biochemistry* **16**, 5329-5341.

Lewin, B. (1975) Units of transcription and translation: sequence components of heterogeneous nuclear RNA and messenger RNA. *Cell* **4**, 77-93.

Lewin, B. (1980) Complexity of mRNA populations; in *Gene Expression 2*, 2nd ed., pp. 694-727, Wiley-Interscience Publications, John Wiley & Sons, New York.

Liang, P. and Pardee, A. B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-71.

Lin, S. and Ying, S.-Y. (1999) Differentially expressed genes in activin-induced apoptotic LNCaP cells. *Biochem. Biophys. Res. Commun.* **257**, 187-192.

Lisitsyn, N., Lisitsyn, N. and Wigler, M. (1993) Cloning the differences between two complex genomes. *Science* **259**, 946-951.

Milner, J. L., Cecchini, E. and Dominy, P. J. (1995) A kinetic model for subtractive hybridization. *Nucl. Acids Res.* **23**, 176-187.

Pardinas, J. R., Combates, N. J., Prouty, S. M., Stenn, K. S. and Parimoo, S. (1998) Differential subtraction display: A unified approach for isolation of cDNAs from differentially expressed

genes. *Anal. Biochem.* **257**, 161-168.

Park, J.-H., Lee, H.-Y., Roh, S. C., Kim, H.-Y. and Yang, Y. M. (2000a) Screening of differentially expressed genes by desferrioxamine or ferric ammonium citrate treatment in HepG2 cells. *J. Biochem. Mol. Biol.* **33**, 396-401.

Park, M.-S., Kim, H.-T., Min, B.-R., Kim, K. and Nam, M.-J. (2000b) Identification of a novel PGE2 regulated gene in SNU1 gastric cancer cell. *J. Biochem. Mol. Biol.* **33**, 184-187.

Sagerstroem, C. G., Sun, B. I. and Sive, H. L. (1997) Subtactive cloning: past, present, and future. *Annu. Rev. Biochem.* **66**, 751-783.

Sive, H. L. and St. John, T. (1988) A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction. *Nucl. Acids Res.* **16**, 10937-10943.

Travis, G. H. and Sutcliffe, J. G. (1988) Phenol emulsion-enhanced subtractive cDNA cloning: isolation of low-abundance monkey cortex-specific mRNAs. *Proc. Natl. Acad. Sci. USA* **85**, 1696-1700.

Wieland, I., Bolger, G., Asouline, G. and Wigler, M. (1993) A method for difference cloning: gene amplification following subtractive hybridization. *Proc. Natl. Acad. Sci. USA* **87**, 2720-2724.

Yang, M. and Sytkowski, A. J. (1996) Cloning differentially expressed genes by linker capture subtraction. *Anal. Biochem.* **237**, 109-114.

Zeng, J., Gorski, R. and Hamer, D. (1994) Differential cDNA cloning by enzymatic degrading subtraction (EDS). *Nucl. Acids Res.* **22**, 4381-4385.