Exontrap

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Contents

1. Background

In the eukaryotic genome most genes are not encoded by a contiguous stretch of DNA but split into "exons". These exons are 10 to many 100 base pairs (bp) long and separated from each other by non-coding "intron" DNA. Only after transcription of the gene into RNA the exon sequences are appended to each other by the splicing mechanism resulting in mature mRNA. Introns, which are cut out of the RNA by the splicing mechanism, are in general much larger (100 bp up to 300 kb) than exon DNA. In addition, two neighboring genes are separated by large stretches of non-coding DNA ranging from 2 to 200 kb. Therefore, the vast majority of the eukaryotic genome is non-coding DNA. Without a specific probe it is thus difficult to find unknown genes in the genome. Exontrap allows the selective cloning of exon sequences from large genomic DNA fragments. These cloned exons can serve as probes to search for new genes in the eukaryotic genome. Exontrap is a new vector system for the selective cloning of exons. Intron sequences are removed before cloning considerably reducing the efforts of further protocols like, for example, sequencing the cloned DNA. Furthermore, the exontrap vector system enables the identification of a eukaryotic gene. The exontrap vector can be used to derive an exon library from a genomic library of a eukaryotic chromosome or a chromosomal region. This exon library could then, for example, be screened for cell-type-specific genes with radiolabeled cDNA from a panel of differentiated cells. The exontrap vector system allows an alternative route to the identification of a gene and the direct cloning of its central sequence.

2. Function of Exontrap

The exontrap function is based on a cloning vector, which includes a 5´ and a 3´ exon separated by a 600 bp intron sequence. This intron sequence contains a polylinker (multiple cloning site) with many unique restriction sites. The eukaryotic genomic DNA of interest is cloned into the polylinker (Figure 1). If the cloned DNA fragment contains an exon in the correct orientation, the vector expresses an RNA, in which the intron sequences originating from the vector as well as those being introduced are removed; only the exons are kept. This mature mRNA with only the exon sequences can then be reverse transcribed into DNA using a primer specific for the 3´ exon. The DNA is amplified *in vitro* and cloned.

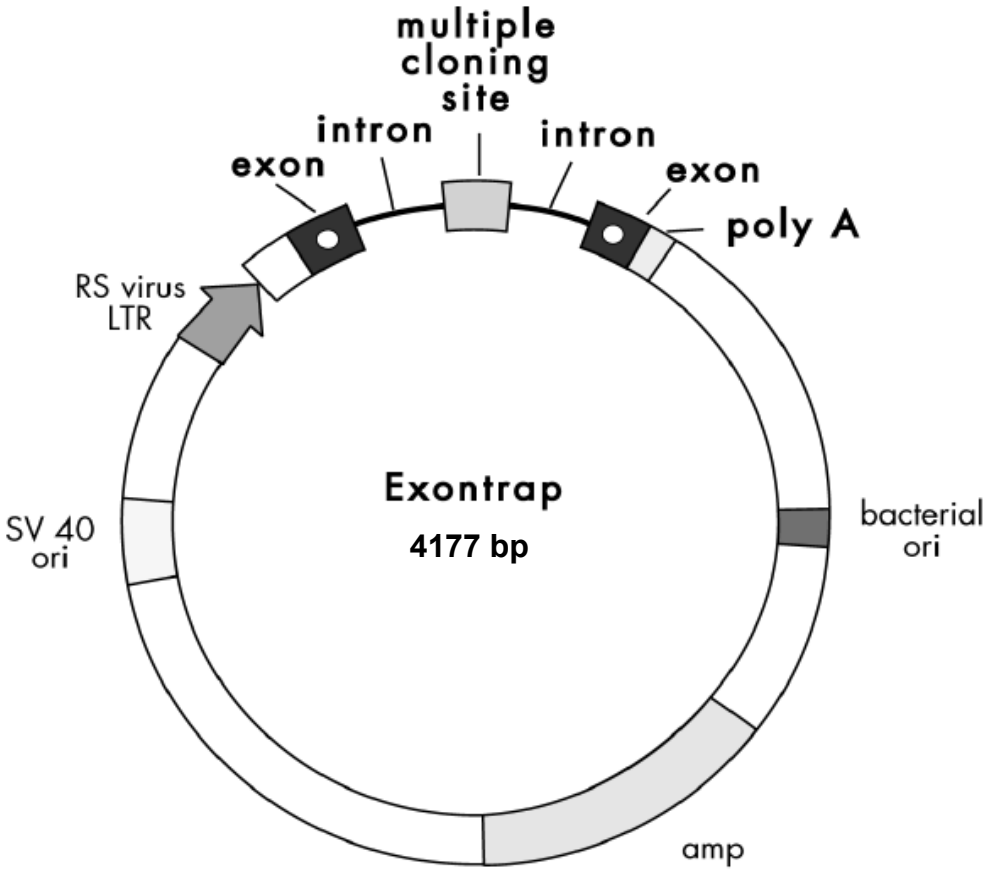


Figure 1: Map of Exontrap vector.

This function is realized by virtue of a shuttle vector (Figure 1) containing prokaryotic as well as eukaryotic genetic elements for replication (i.e. the vector can propagate in bacteria and mammalian cells as well). The intron containing multiple cloning site is framed by the 5´ splice donor site and by the 3´ splice acceptor site of an eukaryotic exon. This vector region is expressed under control of the eukaryotic strong „Long Terminal Repeat“(LTR) promoter of the Rous Sarcoma Virus (RSV) followed by a short stretch of the eukaryotic gene phosphatase. The 3´ splice acceptor site is followed by a 3´ polyadenylation site (poly A). These sequences are cloned into a vector comprising the ampicillin resistance gene and the prokaryotic pBR322 origin of replication as well as the SV40 origin of replication allowing its replication also in eukaryotic cells (if they express the large T antigen).

The exontrap vector expresses a poly A+ RNA containing the non-functional phosphatase sequence followed by the 5´ and the 3´ spliced exons. Between these two exons any inserted exon (if cloned in the correct orientation) will be expressed and can be cloned. The cloned exons can, for example, serve as probes which drastically facilitate the search for new genes in the eukaryotic genome.
The multiple cloning site with unique restriction sites is displayed in Figure 2. The primers are shown in Figure 2. The exon isolation and cloning is described in detail in Figure 4. All steps required in the protocol are listed in chapter 3.

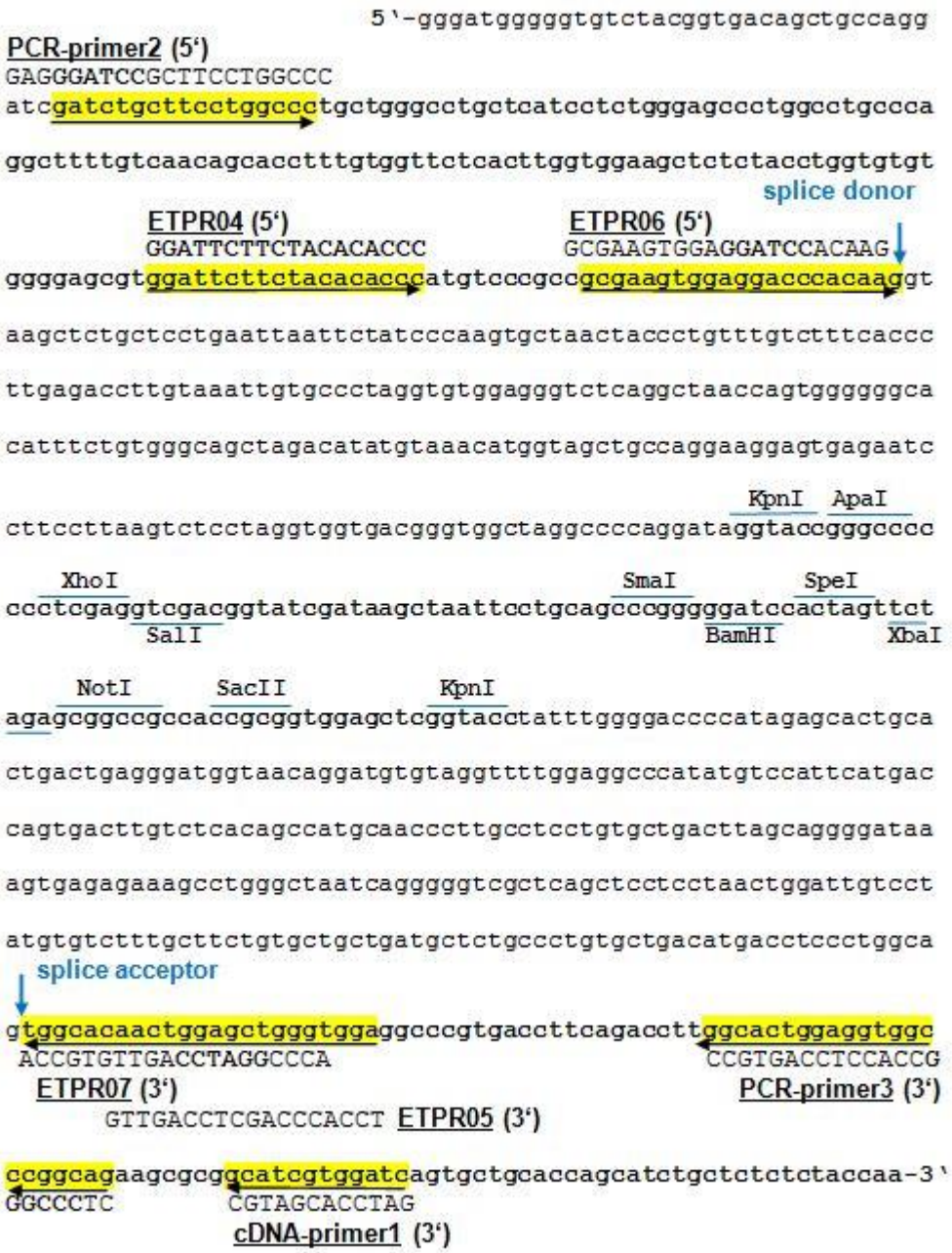


Figure 2: Position of the primers on the pET01 vector.
cDNA-primer 1: cDNA synthesis (3')
Primers for PCR amplification and cloning: PCR-primer 2 (containing a BamHI site) and PCR-primer 3 (containing a SmaI site; **Sequencing primers:** ETPR04 (5') and ETPR05 (3'); **Primers for amplification of exons and cloning:** ETPR06 (containing a BamHI site) and ETPR07 (containing a BamHI site); Multiple cloning site with convenient and unique cloning sites. Please note the presence of two KpnI sites.

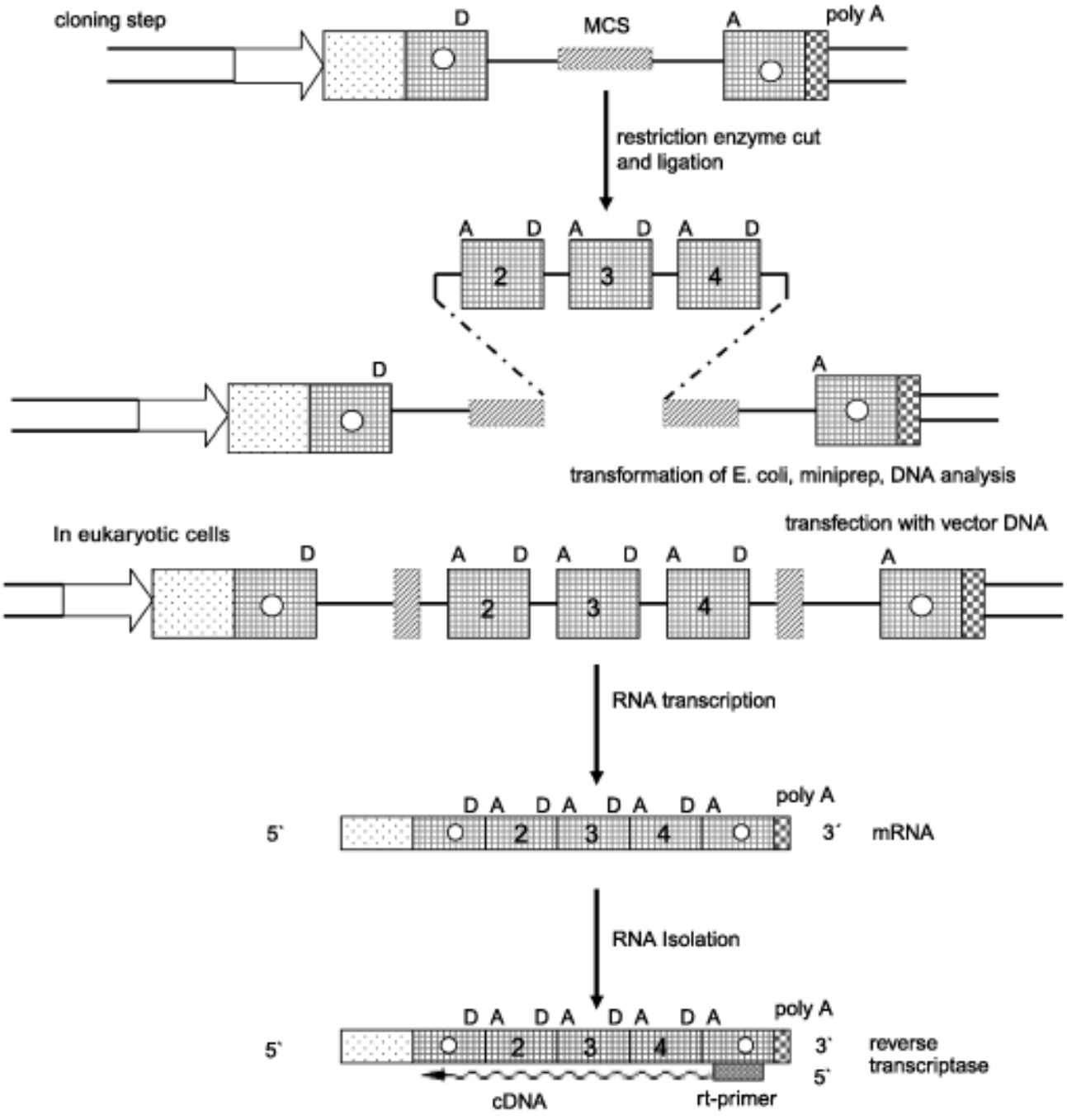


Figure 3: Outline of exon trapping procedure.

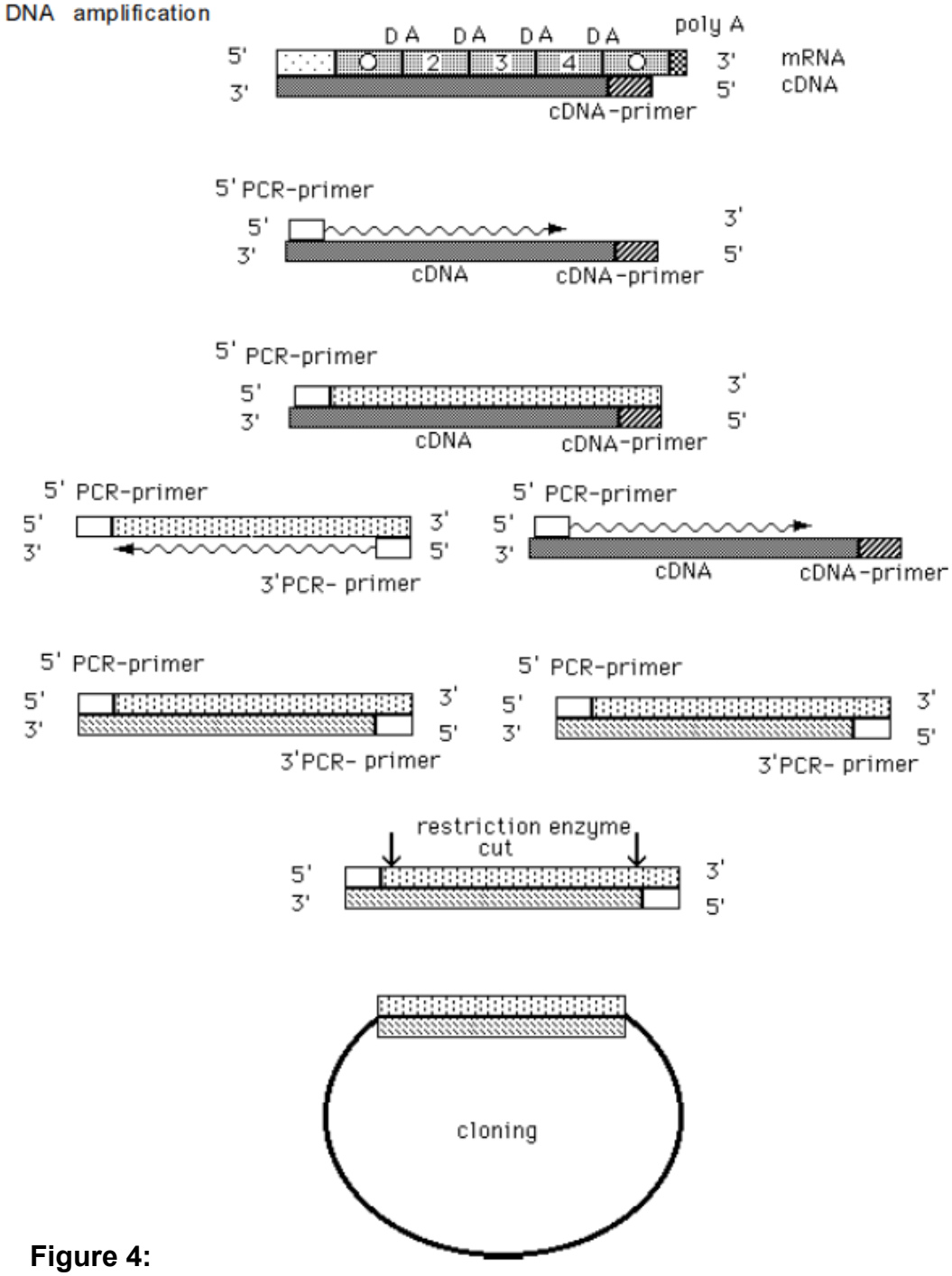
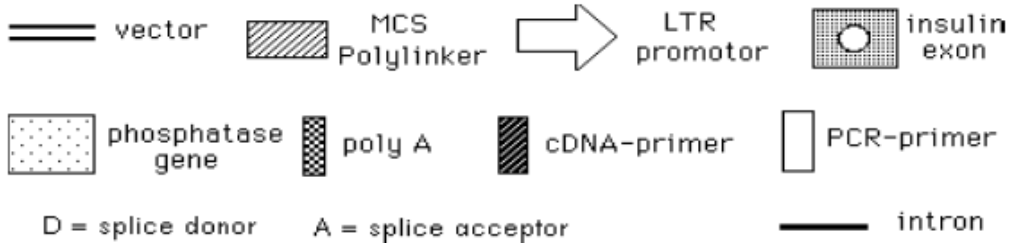

DNA amplification
poly A
D A D A D A D A
2 3 4
5'
3'
3'
5'
mRNA
cDNA
cDNA-primer
5' PCR-primer
cDNA
cDNA-primer
3'PCR- primer
restriction enzyme cut
cloning

Figure 4:


vector
MCS Polylinker
LTR promotor
insulin exon
phosphatase gene
poly A
cDNA-primer
PCR-primer
D = splice donor
A = splice acceptor
intron

3. Protocol

3.1 Summary

The DNA fragment of interest is cloned into the multiple cloning site of the Exontrap vector in both orientations (a general scheme is displayed in Figures 3 and 4). The orientation of the insert is determined by a restriction or sequence analysis. Two recombinant vectors are then expressed transiently in eukaryotic (for example COS-7) cells. After two days total RNA is isolated from the transfected cells. Only those mature mRNAs which contain the bordering vector exon sequences are reverse transcribed into cDNA by using a specific oligo primer complementary to a sequence of the 3´ bordering exon. This single-stranded cDNA is then amplified by polymerase chain reaction (PCR) using a pair of primers from the 5´ and 3´ bordering exons, respectively. These primers create restriction sites (BamHI and SmaI) which can be used for further cloning. Additional pairs of primers are available for sequencing the exons (sequencing primers ETPR04 and ETPR05) and for only the exons themselves (PCR primers ETPR06 and ETPR07). The amplified DNA fragment is then analyzed on an agarose gel. DNA fragments with unknown exons trapped between the two bordering exons are detected by a size shift when compared to the amplified DNA fragment containing only the bordering exons. The amplified size selected DNA fragments are then cut at restriction sites which are introduced at both ends by the primers used for PCR. The digested DNA fragments (with restriction site overhangs) are finally cloned for further analysis (for example, sequencing) into appropriate vectors. Details are listed in the following; buffers and solutions used are given in chapter 6. If alternative protocol steps are successfully applied in your laboratory, please change or modify the following suggested protocol.

3.2 Cloning of the gene of interest

The vector is cut with a convenient restriction enzyme in the multiple cloning site (see Figure 2). (Depending on cloning strategy: to avoid self-ligation, the ends of the vector or the ends of the gene can be dephosphorylated). The gene is ligated into the linearized Exontrap vector and the recombinant plasmid is used to transform *E. coli*. DNA minipreps are made from selected single colonies. The orientation of the cloned DNA in the plasmid is determined by restriction analysis or by partial sequencing. For unknown DNA both orientations are further processed in two parallel transfections; otherwise only the correct orientation is selected. 20 µg of plasmid DNA are required for transfection (see chapter 3.3).

3.3 Harvesting the eukaryotic COS cells

The following protocol is listed for one transfection experiment. Several transfection experiments can be performed in parallel. COS-7 cells (SV40 transformed African green monkey kidney cells, ATCC CRL 1651) are recommended for the transfection. The eukaryotic cells grow adherent to a density of 10^6 cells per culture plate (9 cm plates with 10 ml medium) in cell culture medium (see 6.1). To harvest COS cells, the medium is aspirated off and the plate is washed once with 10 ml sterile PBS (6.2) or cell culture medium (6.1). Then 2 - 4 ml of trypsin-EDTA solution (6.3) is added to the plate. After 5 to 10 minutes incubation at room temperature or 37 °C the cells detach from the plate. The cells are suspended and the solution is collected in a 15 ml tube. The plate is washed with about 10 ml cell culture medium (6.1) and added to the cell suspension in the 15 ml tube. The cells are pelleted by centrifugation for 10 minutes at 250 - 300 g at room temperature. The pellet is dissolved in 0.5 ml Tris saline buffer (6.4; for the DEAE dextran method see chapter 3.4.2) or 0.5 ml cell culture medium (6.1;

for the electroporation method (see chapter 3.4.)). The cell number is counted (for example with a haemocytometer, using trypan blue). The cell suspension (in Tris saline buffer or cell culture medium) is adjusted to 10^6 cells per ml buffer. 1 ml of the cell suspension (10^6 cells) is pipetted into a 15 ml tube or 1.5 ml tube (depending on the method to follow).

3.4 Transfection of the eukaryotic COS cells with recombinant vector DNA

The transfection can be made either by the electroporation or, alternatively, by the DEAE dextran method. Both methods obtain the same yield.

3.4.1 Electroporation method

The cell suspension (in cell culture medium) is kept at 0 °C on ice. 10 - 20 µg of the plasmid DNA (circular recombinant Exontrap DNA) diluted in TE buffer (chapter 6.5) at pH 7.5 is mixed with 1 ml of the cell suspension in a 1.5 ml reaction tube by vortexing. The concentration of the DNA should be about 0.1 to 1.0 mg/ml. The mixture is pipetted into the pulse cuvette and kept at 0 °C on ice until the electroporation is performed. Electroporation is made for example with a Gene Pulser (BioRad) at 1200 Volt and 25 µF. After electroporation, 10 ml of cell culture medium (chapter 6.1) is added and the cell suspension is kept for 2 days in 9 cm plates in the incubator at 37 °C, 5% CO_2.

3.4.2 DEAE dextran method

The cell suspension (in Tris saline buffer) is kept at 37 °C until the transfection mix is added. Circular recombinant Exontrap DNA is diluted to 100 ng/µl in TE buffer (chapter 6.5) at pH 7.4. 50 µl of this DNA solution (5 µg) is mixed with 500 µl transfection buffer (chapter 6.6) in a 1.5 ml reaction tube and mixed by vortexing. This mixture is incubated for 1 hour at 37 °C. After incubation, the transfection mixture (550 µl) is added to the 1 ml cell suspension (10^6 cells) in the 15 ml tube, the solution is mixed well and kept for 90 minutes at 37 °C in the incubator (under 7% CO_2). After this incubation, 10 ml of cell culture medium (chapter 6.1) is added and the cells are pelleted at 250 to 300 g for 10 minutes. The pellet is resuspended in 10 ml culture medium. The cell suspension is kept for 2 days in 9 cm plates in the incubator.

3.5 RNA preparation

3.5.1 Cell treatment

To harvest the transfected cells, the medium is aspirated off and the plate is washed once with 10 ml of sterile PBS (chapter 6.2) or cell culture medium (6.1). To each plate 2 - 4 ml of trypsin EDTA solution (6.3) is added. After 5 to 10 minutes incubation at room temperature or 37 °C, the cells detaching from the plate are transferred into a 15 ml tube. The plate is then washed twice with 5 ml PBS and both washing solutions (10 ml) are combined in the 15 ml tube. The cell suspension is centrifuged at 250 - 300 g for 10 minutes, the pellet is taken up in 1 ml PBS at 4 °C, transferred into a 1.5 ml tube and washed twice in 1 ml PBS (centrifugation at 2,000 rpm for 30 seconds in a microfuge).
After the last centrifugation the cell pellet is loosened, resuspended in 100 µl RNA extraction buffer (6.7) and mixed well by vortexing. Cells are lysed by the addition of another 100 µl of RNA extraction buffer (6.7) containing 1% Nonidet P-40 (NP-40, Sigma) and then incubated at 4 °C for 5 minutes. The cell debris is pelleted at 12,000 rpm for 1 minute at 4 °C in a microfuge. The RNA-containing supernatant is transferred to a new 1.5 ml tube, mixed with

200 µl PK buffer (chapter 6.8) and incubated for 30 minutes at 37 °C in the presence of 50 µg/ml proteinase K (final concentration).

3.5.2 Phenol extraction and DNA degradation

After addition of 400 µl phenol/chloroform (1:1 (v/v)), the tube is vortexed and centrifuged for 5 minutes at 12,000 rpm in a microfuge. The aqueous phase is taken off and transferred into a new 1.5 ml tube. This phenol extraction is repeated. The aqueous phase is mixed with 400 µl cold (0 °C) isopropanol and kept on ice for 30 minutes. The RNA is centrifuged at 13,000 rpm for 10 minutes at 4 °C, the RNA pellet is washed with 70% ethanol. The 70% ethanol solution is removed completely, the RNA pellet is dried (for 10 minutes at room temperature) and then dissolved in 100 µl distilled water (10 minutes at 56 °C). From this solution 1/7 volume can be used directly for cDNA synthesis.

Removal of DNA (helpful, but not necessary):

The DNA still present in the RNA solution is degraded by the addition of 100 µl DNase mix (chapter 6.9) and incubation for 15 minutes at 37 °C. The DNA digestion is stopped after 15 minutes by the addition of 20 µl stop solution (6.10). Then, 300 µl phenol/chloroform (1:1 (v/v)) is added, the solution is vortexed and the phases are separated by centrifugation at 13,000 rpm for 5 minutes. The aqueous RNA containing solution is transferred into a new RNase-free 1.5 ml tube.

The RNA is precipitated by addition of 25 µl 3 M sodium acetate at pH 5.2 and 600 µl ethanol (96%), incubation at -20 °C for 30 minutes or overnight. After precipitation, the tube is centrifuged at 13,000 rpm for 20 minutes. The RNA precipitate is dissolved in 200 µl TE (ch. 6.5) at pH 7.2. To this RNA solution 500 µl ethanol is added. This mixture is stored at -20 °C.

3.6 Synthesis of cDNA

cDNA synthesis and PCR amplification are performed in the same buffer. 100 µl RNA/ethanol solution (one seventh of the RNA preparation) are mixed with 10 µl 3 M Na-acetate, pH 5.2 in a 1.5 ml reaction tube, mixed and kept for 5 minutes at 4 °C. The RNA is then pelleted at 13,000 rpm for 10 minutes. The RNA pellet is washed with 1 ml 70% ethanol followed by centrifugation at 13,000 rpm for 10 minutes, dried and resuspended in 20 µl H_2O. After 20 minutes on ice, 20 µl cDNA synthesis/PCR solution (chapter 6.11) is added to the 20 µl RNA solution. cDNA synthesis is performed at 37 °C for 1 hour using reverse transcriptase from AMV (avian myeloblastosis virus).

3.7 Amplification of cDNA by PCR

After cDNA synthesis,

1 µl 10 x PCR buffer (6.12)
2 µl PCR-primer 2 (10 µM; see 6.14)
2 µl PCR-primer 3 (10 µM; see 6.14)
5 µl H_2O

are added to the 40 µl cDNA synthesis/PCR solution. These two primers (PCR-primer 2 and 3) create 2 restriction sites (BamHI and SmaI) which can be used for cloning of the amplified exons (see 3.8). The total 50 µl solution is pipetted into a MoBiTec PCR tube (no need to cover with paraffin oil) and incubated at 95 °C for 5 minutes. Then, the reaction tube is taken out of the 95 °C bath, 0.5 units Taq polymerase is added and the tube is placed into a thermocycler. The polymerase chain reaction is performed with the following parameters:

25 to 30 cycles of
0.5 to 1 minute at 94 °C,
0.5 to 1 minute at 57 - 60 °C and
0.5 to 1 minute at 72 °C.

(At 57 °C we observed higher yield but more unspecific products, at 60 °C we observed less yield but little unspecific products).

After the last cycle, the tubes are incubated for further 10 minutes at 72 °C. After PCR, 10 µl of the aqueous reaction mix (one fifth of the 50 µl total volume) is mixed with sample buffer including gel dye markers and analyzed on an agarose gel.

3.8 Cloning and sequencing of the PCR fragments

20 µl of a PCR sample including the amplified exons (identified on the agarose gel) is digested with the restriction endonucleases BamHI and SmaI. The restricted DNA fragments are cloned into vectors (for example pUC19, pTZ19, pMEX, pAX, etc.). Alternatively, the amplified DNA fragments can be filled-in using Klenow enzyme. These blunt-ended fragments can be digested with BamHI and SmaI for cloning. The cloned fragments can be enzymatically sequenced using the 5´ and 3´sequening primers (ETPR04 and ETPR05). The inserted exons themselves can be amplified using the PCR primers ETPR06 and ETPR07.

4. General Comments

4.1 Working with RNA

1. All glass ware should be treated by heating to 220 to 240 °C for more than 2 hours.
2. Sterile plastic ware should be autoclaved for 30 min.
3. Solutions, water and buffers should be stirred with DEPC (diethylpyrocarbonate, 50 µl per 100 ml liquid) for some minutes and then cooked for 20 min (or autoclaved). SDS, Triton X-100, and glycerol cannot be treated with DEPC.
4. All materials, which cannot be autoclaved, should be treated for 10 minutes with 0.1 M NaOH and then washed with RNase-free water.
5. Lab equipment should be washed with ethanol.
6. Wear gloves.

(see also: Sambrook *et al.*, 2000; Molecular Cloning: A Laboratory Manual)

4.2 Exons containing particular restriction sites

If the restriction site used for cloning into the Exontrap vector is also present in an exon, this exon will not be trapped. Therefore, exon libraries should be derived from several libraries each of them obtained from a different restriction enzyme digest. In case the exon sequences contain a BamHI or SmaI site, the PCR primer containing these sequences can bind partially also to these sequences and can give additional PCR products. This contamination can be reduced by increasing the temperature during the PCR reaction. Furthermore, when using these sites (BamHI and SmaI) for cloning of the amplified DNA, more than one fragment might be cloned. To avoid this, the amplified fragments can be filled-in using Klenow enzyme followed by blunt-end ligation into a cloning vector.

With the help of the Exontrap vector system, only exons are cloned that contain a splice site on each side.

4.3 Alternative splicing

In case the eukaryotic gene is spliced *in vivo* in different ways ("alternative splicing“), the RNA of the cloned sequences in the Exontrap vector delivered into COS cells will be also spliced accordingly. Thus, also in the Exontrap system all splicing products will be obtained yielding in more than a single PCR product.

4.4 The first and last exon of eukaryotic gene

With the help of the Exontrap vector system, only those exons are cloned which contain a splice site on each side (donor and acceptor site). The first exon containing only a 5´ donor splice site is not trapped by the Exontrap vector. Similarly, the last exon containing only the 3´ acceptor splice site will not be trapped. Most 3´ exons, however, could be cloned if instead of the 3´ PCR primer a poly(dT) primer is used to reverse transcribe the spliced mRNA into cDNA. The cDNA can then be amplified by PCR using the Exontrap vector 5´ PCR primer and poly(dT) as 3´ primer followed by classical cloning procedures.

5. Literature

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6. Buffers

6.1 Cell culture medium:

10% FCS (fetal calf serum)
100 u/ml penicillin/streptomycin
2 mM glutamine
0.05 mM ß-mercaptoethanol
1 x RPMI 1640 (Flow Lab)

The FCS is heat-inactivated (at 56 °C for 30 minutes).

6.2 PBS:

0.15 M NaCl
0.1 M phosphate buffer (KH_2PO_4, K_2HPO_4) pH 7.2

6.3 Trypsin-EDTA-solution:

0.5 g Trypsin
0.2 g EDTA
0.85 g NaCl
add 1000 ml sterile distilled water.

The ready-mixed trypsin EDTA solution from Gibco/BRL (#043.05300H) can be used.

6.4 Tris saline buffer:

8 g NaCl
0.38 g KCl
25 mM Tris/HCl at pH 7.4
add 1000 ml distilled water.

6.5 TE buffer:

50 mM Tris/HCl at pH 8.0
1.25 mM EDTA at pH 8.0

6.6 Transfection buffer (10 ml)**:**

6.55 ml RPMI
3.20 ml 50 mM Tris at pH 7.4
0.25 ml DEAE dextran, 10 mg/ml in distilled water, autoclaved.

6.7 RNA extraction buffer:

0.14 M NaCl
1.5 mM $MgCl_2$
10 mM Tris/HCl at pH 8.6
1 mM dithiothreitol (DTT)
20 mM vanadyl ribonucleoside complex.

6.8 PK buffer:

0.2 M Tris/HCl at pH 8.0
25 mM EDTA at pH 8.0
0.3 M NaCl
2% SDS.

6.9 DNase mix (1 ml) contains**:**

100 µl 10 x DNase buffer (6.13)
1 µl 1 M dithiothreitol (DTT)
500 units RNasin
100 µl (100 units) DNase I (100 µg/ml)
made up to 1 ml with sterile distilled water.

6.10 Stop solution:

200 µl distilled water
24 µl 10% SDS
24 µl 0.5 M EDTA at pH 8.0.

6.11 cDNA synthesis/PCR buffer:

20 µl 2 x cDNA synthesis/PCR solution:

4 µl 10 x PCR buffer
0.4 µl 100 mM DTT
4 µl 2 mM dNTPs
2 µl cDNA-primer 1 (10 µM)
0.4 µl RNasin (10 units/µl)
9 µl H_2O
0.2 µl reverse transcriptase (50 units/µl)

6.12 10 x PCR buffer:

100 mM Tris/HCl at pH 8.3
500 mM KCl
15 mM $MgCl_2$
0.01% gelatin

6.13 10 x DNase buffer:

400 mM Tris/HCl at pH 7.8
100 mM NaCl
60 mM $MgCl_2$

6.14 Primer solutions

Resuspend the primer in sterile distilled water.
To obtain a primer concentration of 10 µM dissolve each primer in a total volume of 25 µl as described below in the table:

Primer	length (bases)	Volume (µl)
cDNA-primer 1	12	25
PCR-primer 2	21	25
PCR-primer 3	22	25

Primer sequences

cDNA-primer 1 (for cDNA synthesis)
5'-GATCCACGATGC-3'

PCR-primer 2 (forward)
5'-GAGGGATCC GCTTCCTGGCCC-3'
BamHI

PCR-primer 3 (reverse)
5'-CTCCCGGGCCACCTCCAGTGCC-3'
SmaI

PCR-primer 2 and 3 are used for amplification of exons and cloning into a vector using BamHI and SmaI sites. Cloned exons can be subsequently verified by sequencing analysis.

ETPR04 (forward, for sequencing of exons)
5'-GGATTCTTCTACACACCC-3'

ETPR05 (reverse, for sequencing of exons)
5'-TCCACCCAGCTCCAGTTG-3'

ETPR06 (forward)
5'-GCGAAGTGGAGGATCCACAAG-3'
BamHI

ETPR07 (reverse)
5'-ACCCGGATCCAGTTGTGCCA-3'
BamHI

The exon probes can be amplified using PCR primers ETPR06 and ETPR07, and after digestion subcloned into a vector using BamHI cloning site.

7. Order Information, Shipping, and Storage

Order#	Product	Quantity
K2010	Exontrap Kit: pET 01 Exontrap vector, 5 µg lyophilized cDNA-primer 1 PCR-primer 2 PCR-primer 3	Kit
PET01	Exontrap vector pET01, lyophilized DNA	5 µg
shipped at RT; store at 4 °C		

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