Exontrap

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

1. Introduction

In contrast to bacteria, most eukaryotic genes do not consist of one single continuous amino acids-encoding stretch of DNA, but are split into encoding “exon” parts and large stretches of noncoding “intron” parts. After transcription of the gene into pre-mRNA, the exon sequences are appended to each other by the splicing mechanism resulting in mature mRNA. The classic approach for getting the coding part of a eukaryotic gene is therefore via the isolation of mRNA. Depending on whether there is any sequence information of the gene of interest known, the cDNA can be used for direct cloning by polymerase chain reaction (PCR) or, alternatively, for constructing a cDNA gene bank (e.g., phage gene bank) and further screening.

The here described Exontrap system uses an alternative route for the identification of eukaryotic genes. It does not involve an initial isolation of cellular mRNA, but allows the selective cloning of exon sequences from large genomic DNA fragments. One big advantage is that genes, which are not transcribed during certain life cycle stages, can also be identified. Exon/intron mapping is greatly facilitated since for the determination of exon boundaries only the trapped exons have to be sequenced and compared to the known gene.
Starting with genomic DNA, an exon library can be derived and screened for cell type-specific gene expression with labeled cDNA from a panel of differentiated cells.

2. The Exontrap System

The Exontrap system is based on a shuttle vector (*E. coli* / eukaryotic cells) with an intrinsic splicing function, allowing selective cloning of exon sequences from large genomic eukaryotic DNA fragments. For trapping exons of genomic DNA fragments, the Exontrap vector pET01 already contains an own 5´ and 3´ exon separated by a 600 bp intron sequence, which contains a multiple cloning site (MCS).
First the genomic fragment of interest, containing introns and exons, is ligated into the multiple cloning site of pET01 and propagated with *E. coli*. The recombinant vector is purified and transfected into eukaryotic cells, e.g., COS cells, and transcribed. The pre-mRNA is processed, i.e., the intron sequences originating from the vector as well as those being introduced, if cloned in the correct orientation, are removed; only the exons are kept in the mature mRNA. After total RNA isolation, the mature mRNA is reverse transcribed into cDNA using a specific primer complementary to a sequence of the bordering exon (included in the kit). The cDNA is amplified by PCR using specific primers (included in the kit), which create restriction sites for further subcloning. The cloned exons can, for example, serve as probes which drastically facilitate the search for new genes in the eukaryotic genome. For an overview on the whole exon trapping and cloning procedure see Fig. 3 and 4. The map of pET01 is shown in Fig.1. The exon-intron-exon part of the sequence with depicted restriction sites of the MCS, cDNA primers, sequencing, and cloning primers is shown in Fig. 2.

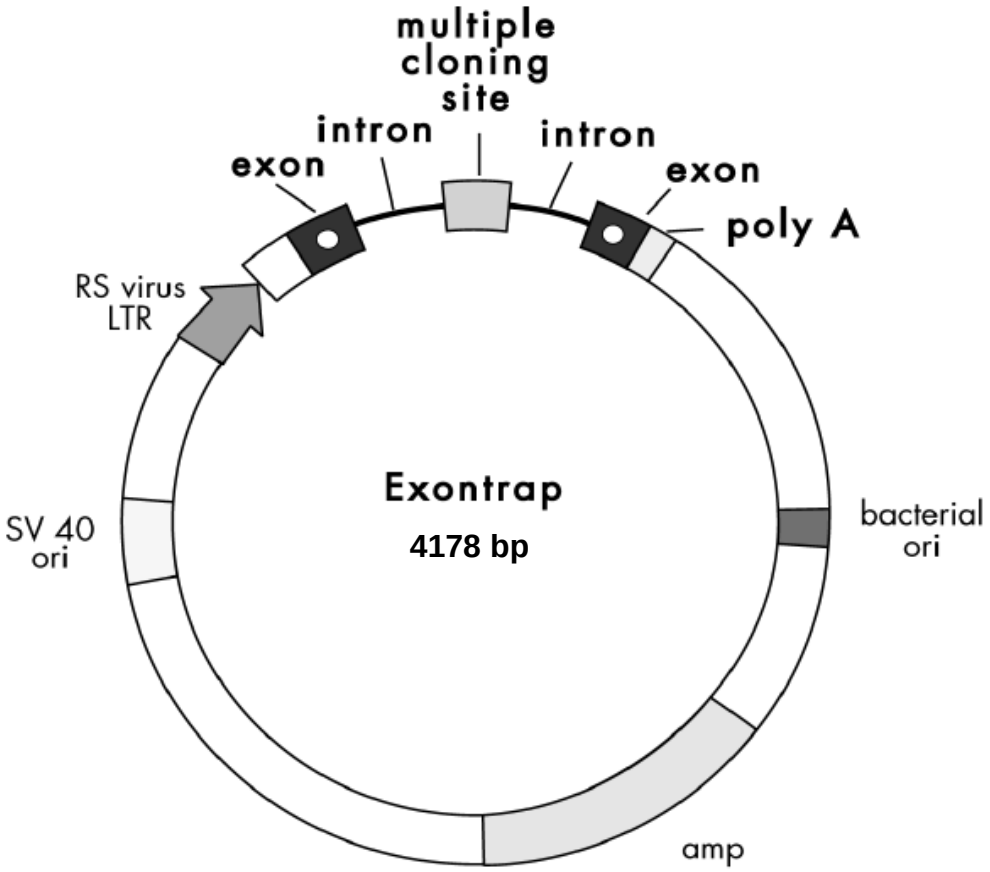

Fig. 1: Features of Exontrap vector.
The intrinsic splicing function of the Exontrap vector is realized by a given exon/intron/exon structure within the vector. The vector-encoded intron sequence contains a multiple cloning site for inserting the genomic fragment of interest. Upstream of the first vector-encoded exon, a truncated part of the eukaryotic phosphatase gene is included. Downstream of the second vector-encoded exon is a polyadenylation signal localized. The transcription of the phosphatase-exon-intron-exon area is under control of the strong „Long Terminal Repeat“ (LTR) promoter of the Rous Sarcoma Virus (RSV). Any genomic DNA that has been inserted into the MCS before, is now included in the synthesized pre-mRNA. After processing, a poly(A)+ mRNA is generated. This contains (5’ to 3’) the non-functional phosphatase gene, the first vector-encoded exon, all exons of the cloned genomic fragment, the second vector-encoded exon, and a polyA tail. All intron sequences are spliced off.
For cloning in *E. coli*, the Exontrap vector contains a bacterial origin of replication, belonging to the ColE1 incompatibility group, and the β-lactamase gene coding for ampicillin resistance. Propagation in eukaryotic cells expressing large T antigens is facilitated through the SV40 origin.

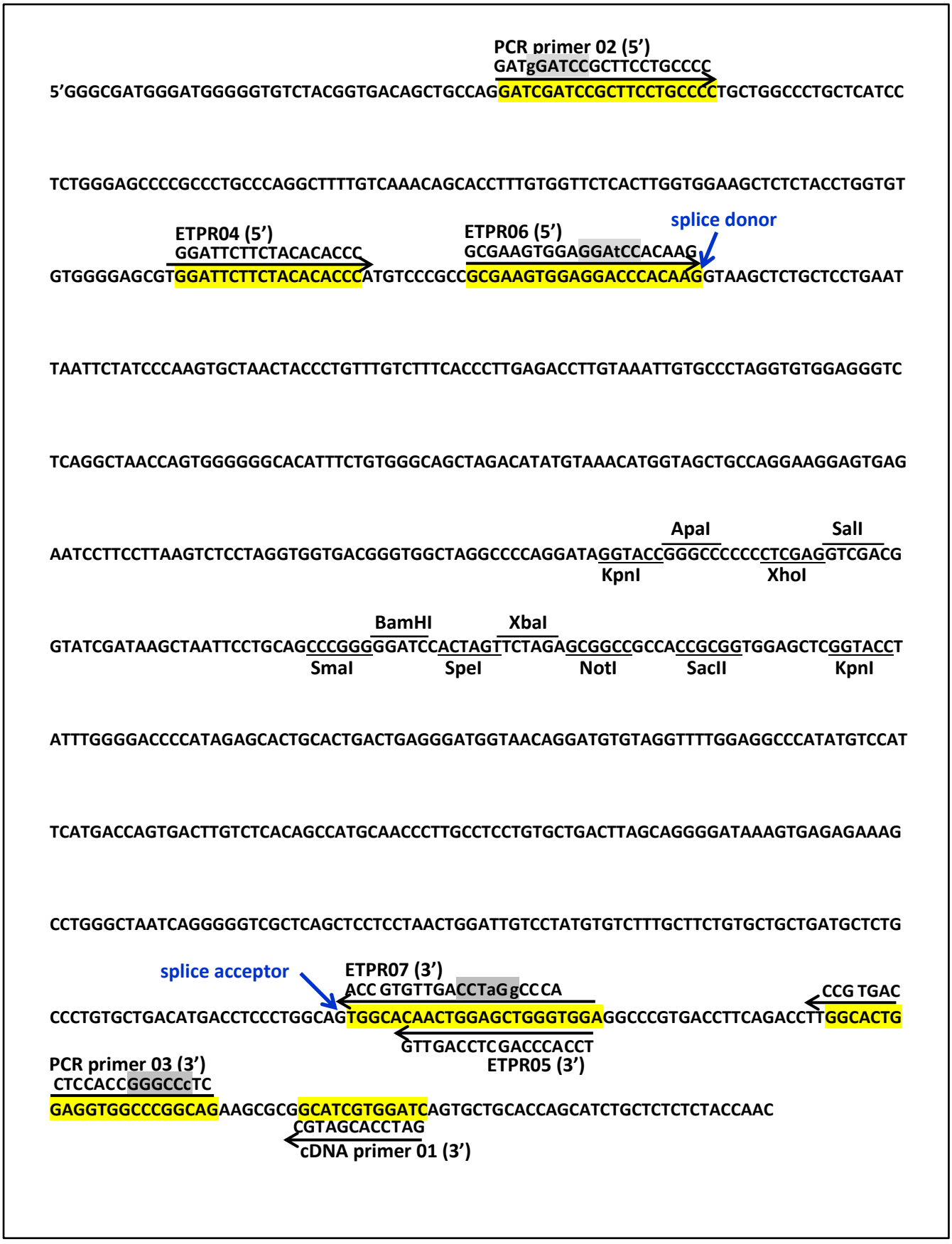

Fig. 2: for explanation see next page.

Fig.2: Exon-Intron-Exon part of pET01.
The splicing sites build the borders between the exons and the intron sequence. The sequence has the following structure:

Exon1 - Splice Donor Site - Intron with MCS - Splice Acceptor Site - Exon2 - polyA site

The sequence of the polyA site is not shown. The restriction sites of the MSC are underlined. Please note the presence of two KpnI sites.

The following primer sequences with their binding sites are depicted in the figure:

cDNA primer 01 for cDNA synthesis (3')

PCR primer 02 and **PCR primer 03**
for PCR amplification and cloning with BamHI and SmaI site, resp.

ETPR04 (5') and **ETPR05 (3')** for sequencing

ETPR06 and **ETPR07** (each containing a BamHI site)
for amplification of exons and cloning

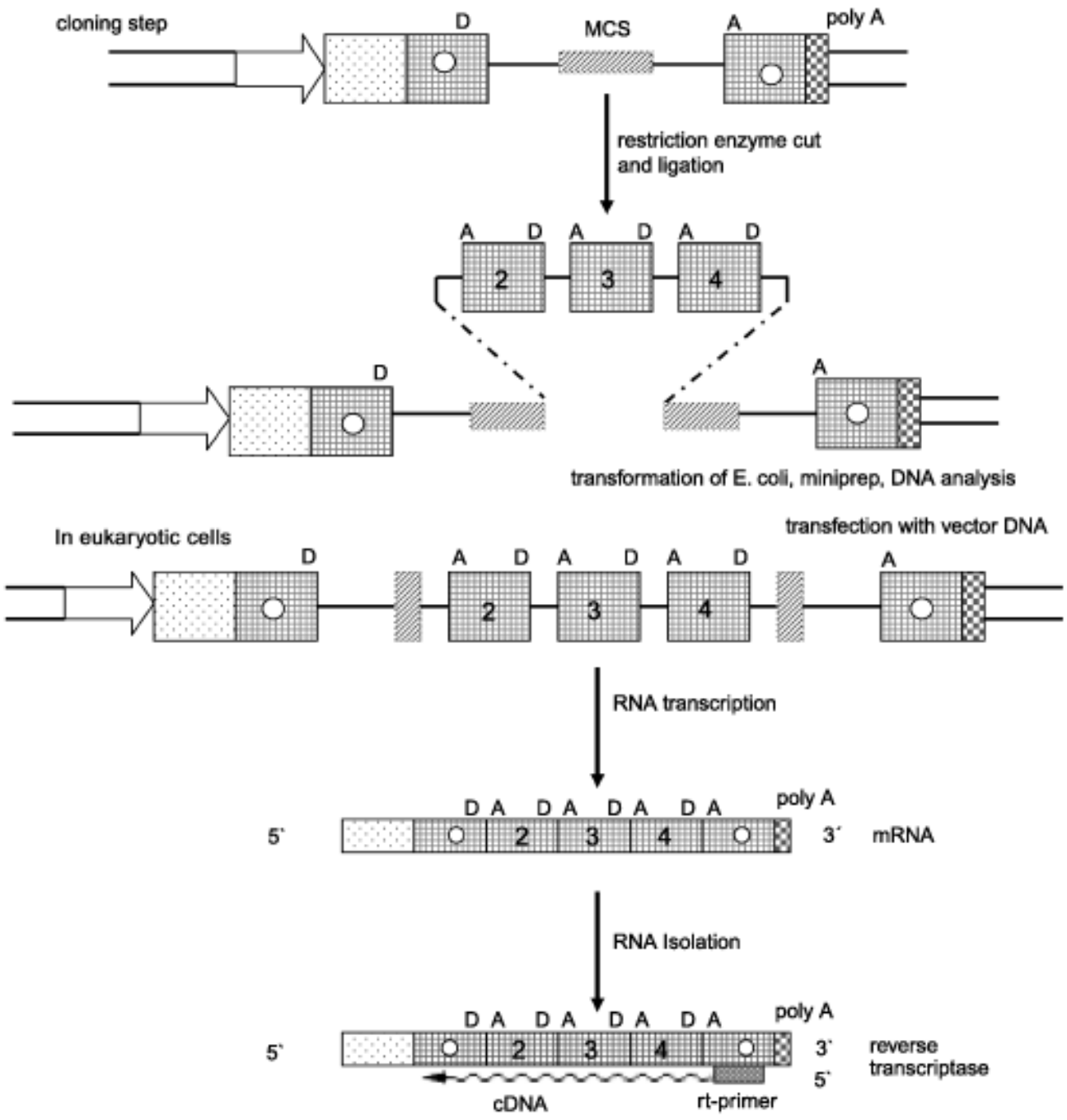

Fig. 3: Outline of exon trapping procedure.
For a detailed description of all steps see 3.1 Procedure of Exon Trapping

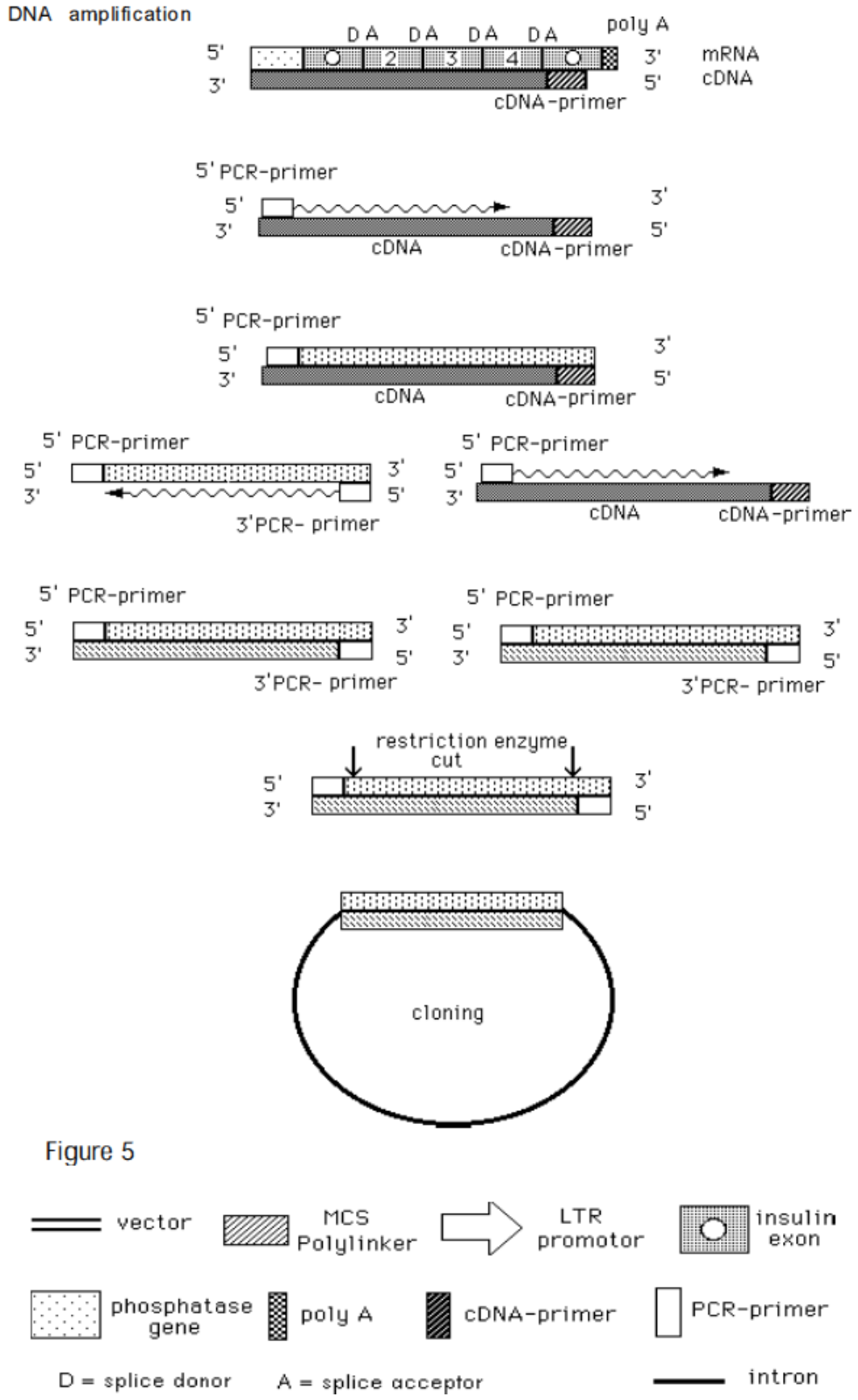

Fig. 4: Outline of exon cloning procedure.
For a detailed description of all steps see 3.1 Procedure of Exon Trapping

3. Protocol

3.1 Procedure of Exon Trapping

The procedure of exon trapping requires the following steps: (a general scheme is displayed in Figures 3 and 4).

- The genomic DNA fragment of interest is cloned into the MCS of the Exontrap vector. For unknown DNA, both orientations of the fragment have to be cloned and processed further in in two parallel preparations (see below).

- Propagate the Exontrap construct with *E. coli* using ampicillin as selection maker.

- Isolate the Exontrap plasmid DNA with a commercially available plasmid DNA miniprep kit. Make sure that each preparation contains only one kind of exontrap constructs with genomic DNA fragments of the same orientation. For unknown DNA, both orientations of the fragment are further processed in two parallel transfections; otherwise only the correct orientation is selected.

- Use the Exontrap construct for transfecting eukaryotic cells. We recommend COS-7 cells (SV40-transformed African green monkey kidney cells, ATCC CRL 1651). Use commercially available transfection reagents. We recommend the following reagents for chemical transfection: *Trans*IT-X2, *Trans*IT-2020, *Trans*IT-LT1, or the Ingenio Electroporation Solution for electroporation (Mirus Bio LLC, distributed by MoBiTec). The optimal post-transfection incubation time usually is 48h.

- After growth the transfected COS-7 cells have to be harvested and the total RNA has to be prepared. This can be done by following the protocol shown below (see 3.2) or by using commercial available solutions or kits (e.g., AquaRNA; MoBiTec; Trizol, Invitrogen; RNeasy, Qiagen)

- The total RNA is used for cDNA synthesis with reverse transcriptase, using an appropriate cDNA synthesis kit and the “cDNA primer 1” provided.

- The generated cDNA is used as template for amplification of the fused exon by PCR. Use the specific primers “PCR primer 02” and “PCR primer 03” (included in this kit) for performing PCR. The primers incorporate specific restriction sites (BamHI and SmaI) for subsequent directional cloning of the amplified exon fragment.

- The amplified exon fragment can be digested with the restriction enzymes BamHI and SmaI and cloned into appropriate *E. coli* vectors for propagation (e.g., pUC19). Alternatively, the amplified DNA fragments can be filled-in using Klenow enzyme and cloned blunt-ended.

- The cloned exon fragment can be sequenced using 5´ and 3´sequening primers ETPR04 and ETPR05. The inserted exon itself can be amplified from the vector construct by using the PCR primers ETPR06 and ETPR07. All primer sequences and binding sites are shown in Fig. 2 (see also 3.3).

3.2 Protocol for RNA preparation

Cell treatment

To harvest the transfected cells, the medium is aspirated off and the plate is washed once with 10 ml of sterile PBS or cell culture medium (for media, buffers, and solutions see chapter 6). To each plate 2-4 ml of trypsin EDTA solution is added. After 5 to 10 min 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 min; 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, and mixed well by vortexing. Cells are lysed by the addition of another 100 µl of RNA extraction buffer containing 1% IGEPAL®CA-630 (formerly Nonidet P-40, Sigma-Aldrich) and then incubated at 4 °C for 5 min. The cell debris is pelleted at 12,000 rpm for 1 minute at 4 °C in a microfuge. The RNA-containing supernatant is transferred into a new 1.5 ml tube, mixed with 200 µl PK buffer, and incubated for 30 min at 37 °C in the presence of 50 µg/ml proteinase K (final concentration).

Phenol extraction and DNA degradation

After addition of 400 µl phenol/chloroform (1:1 (v/v)), the tube is vortexed and centrifuged for 5 min at 12,000 rpm in a microfuge. The aqueous phase is taken off and transferred to a new 1.5 ml tube. This phenol/chloroform extraction is repeated. The aqueous phase is mixed with 400 µl cold (0 °C) isopropanol and kept on ice for 30 min. The RNA is centrifuged at 13,000 rpm for 10 min 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 min at room temperature), and then dissolved in 100 µl distilled water (10 min 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 and incubation for 15 min at 37 °C. The DNA digestion is stopped after 15 min by the addition of 20 µl stop solution. 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 min. 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, pH 5.2, and 600 µl ethanol (96%); incubation at -20 °C for 30 min or overnight. After precipitation, the tube is centrifuged at 13,000 rpm for 20 min. The RNA precipitate is dissolved in 200 µl TE, pH 7.2. To this RNA solution 500 µl ethanol is added. This mixture is stored at -20 °C.

3.3 Primer

An overview on all primers mentioned in this chapter is given in Fig. 2, page 4.

3.3.1 Primers contained in Exontrap Kit:

The primers are provided lyophilized, each aliquot contains 250 pmol. Resuspend each primer in sterile distilled water. To obtain a 10 µM primer solution, dissolve each primer in a total volume of 25 µl.

Primer	length (bases)	Volume (µl)
cDNA primer 01 (3')	12	25
PCR primer 02 (5')	21	25
PCR primer 03 (3')	22	25

PCR primer 02 and 03 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.

Primer sequences:

cDNA primer 01 (for cDNA synthesis)
5'-GATCCACGATGC-3'

PCR primer 02 (5') – (forward; for amplification of cDNA)
5'-GATGGATCC GCTTCCTGCCCC-3'
BamHI

PCR primer 03 (3') – (reverse, for amplification of cDNA)
5'-CTCCCGGGCCACCTCCAGTGCC-3'
SmaI

3.3.2 Additional Primers recommended for sequencing and cloning

Primers ETPR04 and ETPR05 are recommended for sequencing the cloned exon. ETPR06 and ETPR07 are recommended for PCR amplification of exon probes and cloning into a vector using BamHI cloning sites.

Primer sequences:

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

For ETPR05, ETPR06, ETPR07 please see next page.

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

ETPR06 (forward; for exon amplification)
5'-GCGAAGTGGAGGATCCACAAG-3'
BamHI

ETprim07 (reverse; for exon amplification)
5'-ACCCGGATCCAGTTGTGCCA-3'
BamHI

4. General Comments

4.1 Working with RNA

1. All glass ware should be treated by heating to 220 °C to 240 °C for more than 2 h.
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 min 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)

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 which 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 also be 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 a 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 primer02 and poly(dT) as 3’ primer followed by classical cloning procedures.

5. Literature

A. J. Buckler et al., Proc. Natl. Acad. Sci. USA 88, 4005-4009 (1991).

C. Cepko et al., Cell 37, 1053-1 062 (1984).

D. Auch & M. Reth, Nucl. Acids Res. 18, 6743-6744 (1991).

F. Saenger et al., Proc. Natl. Acad. Sci. USA 74, 5463 (1977).

G. E. Moore et al., J.A.M.A. 199, 519-524 (1967).

G. M. Duyk et al., Proc. Natl. Acad. Sci. USA 87, 8995-8999 (1990).

J. E. Schwarzbauer et al., Proc. Natl. Acad. Sci. USA 84, 754-758 (1987).

J. Sambrook et al., Molecular Cloning, Cold Spring Harbour (2000).

J. Sorge & S. H. Hughes, J. Mol. Appl. Genet. 1, 547-559 (1982).

K. Shimotohno & H. M. Temin, Nature 299, 265-268 (1982).

L. Corbo et al., Science 249, 652-655 (1990).

P. Liu et al., Science 246, 813-815 (1989).

R. Dulbecco & M. Vogt, J. Exp. Med. 99, 167-182 (1954).

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 min).

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
ad 1000 ml sterile distilled water.

6.4 TE buffer

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

6.5 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

6.6 PK buffer

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

6.7 DNase mix (1 ml)

100 µl 10x DNase buffer (6.13)
1 µl 1 M dithiothreitol (DTT)
500 units RNase inhibitor
100 µl (100 units) DNase I (100 µg/ml)
ad sterile distilled water

6.8 Stop solution

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

6.9 10x DNase buffer

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

7. Order Information, Shipping, and Storage

Order#	Product	Quantity
K2010	Exontrap Kit: pET 01 Exontrap vector 5 µg cDNA primer 01 PCR primer 02 (5') PCR primer 03 (3') 250 pmol each	Kit
PET01	Exontrap vector pET01, lyophilized DNA	5 µg
shipped at RT; store at 4 °C		

8. Contact and Support

MoBiTec GmbH ◆ Lotzestrasse 22a ◆ D-37083 Goettingen ◆ Germany

Customer Service – General inquiries & orders
phone: +49 (0)551 707 22 0
fax: +49 (0)551 707 22 22
e-mail: order@mobitec.com

Technical Service – Product information
phone: +49 (0)551 707 22 70
fax: +49 (0)551 707 22 77
e-mail: info@mobitec.com

MoBiTec in your area: Find your local distributor at www.mobitec.com