Protocols for Trapping Internal and 3'-Terminal Exons

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The cloning of genes on the basis of their genetic map positions (positional cloning) has proven to be a powerful tool in the growing field of gene discovery.⁽¹⁾ The list of heritable human disease genes successfully isolated by using this strategy is rapidly expanding. Positional cloning can be described as a four-step process: (1) finding genetic linkage between a disease phenotype and genetic markers of known map position; (2) obtaining this region as a set of physically overlapping pieces of cloned DNA using cosmid, P1, P1 artificial chromosome (PAC), bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC) clones;⁽²⁻⁶⁾ (3) screening the set of overlapping clones (known as a contig) for expressed sequences; and (4) using these captured bits of expressed sequence to identify a full-length cDNA clone and the mutation that distinguishes the normal from the mutant allele associated with the genetic disease. Because genetic and physical maps of the human genome are being developed rapidly, perhaps the most difficult part of the positional cloning strategy is the actual isolation of candidate genes from the mapped region. Several methods exist to screen genomic clones for expressed sequences, including multispecies blotting, cDNA library screening, and CpG island identification; however, direct cDNA selection and exon trapping are becoming the preferred methods.⁽⁷⁻¹⁸⁾ Direct cDNA selection usually captures larger bits of expressed sequence than does exon trapping, but it requires a high-quality cDNA source (primary cDNA or cDNA library) in which the gene of interest is expressed.^(10,11) Exon trapping, however, extracts exons directly from cloned genomic DNA without the need for cDNA. For this reason, exon trapping can be considered to be a very useful tool for gene discovery and transcript mapping because the selection for expressed sequences is based solely on the presence of splicing recognition sequences. Methods are presented here for the isolation of internal and 3'-terminal exons from YACs and cosmids containing cloned human DNA.

TRAPPING INTERNAL EXONS USING pSPL3—OVERVIEW

The exon trapping vector pSPL3 is an improved version of the plasmid pSPL1 developed by Buckler and colleagues.^(12,13) pSPL3 (Fig. 2, below) contains a multiple cloning site and other modifications that result in a reduction of cryptic splicing as well as a higher transfection efficiency as a result of the incorporation of restriction enzyme (*Bst*XI) half-sites flanking the splice donor and acceptor sites of pSPL3.⁽¹³⁾ Although most published reports on exon trapping have used cosmids as substrates,^(13,19) at least one paper has described a method for trapping exons from YACs.⁽²⁰⁾ We have focused on using pSPL3 for exon trapping YACs, highlighting their special requirements.

A flow diagram describing the internal exon-trapping procedure for YACs is shown in Figure 1: A YAC clone is propagated, and DNA is purified after pulsed-field gel electrophoresis, then digested, and subcloned into the plasmid pSPL3. The subclones are then propagated in *Escherichia coli* and arrayed in pools. After miniprep DNA is made from subclone pools, the DNA is transfected into COS-7 cells. After transient expression, the RNA is harvested and first-strand cDNA is reverse-transcribed using a vector-specific oligonucleotide. Following digestion of the RNA template by RNase H, an initial round of PCR is performed; *Bst*XI is then added resulting in the removal of PCR products that do not contain exons. A second round of PCR is performed, followed by rapid cloning into a phagemid vector using uracil DNA glycosylase.⁽²¹⁾ The following sections describe the details of each step.

Purification and Preparation of YAC DNA for Subcloning into the Exon-Trapping Vector pSPL3

By encapsulating yeast cells in agarose beads prior to treatment with yeast lytic enzyme and lysis buffers, YAC DNA is protected from the shear forces

FIGURE 1 pSPL3 exon trapping of YAC or cosmid DNA. YAC DNA is digested with a restriction enzyme, e.g., *Bam*HI (B). In this example, a single exon (shaded box) is contained in a *Bam*HI fragment. The exon is flanked by splice acceptor (SA) and splice donor (SD) sites. The pSPL3 vector is prepared for subcloning at the same restriction site. The MCS of pSPL3 contains several restriction enzyme sites. The *Eco*RI and *Eco*RV sites are contained within *Bst*XI sites. The solid boxes represent HIV-tat and rabbit β -globin exon sequences in the pSPL3 vector. The splicing donor and acceptor sites of pSPL3 contain *Bst*XI half-sites. These sites form a complete *Bst*XI site after splicing events occur that fail to capture a “trapped” exon and can therefore be used to eliminate vector background in the subsequent PCR. After subcloning genomic DNA into pSPL3, DNA is isolated and transfected into COS-7 cells. Total RNA is isolated for RNA-based PCR analysis. After generation of cDNA, the first round of PCR is performed using the outside primer pair SD6 (TCT GAG TCA CCT GGA CAA CC) and SA2 (ATC TCA GTG GTA TTT GTG AGC). A second round of PCR is performed using the nested primers dUSD2 (CUA CUA CUA CUA GTG AAC TGC ACT GTG ACA AGC TGC) and dUSA4 (CUA CUA CUA CUA CAC CTG AGG AGT GAA TTG GTC G) to provide specificity and to install DNA sequences that allow UDG cloning of the PCR products. Trapped exons are recognized after gel analysis of the PCR products. Occasional but infrequent use of the cryptic splice donor site can be detected by digestion with *Nde*I.

associated with manipulations required in this procedure. Because the yeast chromosomes are enclosed in very small agarose beads, the material is easily pipetted. Following pulsed-field gel electrophoresis and isolation of YAC DNA using β -agarase, the purified YAC is an ideal high-molecular-weight substrate for partial or complete restriction enzyme digestion.

1. Inoculate 200 ml of AHC broth with 10 ml of an overnight culture started from a single colony from a freshly grown AHC plate or from a small amount of material from a frozen glycerol stock.

2. Incubate culture for 24 hr at 30°C, 200 rpm, in a shaking incubator.
3. Decant culture into two 50-ml sterile polypropylene centrifuge tubes and pellet the cells by centrifuging for 10 min at 1600g at room temperature.

Note: While the cells are being pelleted, prepare the following:

- a. Prewarm a 50-ml sterile polypropylene centrifuge tube in 45°C water bath.

- b. Prepare 10 ml of a 1% solution of low-melting-temperature agarose in SE buffer (75 mM NaCl, 25 mM EDTA at pH 8.0), and equilibrate in 45°C water bath.

- c. Equilibrate 25 ml of light mineral oil in 45°C water bath. Equilibrate 100 ml of SE buffer in a 250-ml glass beaker containing a stir bar to 4°C by placing the beaker in an ice bucket containing a slurry of ice and water. Place this on a stir plate set to medium speed. Allow at least 10 min for equilibration of the SE buffer to 4°C.

4. Wash cells three times with 10 ml of SE buffer. Centrifuge cells for 5 min at 1600g at room temperature.

5. Resuspend combined yeast cell pellets in a total of 4 ml of SE buffer.

6. Add yeast cell suspension to a prewarmed 50-ml sterile polypropylene centrifuge tube in the 45°C water bath. Incubate for 3–5 min.

7. Add 2.5 ml of 1.5% low-melting-temperature agarose in SE buffer (45°C) to the yeast cell suspension. Gently swirl to mix.

8. Add 12 ml of light mineral oil (45°C) to the yeast cell suspension. Shake the tube back and forth vigorously for 5 sec to form an emulsion.

9. Steadily pour the emulsion into the cold SE buffer (100 ml at 4°C) while stirring (do not pour into the vortex—pour just inside the beaker. The bead size is influenced by temperature and degree of swirling. Faster swirling and increasing the temperature of the SE buffer from 4°C to 9°C will result in smaller beads).

10. Stir for 5 min. Transfer to two 50-ml centrifuge tubes and centrifuge for 10 min at 1600g at room temperature.

11. Remove mineral oil from the top of each tube with a pipette. Disperse bead pellet (both at the bottom of the tube and immediately below the mineral oil layer at the top) by pipetting through a 1-ml pipette tip after removing a few millimeters from the tip with a razor blade to increase the bore size.

12. Centrifuge again. Remove supernatant with a pipette. Combine agarose bead pellets using a large-bore pipette tip prepared as described above.

13. Centrifuge once more. Remove any remaining supernatant.

14. Add to the agarose bead pellet 0.5 ml of β -mercaptoethanol, 0.5 ml of SE/yeast lytic enzyme (YLE) solution (5 mg of YLE/ml in SE buffer), and adjust the volume to 10 ml with SE buffer (for a final concentration of 35U/ml of YLE). Disperse beads and incubate for 2 hr at 37°C.

15. Centrifuge tubes for 10 min at 1600g at room temperature.

16. Remove supernatant with pipette. Resuspend in 10 ml of proteinase buffer [1% sarcosyl (wt/vol), 25 mM Na₂EDTA (pH 8.0), 500 mg/ml of proteinase K], and incubate for 18 hr at 50°C.

17. Centrifuge for 10 min at 1600g at room temperature. Remove the supernatant with pipette. Resuspend bead pellet in ET buffer (50 mM EDTA, 10 mM Tris at pH 8.0).

18. Centrifuge again, and resuspend the beads in 10 ml of ET buffer containing 0.1 mM PMSF.

19. Centrifuge again; wash twice in 10 ml of ET buffer before storing at 4°C.

Pulsed-Field Gel Electrophoresis of YAC DNA

A YAC will, in most cases, be distinguishable from the yeast chromosomes

after electrophoresis and staining with ethidium bromide. In those cases where the YAC comigrates with a yeast chromosome, a human or mouse YAC can be detected and sized by transferring the pulsed-field gel to a nylon membrane and hybridizing it with labeled high-molecular-weight human or mouse Cot-1 DNA (Life Technologies/GIBCO-BRL cat. nos. 18376-012 and 18440-016, or contact Life Technologies, Inc., for more information). YAC DNA can be purified after pulsed-field gel electrophoresis in the following manner: A sample of beads prepared as described above can be electrophoresed using $1/2\times$ TBE buffer, at 200 V, with a switch time of 20 sec in a 1% LMP-agarose gel for 24 hr to resolve a YAC in the 250-kb size range from neighboring yeast chromosomes. The switch time can be altered to suit the separation requirements: longer times for resolving larger YACs and shorter times for smaller YACs. After electrophoresis, the YAC band is cut out of the gel and purified as follows:

1. Cut out the YAC from the pulsed-field gel with a clean scalpel.
2. Add 1 volume of $1\times$ agarase buffer (10 mM bis-Tris-HCl at pH 6.5, 1 mM EDTA) to the gel slice. Incubate at room temperature for 30 min.
3. Remove liquid from gel slice and add NaCl to 100 mM. Melt at 65°C for 10 min. Measure volume; place at 45°C for 5 min.
4. Add 1 μ l β -agarase I/200 μ l liquified gel solution, and mix thoroughly (Life Technologies/GIBCO-BRL, cat. no. 10195-014). Place at 40°C for 1–2 hr.
5. Place tube on ice for 10 min, then centrifuge at 12,000 rpm for 5 min to pellet any undigested agarose.
6. Remove supernatant to a new tube, add 0.5 volume of ammonium acetate (or 0.1 volume 3 M sodium acetate) and 0.8 volume of isopropanol. Place on ice for 1 hr and centrifuge at 12,000g at 4°C for 20 min to pellet DNA. Alternatively, incubation overnight at 4°C may be preferred if the amount of DNA being precipitated is present in low amounts, that is, $<0.1\ \mu$ g.
7. Wash the pellet with 500 μ l of 70% –20°C ethanol, and centrifuge the tube for 5 min in a microcentrifuge.
8. Decant ethanol. Add TE to pellet and let sit at room temperature for 1 hr to allow DNA to dissolve (especially if fragments are >100 kb) or overnight at 4°C.

Preparing YAC and pSPL3 DNA for Subcloning

1. Digest the YAC and pSPL3 DNA with the same or compatible restriction endonucleases. There are a large number of unique restriction enzyme sites within the multiple cloning sites (MCS) of pSPL3 (Fig. 2) enabling a great deal of flexibility in choice of cloning site.
2. Phenol-extract YAC and pSPL3 DNA after restriction digestion: Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortex, and centrifuge at room temperature for 2 min at 12,000g to separate the phases. Carefully remove the upper aqueous phase and transfer to a fresh microcentrifuge tube.
3. Precipitate the DNA with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol, mix, hold on wet ice for 20 min, microcentrifuge for 30 min, wash with 70% ethanol, air dry, and resuspend each in TE. Resuspend a gel's worth of YAC DNA in no more than 10 μ l (the yield per gel will vary from 1 to 3 μ g, depending on the gel width and thickness).
4. Dephosphorylate the linearized pSPL3 DNA using calf intestinal alkaline phosphatase and supplier's recommendations.

Shotgun Subcloning of YAC DNA into pSPL3

1. Add distilled water to the following components for a final volume of 5

FIGURE 2 Map of pSPL3. The vector contains sequences that enable replication in *E. coli* and COS-7 cell hosts (bacterial and SV40 origins of replication are present). An ampicillin resistance marker (Ap^r) allows for selection of subclones. An MCS that interrupts the HIV-tat intron provides several restriction sites for the subcloning of genomic DNA. After transfection of COS-7 cells, transcription occurs at high levels facilitated by the SV40 promoter (SV40). Processing of the transcript results in removal of the HIV-tat intron via splicing in which the vector exons (which contain sequences from HIV and rabbit β -globin) are combined at the splice donor (SD) and splice acceptor (SA) sites. Cytoplasmic RNA is polyadenylated [pA = SV40 poly(A) addition recognition sequence]. Exons are trapped from genomic DNA cloned into pSPL3 as a result of interaction of the vector splice sites (derived from HIV-tat) with splice sites flanking exons contained in genomic DNA.

μ l: 5–10 ng of pSPL3 restriction enzyme-digested DNA; 10 ng of restriction enzyme-digested YAC DNA; 1 μ l of $5\times$ T4 DNA ligase buffer (Life Technologies/GIBCO-BRL cat. no. 15224-017); 0.5 μ l (at least 0.5 unit) of T4 DNA ligase (Life Technologies/GIBCO-BRL cat. no. 15224-017). Include a control (all the above components without genomic DNA) to assess the level of vector self-ligation.

2. Mix gently and incubate for 1 hr at room temperature.
3. Transform *E. coli* with the ligated DNA using either chemically or electrocompetent cells.
4. Recover at least 500 colonies for every 100 kb of YAC complexity (by adding medium to the transformation plate and using a glass spreader in a circular motion). Isolate DNA by the alkaline lysis procedure.⁽²²⁾ The efficiency of the shotgun subcloning can be assessed by cutting the DNA with the same restriction enzyme that was used to subclone it and comparing the digested source DNA by performing a "Cotblot" (P.E. Nisson and P.C. Watkins, unpubl.). The digested YAC and the pSPL3 library DNA is electrophoresed, transferred to a filter, and hybridized with labeled high-molecular-weight human Cot-1 DNA. If the hybridization pattern of the digested pSPL3 library DNA and the digested YAC DNA are similar, this would indicate that most of the YAC is represented in the pSPL3 library. In addition, the frequency of recombinants can be qualitatively determined by digesting the library DNA with *PvuII*, which cuts pSPL3 twice, yielding a 1.8- and a 4.2-kb band. The MCS is embedded within the 1.8-kb band (Fig. 2). The presence of an intense 4.2-kb vector band, together with a variety of other bands along with the absence of a strong 1.8-kb band, indicates that the nonrecombinant frequency is quite low.

5. Plate 0.1 and 0.01 ml of each transformation on LB plus ampicillin plates to assess the degree of vector religation. Repreparation of dephosphorylated vector is recommended if >10% of the transformants are vector religations.

Transfection of COS-7 Cells

COS-7 cells, a commonly used tissue culture cell line, have a doubling time of ~20 hr. When ordered from the ATCC (CRL-1651), the cells are frozen and should be passaged at least once to establish the appropriate doubling time. A T-70 flask of COS-7 cells is maintained and passaged by trypsinizing the cells and diluting them 1:50 in fresh medium whenever they reach 100% confluence (once or twice each week). Although electroporation can be used to introduce the genomic DNA subcloned in pSPL3 into COS-7 cells, transfection with the cationic lipid LipofectACE (Life Technologies/GIBCO-BRL cat. no. 18301-010) has been found to be comparable in efficiency and less costly in time and materials.⁽²³⁾ Positive and negative (no DNA) transfection controls are recommended. A positive control plasmid containing an internal exon is included in the Life Technologies Exon Trapping System mentioned above.

1. Add 3×10^5 to 5×10^5 COS-7 cells in supplemented DMEM (Dulbecco's modified Eagle medium, 10% fetal bovine serum, glutamine, nonessential amino acids, penicillin, streptomycin) to 3.5-cm tissue culture plates 16 hr before starting the transfection.
2. For each transfection, add an optimized amount of LipofectACE (generally ~3–10 μ l) to 100 μ l of serum-free medium (Opti-MEM, Life Technologies/GIBCO-BRL cat. no. 31985-021), mix gently, and hold at room temperature for 5 min. The optimal amount of LipofectACE and DNA, as well as the media and the confluence of COS-7 cells used, should be optimized according to the supplier's recommendations.
3. For each transfection, place 1–2 μ g of DNA from the plasmid DNA isolation into 100 μ l of serum-free medium.
4. To form DNA-LipofectACE complexes, combine the two solutions prepared in steps 2 and 3, mix gently, and hold for 15 min at room temperature.
5. Prepare the COS-7 cells for transfection by aspirating the supplemented DMEM from the tissue culture plates, adding 1 ml of Opti-MEM to each plate, and incubating for 5 min at 37°C in 5% CO₂.
6. After the DNA-LipofectACE complexes are formed, add 0.8 ml of DMEM to each DNA-cationic lipid from step 4.
7. Remove the Opti-MEM from each plate.
8. Add one DNA-LipofectACE complex per plate.
9. Incubate 5–6 hr at 37°C in a 5% CO₂ incubator.
10. Remove the DNA-LipofectACE complexes by aspiration, and add 2 ml of supplemented DMEM to each plate.
11. Incubate for 16 hr at 37°C in a 5% CO₂ incubator before isolating total RNA. The plates will become nearly 100% confluent after 16 hr growth.

Preparation of Total RNA

The following procedure uses TRIzol reagent (Life Technologies/GIBCO-BRL cat. no. 15596-026), a monophasic solution of phenol and guanidine isothiocyanate; it maintains the integrity of RNA, while disrupting cells and dissolving cell components.^(24,25) **Note:** Do not wash the cells prior to adding the TRIzol reagent.

1. Aspirate medium from well.
2. Add 1 ml of TRIzol reagent per 3.5-cm well.
3. Pass the cell lysate several times through a pipette and transfer to an autoclaved microcentrifuge tube.
4. Hold the sample for 5 min at room temperature.
5. Add 0.2 ml of chloroform per ml of TRIzol reagent and mix vigorously for 15 sec.

Manual Supplement

6. Hold the sample for 2–3 min at room temperature.
7. Centrifuge at 12,000g for 15 min at 4°C.
8. Transfer the upper aqueous phase containing the RNA to a fresh tube.
9. Add 0.5 ml of isopropanol per ml of TRIzol reagent originally added and mix well.
10. Hold for 5–10 min at room temperature.
11. Centrifuge at 12,000g for 10 min at 4°C. The RNA forms a gel-like pellet on the side and bottom of the tube.
12. Remove the supernate.
13. Add at least 1 ml of 75% ethanol per ml of TRIzol reagent originally added and mix well.
14. Centrifuge at 12,000g for 5 min at 4°C.
15. Air dry the pellet. **Note:** Avoid drying completely to enhance subsequent solubilization.
16. Dissolve the RNA in 50 μ l of DEPC-treated distilled water. A 5-min incubation at 50°C followed by a brief vortexing is recommended. RNA yields of 40–50 μ g per 3.5-cm well are obtained.

First-strand cDNA Synthesis

The cDNA synthesis reaction is catalyzed by SuperScript II RNase H[−] Reverse Transcriptase (SuperScript II RT). This enzyme has been modified to eliminate the RNase H activity (normally present in reverse transcriptases) that degrades mRNA during first-strand cDNA synthesis. The use of this enzyme results in higher yields of cDNA.⁽²⁶⁾ Because SuperScript II RT is not inhibited significantly by ribosomal and transfer RNA, it may be used effectively to synthesize cDNA from a total RNA preparation. The residual RNA should be stored at −20°C or −70°C.

In preparation for first-strand cDNA synthesis, set up a program on a thermal cycler that will run for 10 min at 70°C, 30 min at 42°C, and 15 min at 55°C. Preheat the cycler to 70°C and hold. Alternatively, water baths may be used for the following procedures:

1. Add the following components to a microcentrifuge tube: 1 μ l of oligonucleotide SA2 (20 μ M); RNA (1–3 μ g); DEPC-treated water (to a final volume of 12.0 μ l).
2. Incubate the mixture for 5 min at 70°C; place on ice for 1 min. Collect the contents of the tube by a brief centrifugation and add the following components at room temperature: 4 μ l of 5 $\times$ first strand buffer; 2 μ l of 0.1 M DTT; 1 μ l of 10 mM dNTP.
3. Mix gently, centrifuge briefly, and incubate for 2 min at 42°C.
4. Add 1 μ l (200 units) of SuperScript II RT per reaction, mix gently, and incubate for 30 min at 42°C (Life Technologies/GIBCO–BRL cat. no. 18064-014).
5. Incubate for 5 min at 55°C.
6. Add 1 μ l (2 units) of RNase H, mix gently, and incubate for 10 min at 55°C. RNase H (Life Technologies/GIBCO–BRL cat. no. 18021-014) digestion is conducted at a temperature at which RT is inactive. This eliminates the possible synthesis of snap-back structures and second-strand products.
7. Collect the reverse transcription reaction mixture by a brief centrifugation and place on ice. Remove 5 μ l for the primary PCR amplification described below.

Primary PCR

In this portion of the protocol, the first-strand cDNA is amplified by PCR using vector-directed oligonucleotides. Following six cycles of amplification,

the reaction mixture is digested with *Bst*XI, resulting in the elimination of two classes of background. PCR product background can result from pSPL3-derived molecules that contain only vector sequences or from products resulting from use of the cryptic splice donor (Fig. 2). Vector-only mRNA molecules arise from pSPL3 subclones that contain partial or alternatively spliced exons, exons in the antisense orientation, introns, or from subclones that do not contain an insert. Vector-only mRNA containing the *Bst*XI site is generated via the pairing of the vector splice donor and acceptor sequences. These molecules are converted into cDNA, made double-stranded, and digested with *Bst*XI to greatly reduce the entry of vector-only molecules into the secondary PCR reaction. This permits a higher complexity of DNA to be used per transfection than with the original vector, pSPL1.⁽¹²⁾ pSPL3 also contains *Bst*XI sites at either end of the multiple cloning site. Digestion with *Bst*XI at these sites results in reduction of products that result from the utilization of the cryptic splice donor site. Normally, when an exon is trapped, the MCS is spliced out, unless the cryptic splice donor site located 3' to the MCS is used (Fig. 2).

1. Add the following components to a 0.5-ml tube (this reaction and the secondary reaction can be scaled down to 10 μ l using 0.2-ml tubes): 5 μ l of first-strand cDNA reaction; 5 μ l of 10 $\times$ *Taq* DNA polymerase buffer; 1.5 μ l of 50 mM MgCl₂; 1 μ l of 10 mM dNTP; 2 μ l of oligonucleotide SA2 (20 μ M); 2 μ l of oligonucleotide SD6 (20 μ M). Sterile water to a final volume of 49.5 μ l.
2. Mix the contents of the tube and overlay with a drop of mineral oil.
3. Place the tube in a thermal cycler that has been preheated to 94°C and incubate for 5 min.
4. Reduce the temperature to 80°C and hold; add 2.5 units of *Taq* DNA polymerase.
5. PCR as follows: six cycles (1 min at 94°C, 1 min at 60°C, and 5 min at 72°C); 1 cycle (10 min at 72°C).
6. Hold reactions at 55°C, add 25 units of *Bst*XI to each, and incubate overnight at 55°C. *Bst*XI restricts the DNA in 1 $\times$ PCR buffer.
7. Add 5 units of *Bst*XI to each reaction and incubate for 2 hr at 55°C.

Secondary PCR

1. Add the following components to a 0.5-ml microcentrifuge tube: 5 μ l of *Bst*XI-treated primary PCR product; 4.5 μ l of 10 $\times$ *Taq* DNA polymerase buffer; 1.5 μ l of MgCl₂; 1 μ l of 10 mM dNTP; 1 μ l of oligonucleotide dUSA4 (20 μ M); 1 μ l of oligonucleotide dUSD2 (20 μ M). Sterile water to a final volume of 49.5 μ l. **Note:** An increased final yield has been observed when twice as much primer is used in the first PCR as the second.
2. Mix the contents of the tube and overlay with a drop of mineral oil.
3. Place the tube in a thermal cycler, and bring the temperature to 94°C for 5 min.
4. Lower the temperature to 80°C, hold, and add 2.5 units of *Taq* DNA polymerase.
5. PCR as follows: 30 cycles (1 min at 94°C, 1 min at 60°C, and 3 min at 72°C); 1 cycle (10 min at 72°C).
6. Electrophorese 3–5 μ l of each reaction on a 2% agarose gel to identify which reactions contain potential internal exons.

Cloning the Secondary PCR Products

There are several methods for cloning PCR products, including incorporation of restriction enzyme sites in the oligonucleotides, TA cloning, or using uracil DNA glycosylase (UDG) and modified primers.⁽²¹⁾ The UDG cloning proce-

ture is preferred because it takes about 30 min, avoids the risk of inefficient restriction digestion, cutting of restriction sites within an exon, and the inconsistent performance of TA cloning.

1. Add the following components to a microcentrifuge tube: 1–2 μ l of secondary PCR product (100 ng); 2 μ l of pAMP10 cloning vector (50 ng); 1 μ l of 10 $\times$ *Taq* DNA polymerase buffer; 1 μ l of UDG (1 unit); Distilled water to a final reaction volume of 10 μ l.

2. Mix; incubate 30 min at 37°C.

3. Transform 100 μ l of competent *E. coli* cells with 5 μ l of the annealing reaction and plate on LB plus ampicillin.

Evaluation of Transformants by Colony PCR

This is a rapid method to determine the size of the PCR product that has been cloned.

1. Pick each colony into 50 μ l of the following solution in a 0.5-ml tube (this procedure can also be scaled down to 10 μ l using 0.2-ml tubes): 5 μ l of 10 $\times$ *Taq* DNA polymerase buffer; 1.5 μ l of $MgCl_2$; 1 μ l of 10 mM dNTP; 1 μ l of oligonucleotide dUSA4 (20 μ M); 1 μ l of oligonucleotide dUSD2 (20 μ M); 0.5 μ l of *Taq* DNA polymerase (2.5 units). Sterile water to a final volume of 50 μ l.

2. Place a drop of mineral oil on each sample.

3. Place tubes in a thermal cycler preheated to 94°C and hold for 5 min.

4. PCR as follows: 30 cycles (45 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C); 1 cycle (10 min at 72°C).

5. Analyze PCR products on a 2% agarose gel.

The presence of a faint band at 177 bp results from the amplification of pSPL3 sequences; however, bands larger than 177 bp should be analyzed because they may contain internal exons.

Internal Exon Confirmation and Further Uses

After cloning candidate internal exons, there are a number of steps that should be taken to eliminate some common forms of background, including *Alu* and HIV-tat sequences. For convenience, individual colonies should be picked and stored in 96-well microtiter dishes; this increases the speed with which a large number of subclones can be screened for the presence of repetitive sequences such as *Alu* in human and B1 in the mouse. Colonies are propagated and stored in arrays that can be replicated onto filters and subsequently tested by hybridization to labeled high-molecular weight human or mouse Cot-1 DNA. A filter set should be hybridized against labeled HIV-tat intronic sequence (a template for labeling can be generated by PCR amplification of the intronic region of pSPL3). Following identification of repetitive and HIV-tat sequences, pairwise hybridization of filters containing candidate internal exons against labeled colony PCR-amplified candidate clones will rapidly identify a minimal set of clones that can be hybridized to a filter containing either yeast chromosomes or total yeast DNA that has been digested, electrophoresed, and transferred to a filter. Following elimination of yeast sequences, the minimal set can be sequenced and GenBank can be checked using BLAST.⁽²⁷⁾ The BLAST search will not only eliminate those clones that may be derived from the YAC cloning vector, but it will identify sequences in the DNA or protein sequence data base that the internal exon trapping protocol may have identified.

A critical step after the elimination of the various forms of background is determining whether a putative exon is expressed as part of an mRNA molecule. This can be accomplished in one of several ways, including zoo blotting, Northern blotting, or RT-PCR. Expressed sequences tend to be con-

served among related species; therefore, a human exon may hybridize to primate, bovine or even rodent DNA. A zoo blot is therefore a useful indicator of whether a sequence is expressed or not. A more rigorous test for expression, however, is provided by Northern blot analysis. Alternatively, if oligonucleotides are made to a putative exon, positive RT-PCR results can provide a definitive answer. Once a source of RNA is obtained in which a novel exon is expressed, the cDNA sequence can be lengthened using RACE or used as a hybridization probe to directly screen a cDNA library to obtain a full-length cDNA.

TRAPPING 3'-TERMINAL EXONS USING pTAG4

Most genes contain only one or at most a few 3'-terminal exons. This is in contrast to the number of internal exons a gene can contain. Very large genes can have a surprisingly large number of internal exons. For example, the number of exons from the dystrophin gene is 79.⁽²⁸⁾ 3'-Terminal exons also tend to be larger than internal exons,⁽²⁹⁾ and therefore make better hybridization probes than internal exons. The protocol described here is based on the analysis of a cosmid clone; however, this method can be adapted for the analysis of other (P1, YAC, etc.,) cloned DNAs. Recent improvements in the procedure, including DNase I treatment of COS-7 RNA and restriction digestion after primary PCR, result in a significant increase in the efficiency of 3' exon capture.

3'-Terminal exon trapping, like internal exon trapping, consists of four operations: subcloning cloned genomic DNA, transfecting the subcloned DNA into COS-7 cells, purifying the mRNA from the COS-7 cells, RT-PCR with vector-directed oligonucleotide primers, and UDG cloning. The procedure is depicted in Figure 3. Note that 3'-terminal exons are trapped because they provide flanking splice acceptor and poly(A)⁺ signals. This is in contrast to internal exon trapping, which captures sequences flanked by a splice donor and splice acceptor.

Subcloning into pTAG4

pTAG4 contains a multiple cloning site that can be uniquely cut with a variety of restriction endonucleases (see Fig. 4). For subcloning, the vector should be linearized, dephosphorylated, and ligated to the target genomic DNA. To test for the presence of functional 3'-terminal exons, either individual subclones or pools of subclones can be used. Use the same protocols to prepare vector and target DNA as described above of for internal exon trapping. When subcloning human or mouse genomic DNA that is cloned in a vector that uses ampicillin resistance for selection, digested genomic DNA clones should be dephosphorylated and ligated into linearized (nondephosphorylated) pTAG4. Alternatively, one can purify the vector away from the insert or choose an enzyme that will inactivate the ampicillin resistance gene (e.g., *PvuI*). Otherwise, a major fraction of the subclones will be religated genomic vector or pTAG4 containing additional copies of the β -lactamase gene from the starting vector.⁽³⁰⁾

Ligation and Transformation: Shotgun Subcloning of Cosmid DNA into pTAG4

1. Add distilled water to the following components for a final volume of 5 μ l: 50–100 ng of pTAG4 restriction enzyme-digested DNA; 100–200 ng of restriction enzyme-digested cosmid DNA; 1 μ l of 5 $\times$ of T4 DNA ligase buffer; 0.5 μ l (at least 0.5 unit) of T4 DNA ligase. Final volume of 5 μ l. Include a

Manual Supplement

FIGURE 3 Flow diagram of 3'-terminal exon trapping of a cosmid using pTAG4. YAC DNA is digested with a restriction enzyme, e.g., *EcoRI*. In this example, a single exon (shaded box) is contained in an *EcoRI* fragment. The exon is flanked by splicing acceptor (3') and polyadenylation sites. The pTAG4 vector is prepared for subcloning at the same restriction site. The MCS of pTAG4 contains 38 restriction enzyme sites. After subcloning genomic DNA into pTAG4, DNA is isolated and transfected into COS-7 cells. Total RNA is isolated and treated with DNase I before RNA-based PCR analysis. After generation of cDNA with adapter primer AP2 (AAG GAT CCG TCG ACA TC (T)₁₇), the first round of PCR is performed using the outside primer pair UAP2 (CUA CUA CUA CUA AAG GAT CCG TCG ACA TC) and SV40P (AGC TAT TCC AGA AGT AGT GA) followed by restriction digestion. A second round of PCR is performed using primer UAP2 and the nested primer dUAd2 (CAU CAU CAU CAU CAG TAC TCT TGG ATC GGA) to provide specificity and to install DNA sequences that allow UDG cloning of the PCR products. Trapped exons are recognized after gel analysis of the PCR products.

control (all the above components without cosmid DNA) to assess the level of vector self-ligation.

2. Mix gently and incubate for 1 hr at room temperature.
3. Transform *E. coli* with the ligated DNA.

4. Recover DNA by the plate scraping method described above from at least 100 colonies for every cosmid to be tested. **Note:** The efficiency of the shotgun subcloning can be assessed by cutting the pTAG4 library DNA with the same restriction enzyme that was used to subclone it and comparing it with the digested cosmid DNA by agarose gel electrophoresis.

5. Plate 0.1 and 0.01 ml of each transformation on LB plus ampicillin to assess the degree of vector religation. Repreparation of the dephosphorylated vector is recommended if the nonrecombinant frequency is >10%. The methods for COS-7 transfection and preparation of total RNA are the same as those used for the internal exon trapping method described above. Include mock and pTAG4 DNA transfections as controls.

FIGURE 4 Restriction map of pTAG4. The vector contains sequences that enable replication in *E. coli* and COS-7 cell hosts (bacterial and SV40 origins of replication are present). An ampicillin resistance marker (Ap^r) allows for selection of subclones. An MCS, located in the intron to the right of the adenovirus-2 exon 2, provides many restriction sites for the subcloning of genomic DNA (see side bar). After transfection of COS-7 cells, transcription occurs at high levels facilitated by the SV40 promoter (SV40 early region). Processing of the transcript results in removal of adenovirus introns via splicing and the appropriate vector splice donor (SDv or SDc) pairs with a splice acceptor in the subcloned genomic DNA (SDv is used if DNA is subcloned into sites 5' of CvnI in the MCS, SDc is used if DNA is subcloned into sites 3' of CvnI). If the subcloned genomic DNA contains a polyadenylation site, RNA is polyadenylated. 3'-Terminal exons are trapped from genomic DNA cloned into pTAG4 as a result of the interaction between a vector splice donor site (derived from adenovirus-2) and a splice acceptor site flanking an exon contained in genomic DNA.

DNase I Treatment

DNase I treatment of total RNA in combination with restriction digestion following the first PCR (see below) eliminates the major form of background that has been observed using the trapping vector pTAG4 (P.E. Nisson, unpubl.).

1. Resuspend RNA from a 3.5-cm dish ($\sim 10^6$ cells) in 50 μ l of DEPC-treated water.
2. Combine 17 μ l of the RNA with 2 μ l of 10 $\times$ DNase I buffer and 1 μ l of DNase I (Life Technologies/GIBCO–BRL cat. no. 18068-015).
3. Incubate at room temperature for 30 min; 65°C for 5 min.
4. Phenol/chloroform extract, ethanol precipitate, wash with 70% ethanol, dry, and resuspend in 17 μ l of DEPC-treated water.

cDNA Synthesis

RNA samples that provide positive signals can be useful as controls for cDNA synthesis and primary PCR. (For more information about SuperScript RT II, see First-Strand cDNA Synthesis, above).

1. Add the following components to a microcentrifuge tube:

Component	Amount
Adapter primer (AP2; 10 μ M)	2 μ l
RNA	1–3 μ g
DEPC-treated water	to a final volume of 12 μ l

2. Incubate the mixture for 5 min at 70°C to denature the RNA and chill for 1 min on ice. Collect the contents of the tube by brief centrifugation and add the following components:

Component	Volume (μ l)
5 $\times$ first strand buffer	4
0.1 M DTT	2
10 mM dNTP mix	1

3. Mix gently and collect the solution by brief centrifugation. Incubate for 2 min at 42°C.
4. Add 1 μ l (200 units) of SuperScript II RT, mix gently, and incubate for 30 min at 42°C (Life Technologies/GIBCO–BRL cat. no. 18064-014).
5. Incubate for 5 min at 55°C.
6. Add 1 μ l of RNase H, mix gently, and incubate for 10 min at 55°C. **Note:** RNase H digestion is conducted at a temperature at which SuperScript II RT is inactive. This eliminates the possible synthesis of snap-back structures and second-strand products.
7. Collect the reverse transcription reaction mixture by brief centrifugation and place on ice. Remove 1 μ l for the primary PCR amplification.

Primary PCR

1. Add the following components to a microcentrifuge tube:

Component	Volume (μ l)
RT reaction mixture	1
10 $\times$ <i>Taq</i> buffer (500 mM KCl, 100 mM Tris at pH 8.3)	5
50 mM MgCl ₂	1.5
10 mM dNTP mix	1
Oligonucleotide SV40P (10 μ M)	2.5
Universal amplification primer (UAP2; 10 μ M)	2.5
Distilled water	36

2. Mix the contents of the tube and overlay with a drop of mineral oil.
3. Place the tube in a thermal cycler that has been preheated to 94°C and incubate for 5 min.
4. Reduce the temperature to 80°C and pause; then add 2.5 units (0.5 μ l) of *Taq* DNA polymerase per tube.
5. Perform 10 cycles of PCR amplification as follows: Denature 94°C for 45 sec; anneal 55°C for 45 sec; extend 72°C for 1 min.
6. Incubate for an additional 10 min at 72°C and then hold the reaction at 4°C.

Restriction Digestion of Primary PCR Reaction

A second modification to the original protocol for 3'-terminal exon trapping is restriction enzyme digestion following the first PCR.⁽¹⁸⁾ A major form of background, presumably derived from the contamination of the COS-7 RNA with pTAG4 subclone DNA, can be controlled by two treatments: (1) DNase I treatment of RNA and (2) restriction enzyme digestion following the first PCR (P.E. Nisson, unpubl.). If the intervening sequence from the pTAG4 vector is present, then the restriction site that was used for subcloning will also be present and susceptible to restriction cleavage. This cleavage will prevent these molecules from being amplified.

1. Combine 17 μ l of the primary PCR reaction with 2 μ l of the appropriate 10 $\times$ restriction enzyme buffer and 1 μ l of the restriction enzyme used for subcloning into pTAG4.
2. Incubate for 1 hr at 37°C, dilute the reaction to 170 μ l with water, and use 1 μ l of this for the secondary PCR reaction.

Secondary PCR

There are two reasons for performing this reaction: (1) the secondary PCR amplification uses a primer (dUAd2) that is nested 3' to SV40P, which helps ensure the specificity of the amplified exons, and (2) the secondary primers contain dUMP residues for efficient directional UDG cloning into the pAMP1 vector.⁽²¹⁾

1. Add the following components to a 0.5-ml microcentrifuge tube:

Component	Volume (μ l)
Primary PCR reaction	1
10 $\times$ <i>Taq</i> buffer	5
50 mM MgCl ₂	1.5
10 mM dNTP mix	1
UAP2 (10 μ M)	2
Oligonucleotide dUAd2 (10 μ M)	2
Distilled water	37.5

2. Mix the contents of the tube and overlay with a drop of mineral oil.
3. Place the tube in a thermal cycler that has been preheated to 94°C and incubate for 5 min.
4. Lower the temperature to 80°C, pause, and add 2.5 units (0.5 μ l) of *Taq* DNA polymerase per tube.
5. Perform 30 cycles of PCR amplification as follows: Denature 94°C for 45 sec; anneal 55°C for 45 sec; extend 72°C for 1 min.
6. Incubate for an additional 10 min at 72°C and then hold the reaction at 4°C.

Agarose Gel Electrophoresis and Analysis of Secondary PCR Products

Electrophorese at least 3 μ l of each reaction in a 2% agarose gel. Compare the PCR products in the experimental lanes with the pTAG4 and mock transfection lanes. Occasionally, a faint pTAG4 PCR product may appear as an ~0.9-kb band with a smear. Also, in some cases, products derived from COS-7 RNA will be seen. All products other than the pTAG4 and COS-7 products are potential 3' exons and can be subcloned into pAMP1; alternatively, individual PCR fragments can be isolated. PCR products can be reamplified, sized, and labeled for use as a hybridization probe using colony PCR.

Cloning and Analyzing the Secondary PCR Products

The same options described for internal exon trapping are available for the analysis of secondary PCR products after 3'-exon trapping. After subcloning putative 3' exons, sequence analysis can indicate whether the trapped sequences are real 3' exons. The first criterion is that one of the pTAG4 splice donors has paired with a splice acceptor that flanks novel sequence. The second criterion is observing one of the major polyadenylation signal sequences (AATAAA or ATATAA) 10–30 nucleotides 5' to the polyadenylation site. Finally, if a novel sequence is expressed as mRNA as shown by Northern blotting or RT-PCR, the case will be strengthened that a trapped sequence is a bona fide 3' exon.

In summary, methods for capturing internal and 3'-terminal exons are described. A procedure for trapping internal exons from YACs is presented, followed by a protocol for capturing 3'-terminal exons from cosmid DNA. For the 3'-terminal exon-trapping procedure, methods for DNase I treatment of RNA and restriction enzyme digestion following the primary PCR have been developed and shown to greatly reduce background. These improvements have been integrated into the 3'-terminal exon-trapping protocol.

Note: Kits for internal (cat. no. 18449-017) and 3'-terminal exon trapping (cat. no. 18439-018) are available from Life Technologies/GIBCO-BRL, Inc.

ACKNOWLEDGMENTS

We acknowledge the supportive information provided by conversations with Alan Buckler, Deanna Church, Eric Green, David Krizman, and Michael North.

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Protocols for trapping internal and 3'-terminal exons.

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Genome Res. 1994 4: S24-S39

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