phocytes), or prostate (glandular epithelium) tissue. To improve sensitivity yet maintain specificity, a nested PCR was used. The samples were amplified for 50 cycles, as described earlier with a primary primer set. The primer reaction (1 μ L) was removed and amplified for an additional 45 cycles. Consistent with previous results, PSA was only detected in prostate and not in lung or tonsil tissue (Figure 2C) (10). Analogous results have been found with the specific amplification of β B1 crystallin from microdissected lens epithelia but not from optic nerve or sclera (data not shown). To date, all primer sets tested have yielded RT-PCR products. These have included GAPDH, PSA, PSMA, EMP2, β -actin (5) (not shown), and β B1 crystallin (not shown).

Here, we describe a simple and rapid one-step RT-PCR protocol from laser-captured microdissected tissue. Notably, as a method for rapidly screening dissected cells for gene expression, this protocol has the advantage of decreased manipulation of the sample because no RNA extraction step is necessary. Because of this, the likelihood of losing RNA during this microisolation protocol is diminished. Currently, the technique described here is suitable for rapid screening of microdissected tissue samples but not quantitation. Nevertheless, this protocol demonstrates sensitivity (e.g., detecting signals from as few as 8–10 cells) and specificity (e.g., detecting tissue-specific gene products). We anticipate that this assay can be adapted for a more quantitative approach when combined with real-time PCR technology.

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Madhuri Wadehra, Jonathan Braun, and Lee Goodglick
UCLA School of Medicine
Los Angeles, CA, USA

T Vectors With Endoglucanase A (*celA*) Gene for Direct Detection of PCR Clones

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The dsDNA products obtained by PCR with *Taq* DNA polymerase usually contain a single 3' unpaired dATP residue gratuitously added by the DNA polymerase to its product (3). Accordingly, T vectors containing single 3'-terminal thymidine overhangs produced by enzymatic tailing or digestion, such as *XcmI* endonuclease, have been widely used to allow efficient cloning of PCR products (1,8). In theory, T vectors should not give rise to nonrecombinant clones after transformations in which no PCR product has been inserted. However, such background clones are frequently encountered in real cloning situations so that the detection of the correct clones out of many transformants is very cumbersome and time consuming. In recent years, numerous T vector systems have been developed to identify fast and efficiently the PCR clones in which the β -galactosidase or GFP gene was included as a marker (4,6).

Endoglucanase A (i.e., cellulase A or *celA*) is a member of the glycosyl hydrolase family (EC 3.2.1.4) that catalyzes the hydrolysis of glycosidic bonds in cellulose. A novel gene cloning system, the pCEL1™-Cellomix™ kit developed by RNA, Inc. (Suwon, Korea), utilizes a *celA* gene isolated from *Clostridium thermocellum* as a marker (2). Cellulase expressed by the *celA* gene forms a white circle around the colony in agar plates containing a chromogenic substrate, Cellomix. If the *celA* gene is disrupted by an inserted DNA, then no circle forms around the colony because of the insertional inactivation of the gene.

Based on the pCEL1-Cellomix kit, we describe here the constructions of two new T vectors with the *celA* gene for a direct detection of PCR product clones. Both of these T vectors, pCEL-T1 and pCEL-T2, contain two *XcmI* sites in the middle of multiple cloning sites (MCSs) of the *celA* gene in frame.

Benchmarks

The digestion of each vector with endonuclease *XcmI* generates the 3' T overhang vector suitable for PCR product cloning (Figure 1).

The pCEL-T1 vector was constructed by ligating the pCEL1 vector with a 53-bp fragment isolated from the pHNT vector, which was originally developed as a GFP-T vector (6). Similarly, the pCEL-T2 vector was constructed by ligating the pCEL1 vector with a 61-bp fragment isolated from the pDK101 vector, which was designed to produce a 3' T overhang by endonuclease *XcmI* digestion (5). Standard cloning methods were used for this purpose, and the ligated DNA mixtures were then used to transform the *E. coli* strain DH10B™ (Invitrogen, Carlsbad, CA, USA).

Minipreps from 10 randomly picked colonies for each construct identified several candidate clones containing a 53- or 61-bp fragment in the mother pCEL1 vector. These clones were further tested for cellulase enzyme activities using LB-Cellomix plates as described by the supplier (0.15 mL Cellomix/1 mL LB), and all of them showed clear white circles around them. Sequencing with an M13/pUC forward primer also demonstrated that a 53- or 61-bp fragment was correctly inserted into pCEL1 vector (data not shown).

The 3' T overhang T vectors from pCEL-T1 or pCEL-T2 were prepared by cutting each plasmid with *XcmI*. After digestion, the vectors were concentrated by ethanol precipitation, redissolved in water, and used for ligation without any additional steps. To prepare the PCR product DNA for cloning, forward (5'-GAAGACGAGTGGATGCGGATAA-3') and reverse (5'-GAGCCCCAACTG CCCTGAAC-3') primers were designed to amplify a 580-bp mouse secretogranin II gene fragment from mouse cDNAs (7). PCR was performed as follows in the recommended buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.2 mM each dNTP using 10 μM each primer, 10 ng cDNA template, and 1 U *Taq* DNA polymerase in a total reaction volume of 50 μL. PCR cycles were 2 min at 95°C, then 35 cycles of 30 s at 94°C, 30 s at 57°C, 45 s at 72°C, and an additional 7 min at 72°C after the last cycle. Approximately 100 ng PCR product, 10 ng *XcmI*-cut T vector, and 1 U T4 DNA lig-

ase were mixed and incubated at 16°C in a 10-μL reaction volume. Five microliters ligation mixture were used to transform the *E. coli* DH10B. The cells were plated on LB-Cellomix supplemented with 50 μg/mL ampicillin.

The ratio of circled to non-circled colonies varied from 1:1 to 1:3 and seemed to depend most on the vector preparation and amount of PCR product used for ligation. Minipreps prepared from the 20 non-circled (recombinant) and the 10 circled colonies (nonrecombinant) were digested with *XbaI* and *HindIII* for pCEL-T1 or with *NcoI* for pCEL-T2, which is supposed to raise the inserted DNA from the T vector. Figure

2 illustrates an example of miniprep analysis using the pCEL-T1 vector. As expected, all 20 non-circled clones demonstrated a 580-bp insert while 10 circled clones showed a 100-bp fragment representing just a MCS region.

These new T vectors described here allow easy and rapid screening of the PCR product clone simply by observing the circle formation around the transformants on the light-box or even with the naked eye. The disruption of the *celA* gene in our T vectors, due to an inserted PCR fragment, prevented hydrolysis of the blue-colored Cellomix substrates so that no circle formation would be observed around the recombinant clones.

Figure 1. Schematic overview of constructions of two pCEL-T vectors (A) and their partial nucleotide sequences spanning two *XcmI* sites (B). The 53-bp *NorI* DNA fragment isolated from pHNT was inserted into the *NorI* site of the pCEL-1 vector, constructing the pCEL-T1 vector. Likewise, the 61-bp *SpeI-SphI* DNA fragment isolated from pDK101 was inserted into the pCEL-1 vector cut with *XbaI* and *SphI*, constructing the pCEL-T2 vector. The pCEL-T1 and pCEL-T2 vectors contain two different MCS. Both contain two *XcmI* restriction sites to generate 3'-terminal thymidine overhangs. The PCR DNA cloned with these T vectors can be easily released by digestion with various restriction enzymes present in MCS as shown here. The two *XcmI* sites are underlined. Abbreviations: Xc, *XcmI*; N, *NorI*; Se, *SpeI*; and Sh, *SphI*.

Benchmarks

There are additional advantages when *celA* is used as a marker gene for the cloning of PCR products. Firstly, detection based on cellulase enzyme activities is more accurate when compared with the GFP-based fluorescence or β -galactosidase white/blue assay. GFP and β -galactosidase sometimes show considerable basal activities so that false-positive colonies are frequently

encountered, making it difficult to identify true recombinants. Secondly, this T vector system does not require any exposure to the harmful UV irradiation or hazardous organic solvents such as dimethylformamide. Moreover, Cellomix, the chromogenic substrate that should be used with this T-vector system is much cheaper than the expensive X-gal. Thirdly, this T-vector system can be ap-

plied to various *E. coli* strains since cellulase is not toxic to bacteria; there is a wide range of bacterial strains to choose from. Finally, two different versions of the pCEL-T vector allow more flexible manipulation of the cloned PCR product for downstream applications.

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Seok Bean Song, Kee Kwang Kim, and Kyoong Eon Kim
Chungnam National University
Taejeon, Korea

Figure 2. Cellulase activity of the *E. coli* transformants on LB-Cellomix (A) and the detection of inserts in plasmids prepared from non-circled recombinant colonies (B) or from circled nonrecombinant colonies (C). (A) A typical non-circled colony and a circled colony on a Cellomix-containing plate are indicated by an arrow and arrowhead marks, respectively. (B) Twenty non-circled colonies were chosen for miniprep analysis. Five microliters of prepared plasmid DNA were digested with *XbaI* and *HindIII* to release the 580-bp insert (lanes 1–20). As expected, all 20 colonies demonstrated the correct insert. (C) Likewise, 10 circled colonies were chosen and subjected to miniprep analysis. The MCS region that originated from pCEL-T1 cut with *XbaI* and *HindIII* is indicated by 100 bp. Abbreviations: M, DNA size marker, and C, linearized pCEL-T1 as a control.