Generation of Full-Length cDNA Library from Single Human Prostate Cancer Cells

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Several existing methods to construct cDNA libraries require several thousand cells and involve the lengthy procedure of reverse transcription (RT), restriction, adaptor ligation and vector cloning (5,6), which usually fail to maintain the completeness of a cDNA library, resulting in a loss of rare cDNAs. However, gene expression analysis of specific cell populations within a heterogeneous tissue is essential for research in vivo, requiring a method to generate cDNA libraries from a very small number of specific cells. The generation of amplified antisense RNA (aRNA) by incorporating an oligo(dT) primer coupled to a T7 RNA polymerase promoter sequence during RT has been developed to increase transcriptional copies of mRNAs from a limited amount of promoter-linked cDNAs (2,4). However, the aRNAs prepared from a single live neuron has been reported to cover 50%–75% of the total mRNA population (1,2), indicating that rare mRNAs were not protected during the amplification procedure.

Here, we present a novel method for generating cDNA libraries that combines in situ RT (3) with amplified aRNAs followed by polymerase chain reaction (PCR) amplification. To protect mRNAs from degradation, the initial steps of in situ RT, 3′ oligonucleotide tailing and aRNA amplification are performed on fixed and permeabilized cells. Subsequent RT-PCR generates full-length cDNA libraries in a quantity easily manipulatable for gene expression analysis; the resultant cDNAs are amplified to total more than 10^9 the amount of original mRNAs.

The method can be broken down into six steps (Figure 1, a–f): (a) cell fixation and permeabilization, (b) first RT, (c) terminal transferase tailing and cDNA double-stranding, (d) in-cell transcription, (e) second RT and (f) PCR amplification.

Cell fixation and permeabilization (3). We have used LNCaP cells, cultured in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 2% fetal calf serum, which were trypsinized and suspended in 1 mL of ice-cold 10% formaldehyde solution in 0.15 M NaCl. After a 1-h incubation on ice with occasional agitation, the cells were centrifuged at 11 000× *g* for 2 min and washed three times in ice-cold phosphate-buffered saline (PBS) with vigorous pipetting. The collected cells were permeabilized in 0.5% Nonidet® P-40 (NP40; BDH Ltd., Poole, Dorset, England, UK) for 1 h with frequent agitation. After three washes in ice-cold PBS containing 0.1 M glycine, the cells were resuspended in 1 mL of the same buffer, aliquotted and stored at -70°C for up to a month.

First RT. For RT of mRNAs in cells, twenty fixed cells were picked up under microscopy and suspended in 20 µL of ice-cooled diethyl pyrocarbonate (DEPC)-treated double-distilled (dd)-H_2O. An RT reaction (50 µL) was prepared, containing the above cold cells, 5 µL of 10× RT buffer [1.2 M KCl, 0.5 M Tris-HCl, 80 mM $MgCl_2$, 50 mM dithiothreitol (DTT), pH 8.1 at 42°C], 25 pmol oligo(dT)-T7 primer (2), 0.2 mM of each dNTP and 80 U RNase inhibitor. After 6 U *C. therm*. Polymerase with Reverse Transcriptase Activity (Roche Molecular Biochemicals, Indianapolis, IN, USA) were added, the RT reaction was vortex mixed and incubated at 55°C for 2 min and then 68°C for 1 h. The cells were washed once with PBS and collected with an Amicon® Microcon-50 Microconcentrator (Millipore, Bedford, MA, USA).

Terminal transferase tailing and

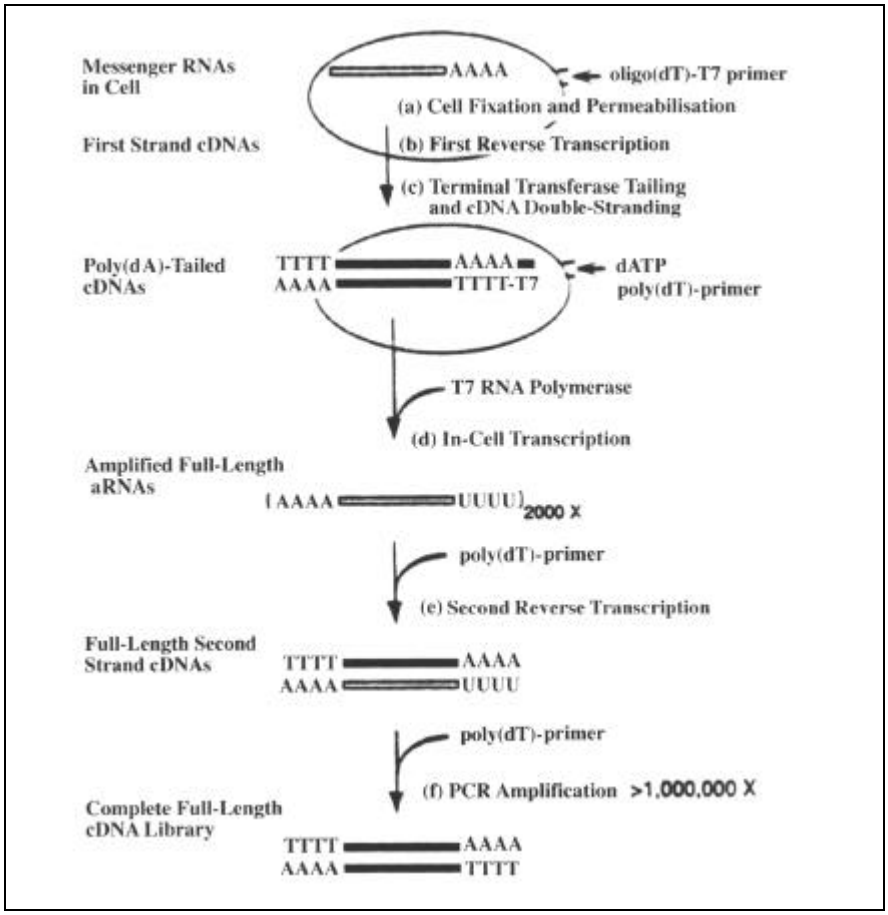

Figure 1. Flowchart of current method for generating a full-length cDNA library from single cells. Cell fixation and permeabilization protect mRNA from degradation and allow enzymes to penetrate cells. Upon RT using an oligo(dT)-T7 promoter and tailing with poly(dA) oligonucleotide, the cDNA library was amplifiable by in-cell transcription and PCR. The final PCR products yield one billion times the amount of original mRNAs.

cDNA double-stranding. To increase the intracellular copies of whole mRNAs, the T7 promoter region of the poly(dA)-tailed cDNAs served as an initial template for transcriptional amplification by T7 RNA polymerase (Roche Molecular Biochemicals) (2). The cells collected from the first RT were suspended in a 50-µL tailing reaction solution, containing 0.2 mM dATP and 10 µL of 5× tailing buffer (Roche Molecular Biochemicals). The mixture was denatured at 94°C for 3 min and then chilled on ice immediately for mixing with 20 U Terminal Transferase (Roche Molecular Biochemicals), followed by further incubation at 37°C for 20 min. The reaction was stopped by warming to 94°C for 3 min, mixed with poly(dT) 26-mer primer and chilled on ice immediately; this formed poly(dA)-tailed cDNAs. The double-stranding of the poly(dA)-tailed cDNAs was completed by adding 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 6 U *C. therm*. polymerase at 55°C for 2 min and then at 70°C for 5 min. As few as one cell in 5 µL of the tailing reaction can be used to accomplish aRNA amplification.

In-cell transcription. The in-cell transcription reaction mixture (50 µL) contained 10 µL of 5× transcription buffer (Roche Molecular Biochemicals), 80 U RNase inhibitors, 0.5 mM of each rNTP and 2000 U T7 RNA Polymerase (Roche Molecular Biochemicals). After a 3-h incubation at 37°C, the cDNA transcripts were isolated from both cells and supernatant and used for another round of RT.

Second RT. The second RT reaction consisted of 10 µL of 5× RT buffer (Roche Molecular Biochemicals), 0.5 mM of each dNTP, 25 pmol poly(dT) 26-mer, 80 U RNase inhibitor, DEPC-treated ddH_2O and 5 µL of the above aRNA-containing supernatant. After 6 U *C. therm.* polymerase were added, the RT reaction was vortex mixed and incubated at 55°C for 1 min and then 68°C for 1 h.

PCR amplification. The resulting products of the second RT were directly used for the PCR (50 µL), which contained 5 µL of 10× PCR buffer (Roche Molecular Biochemicals), 0.2 mM of each dNTP, 25 pmol poly(dT) 26-mer primer, 2 µL of above RT product and 3.5 U of Taq/Pwo DNA Polymerase Mixture (Expand™ Long Template PCR System; Roche Molecular Biochemicals). The PCR underwent thirty cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 4 min.

The quality of the final amplified cDNA library (20 µL) was assessed on a 1% denaturing-formaldehyde-agarose gel. It ranged from 300 bp to above 7.4 kb, demonstrating the completeness of cDNA libraries generated by this method (Figure 2A). We observed that mRNA was well preserved by cell fixation and RNase inhibitors during the amplification process. With an oligo-(dT)-promoter sequence as primer, the intracellular mRNAs were reverse transcribed into cDNAs, and consequently the cDNAs were used to transcribe aRNAs. A terminal transferase reaction was performed so that a poly(dA) tail was added to the furthermost 3′ end of the first-strand cDNAs for full-length generation by complementary poly(dT) primers. An RNA polymerase reaction from the promoter region of the double-stranded cDNAs generated full-length aRNAs, which were amplified approximately 1500- to 2000-fold (2). Subsequently, the full-length aRNAs were reverse transcribed and further amplified with PCR extension of poly(dT) primers, by which another one million-fold amplification was achieved. The resulting product represents a complete library enriched in full-length cDNAs from the individual fixed cells. Furthermore, with this method, as few as a single cell can be used to produce a complete cDNA library. We have routinely cloned specific gene products from this cDNA library using PCR amplification with related primers (not shown); we have also detected Rb (4.9 kb), p21 (2.3 kb), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.7 kb) and β-actin (2.2 kb) cDNAs by Southern blot analysis, shown to be relatively the same size as their original mRNAs (Figure 2B).

Although the above procedure was shown to synthesize a cDNA library from cultured human prostate cancer cells, the method can also be used to analyze gene expression patterns of specific cells in vivo. A challenge in studying the organogenesis and pathogenesis of complex organs such as the

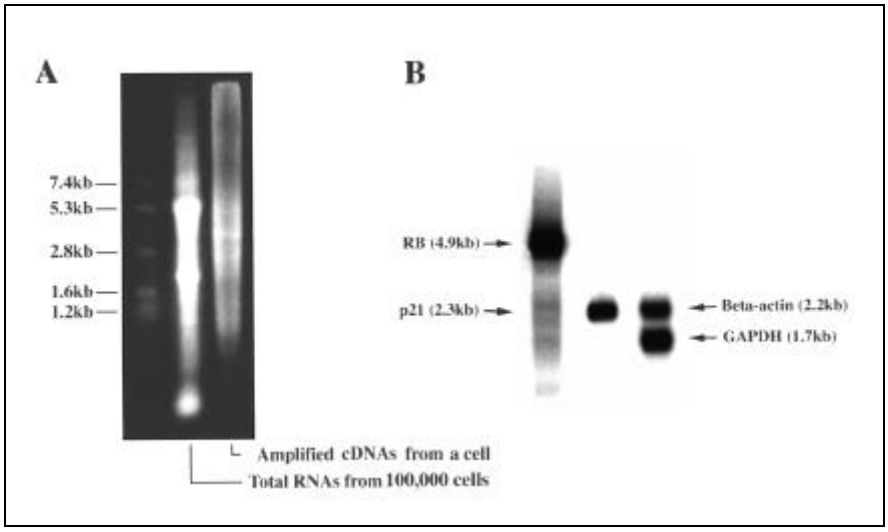

Figure 2. (A) 1% formaldehyde-agarose gel analysis of cDNA library from different numbers of cells. Lane 1, RNA markers from 1.2–7.4 kb; lane 2, total RNA repertoire from 100 000 cells; lane 3, cDNA library from a few single cells. cDNA library obtained from single cells was sufficient to produce a smear visualizable upon staining with ethidium bromide. (B) Southern blot analysis of Rb, p21, β-actin and GAPDH in cDNA library generated from LNCaP cells. After electrophoresis, the cDNA library was denatured and transferred onto a nylon membrane and UV cross-linked. Specific cDNA probes were radiolabeled by Prime-It® II Kit (Stratagene, La Jolla, CA, USA) with [^{32}P]dATP (>3000 Ci/mM; Amersham Phamacia Biotech, Piscataway, NJ, USA). Hybridization was carried out in QuikHyb® (Stratagene) for 4 h at 68°C according to the manufacturer's protocol and washed in 2% standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 20°C for 15 min, then in 0.1% SSC/0.1% SDS at 55°C for 30 min before autoradiography.

prostate is that these organs are composed of heterogeneous cell populations (i.e., epithelial vs. stromal, hormone-dependent vs. hormone-independent). The analysis of gene expression at the single cellular level would aid in our understanding of molecular mechanisms involved in a variety of developmental and pathological processes.

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Multistep-Touchdown Vectorette-PCR—A Rapid Technique for the Identification of IVS in Genes

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The determination of the genomic organization of eukaryotic genes often requires additional intronic sequence information, since the majority of gene sequences have been published on cDNA level. An efficient method for characterizing unknown (intronic) DNA sequences adjacent to known sequences is vectorette-polymerase chain reaction (PCR) (1). In this paper, we found that both, the yield of DNA and its specifity, were substantially improved when a touchdown-PCR was performed in two successive steps and combined with the use of a widely used genomic DNA library as template (5). This method was used to establish unknown intronic sequences of a human potassium channel gene, *Herg*, in which mutations are associated with cardiac arrhythmias and sudden cardiac death (3,8). Thus, the knowledge of the complete exonic sequence including the splice donor and acceptor sites, the Lariat sites and additional, adjacent intronic sequences for primer annealing is essential for effective mutation detection and genetic counseling. Hereby, the complete sequences of the often large introns have not been explored in detail, because they are unlikely to bear disease-causing mutations. Using "multistep-touchdown vectorette-PCR" (MTV-PCR), we were able to establish the complete genomic structure of *Herg* for routine genetic analysis in a solid and rapid way. From our experience, MTV-PCR seems to be widely applicable for any other gene of interest.

MTV-PCR uses a commercially available genomic DNA library that is based on five single adaptor-ligated cloned libraries (CLONTECH Laboratories, Palo Alto, CA, USA) in which to a digest of human DNA (using *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I separately) an adaptor sequence for PCR primer annealing has been ligated (9,10). Each of the five libraries was used as a template in the first of two successive PCR assays.

In the outer PCR, an adaptor primer and an exonic Herg primer (binding approximately 70 bp from a putative exon-intron boundary) were used (Figure 1). Each reaction mixture (50 µL) contained 1 µL of library DNA, 20 µM

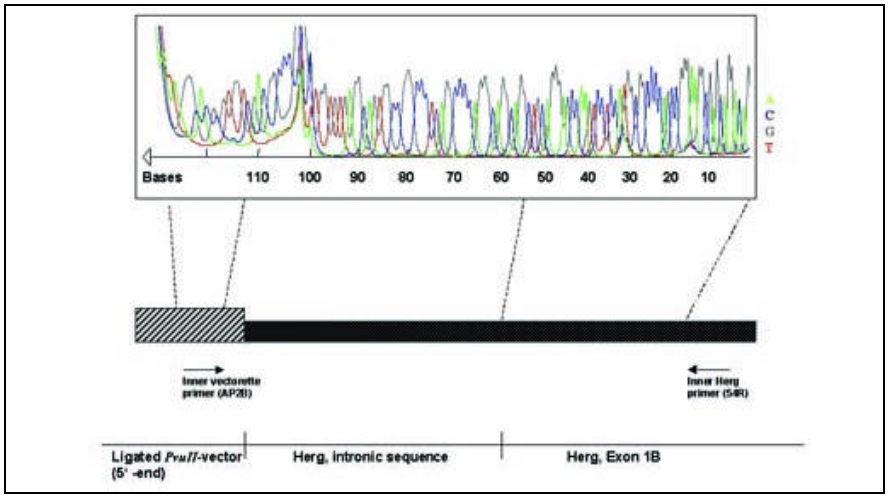

Figure 1. Schematic diagram of the obtained sequences flanking exon 1B of *Herg*. After amplification with MTV-PCR, the PCR product of the inner PCR (using primers AP2B and Herg54R) was sequenced. With the *PvuII* genomic library, the 5′ flanking intronic sequences of exon 1B of *Herg* were obtained. The exonic primer Herg54R was derived from the cDNA sequence of the mouse *eag*-related gene (*M-erg*) (7). We identified approximately 65 bp of intronic sequence that were highly homologous to that of mouse DNA (7). AP2B, biotinylated, inner adaptor primer matching the vectorette sequence (see manufacturer's manual); Herg54R, Cy5-labeled, nested primer, 5′-TTCTGGGCCCTGGGCCGCAGA-3′. The 3′ end of the ligated vectorette genomic library and the outer PCR primers are not shown.