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Inverse PCR Approach to Cloning cDNA Ends

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1. Introduction

Since the first report on cDNA cloning in 1972 *(1)*, this technology has been developed into a powerful and universal tool in the isolation, characterization, and analysis of both eukaryotic and prokaryotic genes. But the conventional methods of cDNA cloning require much effort to generate a library that is packaged in phage or plasmid and then survey a large number of recombinant phages or plasmids. There are three major limitations in those methods. First, substantial amount (at least 1 μg) of purified mRNA is needed as starting material to generate libraries of sufficient diversity *(2)*. Second, the intrinsic difficulty of multiple sequential enzymatic reactions required for cDNA cloning often leads to low yields and truncated clones *(3)*. Finally, screening of a library with hybridization technique is time consuming.

Polymerase chain reaction (PCR) technology can simplify and improve cDNA cloning. Using PCR with two gene-specific primers, a piece of known sequence cDNA can be specifically and efficiently amplified and isolated from very small numbers ($<10^4$) of cells *(4)*. However, it is often difficult to isolate full-length cDNA copies of mRNA on the basis of very limited sequence information. The unknown sequence flanking a small stretch of the known sequence of DNA cannot be amplified by the conventional PCR. Recently, anchored PCR *(5–7)* and inverse PCR (IPCR) *(8–10)* have been developed to resolve this problem. Anchored PCR techniques have a common point: DNA cloning goes from a small stretch of known DNA sequence to the flanking unknown sequence region with the aid of a gene-specific primer at one end and a universal primer at other end. Because of only one gene-specific primer in the anchored PCR, it is easier to get a high level of nonspecific amplification by PCR than with two gene-specific primers *(10,11)*. The major advantage of

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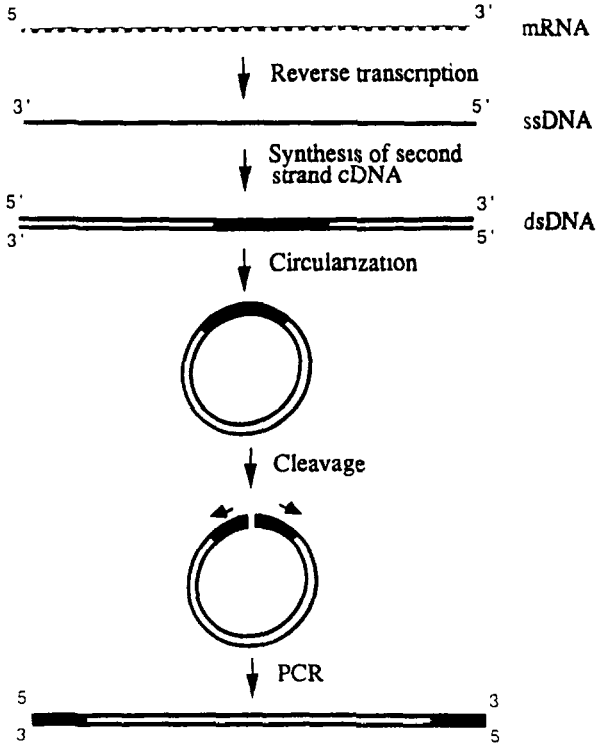

Fig. 1. Diagram of IPCR for cDNA cloning. The procedure consists of five steps: reverse transcription, synthesis of second-stranded cDNA, circularization of double-stranded cDNA, reopen the circle DNA, and amplification of reverse DNA fragment. The black and open bars represent the known and unknown sequence regions of double-stranded cDNA, respectively

IPCR is to amplify the flanking unknown sequence by using two gene-specific primers.

At first, IPCR was successfully used in the amplification of genomic DNA segments that lie outside the boundaries of known sequence *(8,9)*. There is a new procedure that extends this technique to the cloning of unknown cDNA sequence from total RNA *(10)*. Double-stranded cDNA is synthesized from RNA and ligated end to end (Fig. 1). Circularized cDNA is nicked by selected restriction enzyme or denatured by NaOH treatment *(12,13)*. The reopened or denatured circular cDNA is then amplified by two gene-specific primers. Recently, this technique has been efficiently used in cloning full-length cDNAs *(14–16)*. The following protocol was used to amplify cDNA ends for the human stress-related protein ERp72 *(10)* (Fig. 2).

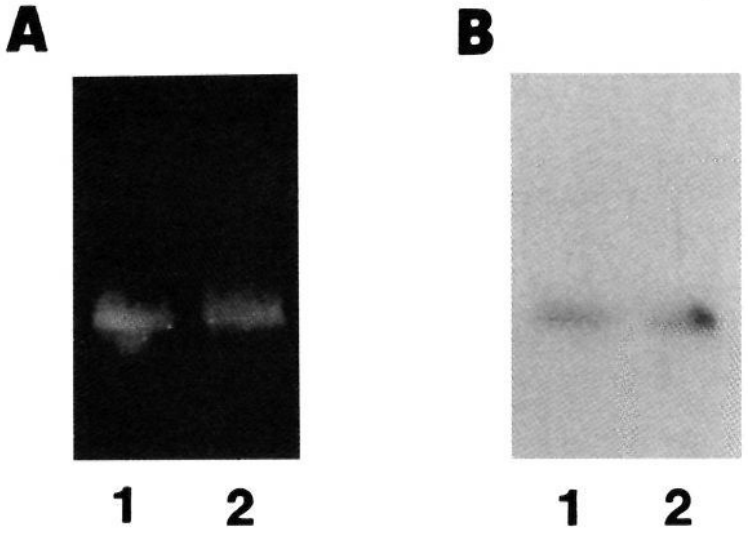

Fig. 2. Application of IPCR to amplifying the joining region (280 bp) from 5' (160 bp) and 3' (120 bp) sequences of human ERp72 cDNA. Amplified DNAs from CCRF/CEM cells sensitive (lane 1) and resistant (lane 2) to cytosine arabinoside stained by ethidium bromide **(A)** or hybridized with ^{32}P-labeled ERp72 cDNA **(B)**. *See* text for the sequences of the primers and the parameters of IPCR.

2. Materials

2.1. First-Strand cDNA Synthesis

1. Total RNA prepared from human CCRF/CEM leukemic lymphoblast cells *(17,18)*.
2. dNTP Mix (10 m*M* of each dNTP).
3. Random primers (Boehringer Mannheim, Indianapolis, IN). Prepare in sterile water at 1 μg/μL. Store at –20°C.
4. RNasin (Promega, Madison, WI).
5. Actinomycin D (1 mg/mL): Actinomycin D is light sensitive and toxic. It should be stored in a foil-wrapped tube at –20°C.
6. Moloney Murine Leukemia Virus (MMLV) reverse transcriptase.
7. 5X First-strand buffer: 250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 50 m*M* $MgCl_2$, 50 m*M* dithiothreitol (DTT), and 2.5 m*M* spermidine. The solution is stable at –20°C for more than 6 mo.

2.2. Second-Strand Synthesis

1. 10X Second-strand buffer: 400 m*M* Tris-HCl, pH 7.6, 750 m*M* KCl, 30 m*M* $MgCl_2$, 100 m*M* $(NH_4)_2SO_4$, 30 m*M* DTT, and 0.5 mg/mL of bovine serum albumin (BSA). The solution is stable at –20°C for at least 6 mo.

2 1 m*M* NAD
3. 2 U/μL RNase H
4 5 U/μL *Escherichia coli* DNA polymerase I.
5 1 U/μL *E coli* DNA ligase
6. Nuclease-free water.
7 T4 DNA polymerase.
8. 200 m*M* EDTA, pH 8.0
9 GeneClean (Bio 101, La Jolla, CA)
10. TE buffer: 10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA Sterile filter.
11. DNA standards: Prepare 1-mL aliquots of a purified DNA sample at 1, 2 5, 5, 10, and 20 μg/mL in TE buffer. Store at –20°C for up to 6 mo.
12. TE/ethidium bromide: 2 μg/mL of ethidium bromide in TE buffer. Store at 4°C for up to 6 mo in a dark container

2.3. Circularization and Cleavage or Denaturation

1. 5X Ligation buffer (supplied with T4 DNA ligase)
2 1 U/μL T4 DNA ligase.
3 4 μg/μL T4 RNA ligase.
4. 15 μ*M* Hexaminecobalt chloride.
5. Phenol.$CHCl_3$:isoamyl alcohol (25:24·1).
6 3*M* sodium acetate, pH 7.0
7 Absolute ethanol
8 70% Ethanol

2.4. IPCR

1. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0 01% (w/v) gelatin
2. 15 m*M* $MgCl_2$
3. Deoxyoligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer and purified by OPEC column from the same company. The primer pairs were selected from the 5' and 3' sequence of the cDNA coding for human ERp72 stress-related protein (5'-primer. 5'-TTC CTCCTCCTCCTCCTCTT-3'; 3'-primer· 5'-ATCTAAATGTCTAGT-3') *(10)*
4. Light mineral oil.
5 *Taq* DNA polymerase.

3. Methods

3.1. First-Strand cDNA Synthesis (19)

Perform reverse transcription in a 25-μL reaction mixture, adding the following components· 5.0 μL 5X first-strand buffer, 2.5 μL dNTP mix, 2.5 μL random primers, 1.0 U RNasin, 1.25 μL actinomycin D, 250 U MMLV reverse transcriptase, 15–25 μg of total RNA (heat denature RNA at 65°C for 3 min prior to adding to reaction), and nuclease-free water to 25 μL final volume.

3.2. Second-Strand Synthesis (20)

1. Add components to the first-strand tube on ice in the following order: 12.5 µL 10X second strand buffer, 12.5 µL NAD (1 m*M*), 0 5 µL RNase H (2 µ/µL), 5.75 µL *E coli* DNA polymerase I (5 µ/µL), 1 25 µL *E coli* ligase (1µ/µL), and 67.5 µL nuclease-free water.
2. Incubate at 14°C for 2 h.
3. Heat the reaction mix to 70°C for 10 min, spin for a few seconds, and then put in ice.
4 Add 4 U of T4 DNA polymerase and incubate at 37°C for 10 min to blunt the ends of double-stranded cDNA
5. Stop the reaction with 12.5 µL of 0.2*M* EDTA and 200 µL sterile water.
6. Concentrate and purify the sample with GeneClean. Resuspend the DNA in 100–200 µL of sterile water.
7. Estimate the DNA concentration by comparing the ethidium bromide fluorescent intensity of the sample with that of a series of DNA standards on a sheet of plastic wrap *(21)* Dot 1–5 µL of sample onto the plastic wrap on a UV transilluminator Also dot with 5 µL of DNA standards. Add an equal volume of TE buffer containing 2 µg/mL of ethidium bromide, and mix by repipetting up and down. Use proper UV shielding for exposed skin and eyes

3.3. Circularization and Cleavage (see Notes 1–4)

1. Set up the circularization reaction mix containing the following components. 100 µL (100 ng DNA) of the purified sample, 25 µL of 5X ligation buffer, and 6 µL of T4 DNA ligase. Finally, add 2 µL of T4 RNA ligase or 15 µL of 15 µ*M* hexaminecobalt chloride (*see* Note 5).
2. Incubate at 18°C for 16 h
3 Boil the ligated circular DNA for 2–3 min in distilled water or digest with an appropriate restriction enzyme to reopen circularized DNA.
4. Purify the DNA sample with GeneClean as described in step 6 in Section 3.2. or extract with water-saturated phenol/$CHCl_3$ and then precipitate with ethanol *(20)*.

3.4. IPCR (see Note 6)

1. Add $^1/_{10}$ of the purified cDNA to 100 µL of amplification mix (22): 10 µL 10X PCR buffer, 10 µL 15 m*M* $MgCl_2$, 10 µL dNTP mix (2.5 m*M* of each), 10 µL 5'-primer (10 pmol/µL), 10 µL 3'-primer (10 pmol/µL), 10 µL cDNA, 39.5 µL nuclease-free water, and 0 5 µL *Taq* DNA polymerase (2.5 µ/µL).
2. Cap and vortex the tubes to mix. Spin briefly in a microfuge. Cover each reaction with a few drops of light mineral oil to prevent evaporation.
3. Put a drop of mineral oil into each well of the thermal cycler block that will hold a tube. Load the reaction tubes.
4. Amplify by PCR using the following cycle profile· 25 cycles· 94°C for 1 min (denaturation), 65°C for 2 min (annealing), and 72°C for 4 min (elongation)

4. Notes

1. For maximum efficiency of intramolecular ligation, a low concentration of cDNA should be used in the ligation mix. High density of cDNA may enhance the level of heterogeneous ligation, which creates nonspecific amplification
2. Cleavage or denaturation of circularized double-stranded cDNA is important since circular double-stranded DNA tends to form supercoil and is poor template for PCR *(23)*. Circularized double-stranded DNA is only good for amplification of a short DNA fragment
3. The following three ways can be considered to introduce nicks in circularized DNA. Boiling is a simple and common way However, because of the unusual secondary structure of some circular double-stranded DNA, sometimes this method is not sufficient in nicking and denaturing circular double-stranded DNA. A second method is selected restriction enzyme digestion The ideal restriction site is located in the known sequence region of cDNA. In most cases, it is difficult to make the right choice of a restriction enzyme because the restriction pattern in unidentified region of cDNA is unknown. If an appropriate enzyme is not available, EDTA-oligonucleotide-directed specific cleavage may be tried *(24,25)*. Oligonucleotide linked to EDTA-Fe at T can bind specifically to double-stranded DNA by triple-helix formation and produce double-stranded cleavage at the binding site
4. Alkali denaturation has been successfully used to prepare plasmid DNA templates for PCR and DNA sequencing *(12,13,26)*. This method should be feasible in denaturing circularized double-stranded cDNA
5. Inclusion of T4 RNA ligase or hexaminecobalt chloride can enhance the efficiency of blunt-end ligation of double-stranded DNA catalyzed by T4 DNA ligase *(27)*.
6. IPCR can be used to efficiently and rapidly amplify regions of unknown sequence flanking any identified segment of cDNA or genomic DNA. This technique does not need construction and screening of DNA libraries to obtain additional unidentified DNA sequence information Some recombinant phage or plasmid may be unstable in bacteria and amplified libraries tend to lose them *(23)*. IPCR eliminates this problem.

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