genomic DNA as template. For example, if the ligation took place between the first and the fifth *Sau*3A1 site in Figure 1, PCR between the primers P1 and P3, and P2 and P4 would be positive. If ligation took place between the second and the sixth sites, it would be negative for P1–P3 but still positive for P2–P4.

The method we report is simple and versatile for a short genomic walk from any known sequence. One can choose how far to walk by picking the PCR products of appropriate size. The same partial digestion/ligation can be used repeatedly to walk from a known sequence in both directions by choosing appropriate PCR primers. It can also be a general method to clone flanking sequences in cases such as determination of translocation break points or integration sites.

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Refinements in Re-amplification and Cloning of DDRT-PCR Products

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Differential display reverse transcription polymerase chain reaction (DDRT-PCR) is a valuable technique for detecting and characterizing altered expression of mRNAs in eukaryotic cells (4). We have used DDRT-PCR to detect murine hepatic mRNAs differentially expressed as a consequence of *N*-nitrosodimethylamine-induced hepatotoxicity (3). However, refinements in various downstream steps of the DDRT-PCR protocol are required for optimal and convenient utilization of DDRT-PCR products. We often encountered poor cDNA re-amplification following recovery of DDRT-PCR products from acrylamide gels. Cloning DDRT-PCR products into plasmid vectors offered additional challenges. We describe preparation of thymidine (T)-overhang vectors for high-efficiency, low-background cloning and a bacterial colony PCR technique for universal screening of plasmids that contain DDRT-PCR cDNA inserts.

Traditionally, after a differentially expressed DDRT-PCR cDNA band is detected on a 7 M urea, 6% polyacrylamide gel, an approximately 5-mm^2 gel slice is excised, and the cDNA is eluted in water, alcohol-precipitated and re-amplified (4). Elimination of the precipitation step and direct re-amplification of DDRT-PCR products is possible at urea concentrations ≤0.35 M, and it avoids problems of poor cDNA recovery and possible PCR contamination. Previously, Raju et al. (6) directly re-amplified 1 µL of eluted cDNA samples using gene-specific primers in a 10-µL PCR mixture. However, when using nonspecific DDRT-PCR primers to re-amplify eluted DDRT-PCR cDNAs, we obtained inadequate and inconsistent amounts of re-amplification products (Figure 1A) using the conditions described (6). When 2 µL of DDRT-PCR product eluates were re-amplified directly in 40 µL PCR mixture (final urea concentration of 0.014 M) for 30 cycles, templates that did not re-amplify at 0.03 M urea generated sufficient product (Figure 1, B and C). We re-amplified 36 of 36 different eluted DDRT-PCR products using these conditions. Several DDRT-PCR templates re-amplified at urea concentra-

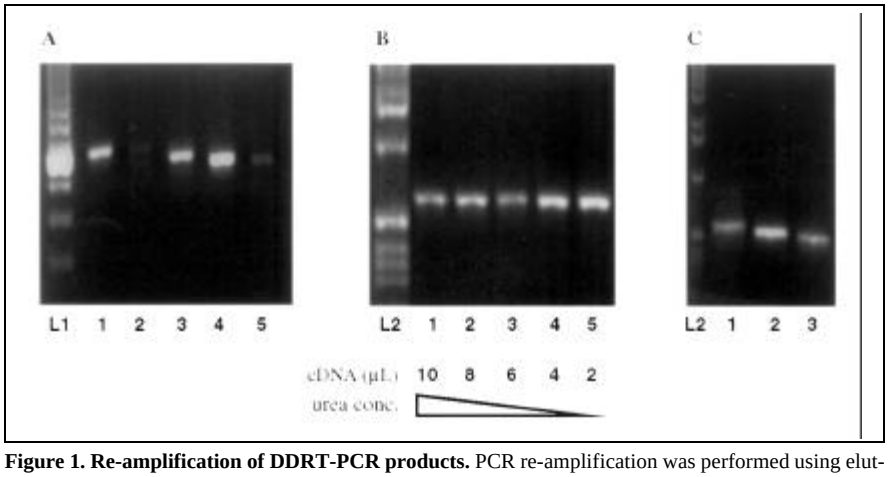

Figure 1. Re-amplification of DDRT-PCR products. PCR re-amplification was performed using eluted cDNA in 40 µL essentially as described (4), except that 200 µM dNTP were used. Re-amplified DDRT-PCR products (20 µL) were resolved on a 1.2% agarose gel and stained with ethidium bromide. L1 and L2 are 100-bp and 1-kbp DNA ladders, respectively. (A) Eluted DDRT-PCR products (10 µL) sized 599 (lane 1), 598 (lane 2), 530 (lane 3), 520 (lane 4) and 519 bp (lane 5) were re-amplified at 0.07 M urea using the HAP-12 and H-T_{11}C primers (GenHunter, Brookline, MA, USA). (B) Lanes 1–5 are direct re-amplifications of the 599-bp DDRT-PCR product (A, lane 1) at 0.07, 0.056, 0.042, 0.028 and 0.014 M, respectively. Note the increased yield of PCR product at 0.014 M urea concentration in lane 5. (C) Lanes 1–3 are direct cDNA amplification products of 598, 530 and 519 bp, respectively, obtained with 2 µL of cDNA-eluted samples at final urea concentration of 0.014 M. Note that the 598- and 519-bp DDRT-PCR products were successfully re-amplified under these conditions but were previously unsuccessful under conditions described in A (lanes 2 and 5).

tions as high as 0.07 M (Figure 1A), suggesting that re-amplification efficiencies of DDRT-PCR products are template-dependent and DDRT-PCR re-amplifications should therefore be optimized for urea.

While a variety of methods have been described for cloning PCR products, many are inefficient for DDRT-PCR products, or are inconvenient for downstream cDNA insert utilization (5). Introduction of 3′ T overhangs in cloning vectors facilitates the cloning efficiency for PCR products because a significant portion of PCR products have 3′-adenosine (A) overhangs (a result of terminal transferase activity of *Taq* DNA polymerase) and therefore readily anneal with T-overhang vectors (5). During preparation of T-overhang vectors using *Taq* DNA polymerase however, a large majority of vectors escape T addition, thus reducing cloning efficiency. An alternative TA cloning strategy has been reported in which *Eco*RV-digested vectors with T overhangs are ligated overnight with T4 DNA ligase, and subsequently separated and purified from slower-migrating, self-ligated vectors that escaped T addition (2). We now describe an efficient TA cloning protocol that is faster and simpler than other TA cloning protocols.

Our TA cloning protocol includes preparation of a blunt-ended vector that lacks 5′ phosphates, but has T overhangs. Vectors that lack 5′ phosphates and escape T addition are unable to self-ligate. In addition, concatamerization of the PCR-generated inserts is prohibited by the 3′-A overhangs, and ligation of unmodified PCR products to unphosphorylated T-overhang vectors is inefficient (1). Therefore, we used T4 polynucleotide kinase (T4PNK) to incorporate 5′ phosphates on the PCR products. This technique does not require overnight ligation during vector preparation and purification steps, increases cloning efficiency and reduces the number of background colonies. Alternatively, it is possible to incorporate T4PNK-treated primers or 5′-phosphate-modified primers into DDRT-PCR products during the re-amplification step.

Re-amplified cDNA products were prepared by adding 16 µL 5× T4PNK buffer (325 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 375 mM KCl, 1.5 mM ATP, 25 mM dithiothreitol [DTT] and 500 µg/mL bovine serum albumin [BSA]), 23 µL sterile water and 5 U of T4PNK (Promega, Madison, WI, USA) directly to the 40-µL PCR re-amplification mixture. The mixture was incubated at 37°C for 30 min, and an aliquot (40 µL) was loaded on a 1.2% agarose gel. The band of interest was purified using a QIAquick™ Column (Qiagen, Chatsworth, CA, USA) and collected in

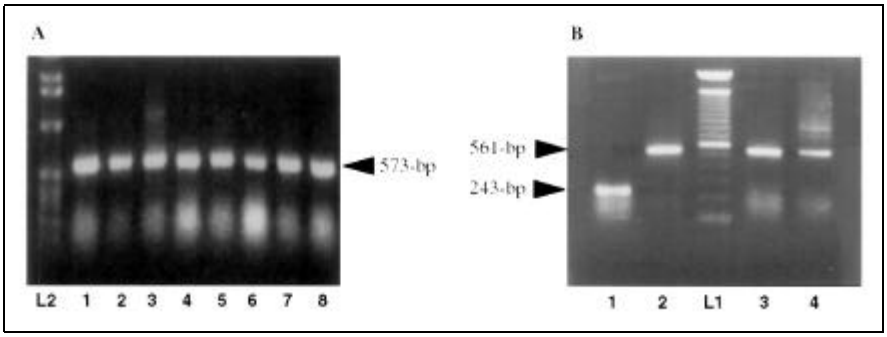

Figure 2. Cloning efficiency using CIAP-treated, T-overhang vectors. Products from DDRT-PCR re-amplifications were treated as described in the text, ligated to *Eco*RV-digested/CIAP-treated/T-overhang vectors and plated overnight. Colonies were randomly picked without blue/white selection and directly added into a 20-µL PCR mixture. PCR conditions were as described in Figure 1 except M13-40 and M13 reverse primers were used. PCR products (10 µL) were resolved on agarose gels as described in Figure 1. (A) A representative colony PCR screening gel demonstrated that all 8 clones selected from the transformation plate had the expected 330-bp DDRT-PCR product insert. Since vector primers were used, the PCR product size increased to 573 bp (243 + 330). (B) Mouse serum amyloid A-1 cDNA was generated by RT-PCR using murine-specific primers. The PCR product was treated as described for DDRT-PCR re-amplification products and ligated into CIAP/T-overhang vector. Clone 1 did not contain a cDNA insert and generated a 243-bp fragment, whereas clones 2–4 contained serum amyloid A-1 cDNA (318 bp), observed as a 561-bp (243 + 318) fragment.

30 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The gel purification step is optional, but recommended to prevent cloning of spurious PCR products that may be generated during PCR re-amplification steps.

For preparation of the T-overhang vector, 80 µg pBluescript® SK(+) plasmid (Stratagene, La Jolla, CA, USA) were digested with *Eco*RV at 37°C for 2 h, and an aliquot was checked for digestion efficiency by agarose gel electrophoresis. When restriction was complete, an aliquot (40 µg) of the *Eco*RV-digested plasmid was treated with 1 U calf intestinal alkaline phosphatase (CIAP; Boehringer Mannheim, Indianapolis, IN, USA) at 37°C for 30 min in the digestion mixture, followed by addition of another 1 U CIAP for 30 min. The *Eco*RV-digested/CIAP-treated vector was phenol-extracted and precipitated with alcohol. For creating T overhangs, the *Eco*RV-digested/CIAP-treated vector (20 µg) was incubated with 2 mM dTTP and 10 U *Taq* DNA polymerase at 72°C for 2 h in 1× PCR buffer (PE Applied Biosystems, Foster City, CA, USA). Finally, the vector was extracted with phenol and alcohol-precipitated.

To check the efficiency of the vector preparation, an aliquot (1 µL) of the *Eco*RV/CIAP/T-overhang vector can be examined for self-ligation by incubating overnight with 1 U T4 DNA ligase at 14°C, then transforming *E. coli* DH5α. If background colonies are observed (>50 colony-forming units [cfu]/µg vector), the vector can be incubated an additional 30 min with 2 mM dTTP and 1 U CIAP, followed by the addition of 10 U *Taq* DNA polymerase for 2 h. Transformation controls for two separate vector preparations confirmed that background colonies were virtually eliminated (<50 cfu/µg vector) by the method described here, making selection of blue/white colonies on 5-bromo-4-chloro-β-D-galactopyranoside (X-gal) unnecessary. An advantage to this method is that it provides the opportunity for identifying and rectifying the source of background colonies before cloning PCR products and without discarding the vector stocks. A vector prepared in this manner is stable at 4°C for at least 2 months.

A bacterial colony screening procedure using vector-specific rather than insert-derived primers was used to identify cDNA-containing clones. Following a standard overnight ligation of vector with DDRT-PCR re-amplification products, transformation into *E. coli* and plating on LB agar without X-gal, bacterial colonies were added without lysis treatment directly into a 20-µL PCR mixture that contained M13-40 and M13 reverse vector-specific primers (200 pmol). The mixture was heated to 94°C for 2 min to lyse the

bacteria, and amplification was performed for 30 cycles (at 94°C for 15 s, 50°C for 60 s and 72°C for 30 s). Using these primers, a PCR product of 243 bp (corresponding to vector sequence only) was observed on agarose gels for clones lacking an insert, whereas colonies containing DDRT-PCR inserts produced bands greater than 243 bp (Figure 2A). The presence or absence of cDNA inserts was confirmed by *Hin*dIII digestion of plasmid minipreparations (unpublished observations). Vector-specific amplification primers have the advantage of distinguishing unsuccessful insert amplifications from colonies lacking the plasmid vector. Moreover, because DDRT-PCR uses multiple primer combinations and generates heterogeneous products, vector-specific primers can be used to screen all DDRT-PCR clones for inserts. In that concatamerization of inserts is a problem with standard blunt-end ligations, vector-specific primers will detect concatamerized inserts by comparing the colony PCR product size to the cloned DDRT-PCR product size. Using our DDRT-PCR cloning method and vector-specific primers for screening, we observed that only 3 of 288 clones contained concatamerized inserts. These data demonstrate that our TA cloning technique reduces insert concatamerizations.

We prepared two separate *Eco*RV/CIAP/T-overhang vector stocks and tested their efficiency in cloning DDRT-PCR products. Based on two separate cloning experiments using 8 randomly picked colonies from each of 8 different DDRT-PCR re-amplifications, we obtained 75% and 91% insert-containing clones. Sequencing of DDRT-PCR clones revealed a single T:A base pair between the *Eco*RV site and DDRT-PCR primer sequences (data not shown), consistent with cloning according to the TA strategy. The cloning and screening technique described here was similarly used to obtain mouse serum amyloid A-1 cDNA following RT-PCR amplification (Figure 2B).

Titration of urea in DDRT-PCR eluates from acrylamide gels as reported here improves both amplification consistency and yield for heterogeneous DDRT-PCR templates. Additionally, our TA cloning and colony PCR screening protocols provide rapid, efficient and inexpensive cloning of DDRT-PCR products into any plasmid vector that can generate blunt ends. These techniques can similarly be used for cloning homogeneous RT-PCR products. These strategies greatly facilitate subsequent utilization of DDRT-PCR products, including sequencing, insert orientation or riboprobe preparation.

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