Published in final edited form as:
Gene. 2008 December 31; 427(1-2): 104. doi:10.1016/j.gene.2008.09.021.

Rapid generation of splicing reporters with pSpliceExpress

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Abstract

Almost all human protein-coding transcripts undergo pre-mRNA splicing and a majority of them is alternatively spliced. The most common technique used to analyze the regulation of an alternative exon is through reporter minigene constructs. However, their construction is time-consuming and is often complicated by the limited availability of appropriate restriction sites. Here, we report a fast and simple recombination-based method to generate splicing reporter genes, using a new vector, pSpliceExpress. The system allows generation of minigenes within one week. Minigenes generated with pSpliceExpress show the same regulation as displayed by conventionally cloned reporter constructs and provide an alternate avenue to study splice site selection *in vivo*.

Keywords

Alternative splicing; High throughput; Recombination; Reporter gene

1. Introduction

A majority of human protein coding polymerase II transcripts is alternatively spliced and an increasing number of human diseases arise from aberrant splice site selection (Buratti et al., 2006; Novoyatleva et al., 2006). Alternative splice site selection is regulated by combinatorial control through an interplay of weakly defined, degenerate cis-elements that makes it currently impossible to accurately predict splicing decisions from sequence data alone (Smith and Valcarcel, 2000; Blencowe, 2006). Furthermore, analysis of cystic fibrosis transmembrane conductance regulator (CFTR) and medium-chain specific acyl-CoA dehydrogenase (MCAD) alternative exons that have a role in cystic fibrosis and MCAD deficiency, respectively, showed that synonymous mutations and SNPs can have a strong effect on alternative splicing and disease outcome (Pagani et al., 2003; Nielsen et al., 2007). Due to this complexity, splicing regulatory elements are typically analyzed experimentally using transient transfection of minigene reporter constructs (Cáceres et al., 1994). Advances in sequencing technology allow routine sequencing of disease candidate genes from patient DNA that often result in the identification of new mutations. These mutations could influence exon usage but require experimental verification to allow predictions. Currently, the laborious construction of splicing reporter minigenes prevent their more widespread and routine use which thus far, has been applied to approximately 80 original minigenes described in the literature (Tang et al., 2005).

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Therefore a rapid and effective cloning system that allows the analysis of splicing events using reporter minigenes would be advantagous.

Site-specific recombination between bacteriophage lambda and *E. coli* chromosomal DNA offers an alternative to traditional cloning using restriction enzymes. This system allows high throughput cloning and was commercially popularized as the Gateway system. Two 25 bp long sequences are the basis of recombination. The attachment B (attB) site resides on the *E. coli* chromosome and the attP site on the lambda DNA. Both sites assemble recombination proteins that catalyze recombination between attB and attP sites, generating attL and attR sites (Weisberg et al., 1983). The system can be changed to allow exchange of DNA fragments flanked by appropriate recombination sites.

To allow a fast analysis of alternative splicing in transfection systems, we generated pSpliceExpress. This vector uses the recombination system to generate splicing reporters from genomic DNA using a single recombination reaction. In addition, we devised pDESTsplice to clone reporter constructs larger than 4kb using two sequential recombination reactions. These vectors allow generation of a minigene within one week, and the minigenes generated by this new method show a similar splicing pattern compared to the ones cloned by conventional restriction enzyme techniques.

2. Material and Methods

2.1 Bacterial strains

For constructs containing the ccdB marker, we used: DB3.1 (Invitrogen, *E. coli* F^- gyrA462 endA1 (sr1-recA) mcrB mrr hsdS20(r_B^-, m_B) supE44 ara14 galK2 lacY1 proA2 rpsL20 (Sm^R) xyl5 Δleu mtl1) strain contains mutation in the DNA gyrase allele (*gyr*A462) which renders the strain resistant to the toxic effects of the *ccd*B gene.

For all other constructs, we used TOP10: (Invitrogen, *E. coli* F^- mcrA Δ(mrr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara- leu)7697 galU galK rpsL (Str^R) endA1 nupG

2.2 Cloning procedures

2.2.1 PCR amplification—The insert can be prepared from genomic DNA by standard PCR using a proofreading Pfx DNA polymerase (Invitrogen). If the PCR template is a plasmid that contains the ampicillin resistance gene, the PCR reaction mixture was treated with *Dpn* I. Depending on the template DNA used for PCR reaction, 5–10 units of *DpnI* was added to the PCR reaction and incubated at 37°C for 2 hours. This treatment degrades the contaminating plasmid DNA (*i.e*. *Dpn* I recognizes methylated GATC sites) and reduces background in the subsequent BP recombination reaction associated with template contamination. Purification of the PCR-amplified DNA is generally not required. In those cases where there is a high background, PCR purification of the products is performed by agarose gel electrophoresis followed by crystal violet staining and gel purification. This step also removes any contaminating template plasmid.

2.2.2 Performing the BP recombination reaction—The 5 μl BP recombination reaction consisted of 20–30 fmoles of the attB containing PCR product, mixed with 25 fmoles of pSpliceExpress vector. 1 μl of 5-fold BP clonase reaction buffer mixture was added. The additional reaction volume was made up with TE buffer, pH 8. The reaction was incubated at 25°C for 1 hour (preferably overnight for fragments larger than 3 kb). To inactivate the enzyme, 0.5 μl of Proteinase K (2mg/ml) solution was added to the reaction, which was further incubated at 37°C for 10 min.

2.2.3 Transforming the Competent cells—Any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1R, TOP10, DH5α™, DH10B™ or equivalent can be used for transformation. Use of *E. coli* strains that contain the F′ episome (*e.g.* TOP10F') for transformation is not recommended. These strains contain *ccd*A gene, which prevents negative selection with the *ccd*B gene. The transformed cells were plated on Ampicillin-supplemented LB plates pre-warmed at 37°C for 30 min

2.2.4 Screening of colonies—Single colonies were inoculated in 5 ml of LB medium overnight and plasmid DNA was isolated. Restriction digestion was performed to confirm the presence of the desired insert. The positive clones were further verified by sequencing.

2.2.5 Cloning minigenes into pDESTsplice—The PCR reaction was carried out as described above. The pDESTsplice vector contains attR1-attR2 recombination sites and cloning into pDESTsplice requires the construction of an entry clone containing attL1 and attL2 recombination sites. The entry clone is generated by performing a BP recombination of the PCR product with the pDONR221 vector, which carries gene for Kanamycin resistance. If the PCR template as well carries a Kanamycin resistance, DpnI treatment is performed as described above..

2.2.6 LR recombination of the entry clone with the pDESTsplice—5.0 μl of a LR recombination reaction was set up similar to the above-described BP recombination. In brief, 25–30 fmoles of entry clone were mixed with 25 fmoles of pDESTsplice. 1 μl of LR clonase reaction buffer mixture was diluted five-fold from its commercially supplied concentration. The additional volume was made up with TE buffer, pH 8.0. The reaction was incubated at 25°C for 1 hour. For most applications, 1 hour will yield a sufficient number of colonies for analysis. The length of recombination reaction can be extended up to 16 hours. For large plasmids (>5 kb), a longer incubation time (*i.e.* 16 hours incubation) yields additional colonies and is recommended.

The reaction was treated with 0.5 μl of Proteinase K (2 mg/ml) for 20 minutes, transformed and plated on Ampicillin supplemented LB-Agar plates and screened for positive clones. The positive clones were confirmed by sequencing to rule out any undesired mutations.

3. Results

3.1 Overview of the procedure

To analyze an exon using pSpliceExpress, the DNA of interest is first amplified using primers that contain the attB1 and attB2 attachment sites. To determine the splicing regulation of an exon in its genomic context, this exon is usually amplified together with its constitutive flanking exons. The recombination of this PCR product with pSpliceExpress generates a reporter minigene (Figure 1A–C). To allow usage of cDNA and genomic libraries that contain inserts flanked by attL sites, we constructed pDESTsplice that can recombine with attL sites (Figure 1D–F). The minigene construct carrying the DNA insert made by either method is then transfected in cell lines of choice. The splicing products are assayed by RT-PCR (Figure 1G). pSpliceExpress and pDESTsplice contain two constitutively expressed insulin exons, which are spliced together in most cases and serve as a positive control. In addition, the vector generates mRNA species that reflect the splicing pattern of the subcloned genomic fragment (Figure 1G).

3.2 Generation of the recombination vectors

pSpliceExpress (Figure 2A) was constructed by amplifying the ccdB/CmR element from the pDONR221 vector (Invitrogen) using the primers: ccdB-Cmr- *X h o I F* :

aaa*ctcgag*agaaacgcaaaaaggc cat c ; c cdB-CmR-*XbaIR*: aaa*ctagag*agctgccaggaaacagcta. The amplicon was cloned into the pCR4 TOPO vector (Invitrogen) and subcloned into the multiple cloning site of Exontrap vector (Mobitec) using *XhoI* and *XbaI* restriction sites. This Exontrap vector system is the best-studied vector for minigene analysis (Tang et al., 2005). It contains multiple cloning sites in the intron of insulin exons derived from *Rattus norvegicus*.

pDESTsplice (Figure 2B) was generated by blunt end cloning of ccdB recombination cassette C1 into the multiple cloning site of Exontrap vector through *SmaI* restriction site.

3.3 Generation of the minigenes

To generate a minigene, the genomic region of interest is amplified using the forward attB1F and reverse attB2R primer containing attB1 and attB2 recombination sites respectively (Figure 1A). Typically, the primer sequences consisted of: (i) four guanine residues at the 5’ end followed by (ii) the 25 bp attB1 site (forward primer) or attB2 site (reverse primer), followed by (iii) at least 18–25 bp of template-specific sequences (Table 1).

We perform the PCR with a proofreading polymerase, (e.g., Pfx DNA polymerase) using optimized PCR conditions (<30 cycles). As template, we use the genes cloned in BAC clones, which are commercially available. This strongly reduces primer mispriming and aberrant PCR products.

Since the amplification primers contain significant amounts of non-target sequences, in some genes we encountered undesired PCR products. This problem was especially apparent when we used genomic DNA. In these cases, we perform a two-step PCR procedure. First, the reaction is performed with a primer that is template specific and contains a part of the *attB* sequence at the 5’ end. The first PCR is then used as a template for the second PCR with adapter primers having a complete *att*B sequence. Template-specific primers for the first PCR reaction are designed with twelve bases of the attB1 or attB2 site on the 5’ end of each primer (attB1nestedF and attB1nestedR; Table 1)

For the second PCR reaction, adapter primers are designed to generate the complete *att*B sequences (attB1adapterF and attB2adapterR; Table1). The identity between adapter primers and template-specific primers has been underlined.

This alternative method allows smaller primers to be synthesized. Only the first set of primers (template-specific primers) are specific for a new minigene. The second set of primers (adapter primers) are used repeatedly for different minigene cloning projects.

To generate the splicing reporter, 25 fmol of the PCR product is incubated with an equimolar amount of the pSpliceExpress vector in the presence of the BP recombinase, which results in a reporter minigene. The reaction is performed in a 5 μl volume for at least 16 hours.

3.4 Recombination efficiency

The recombination reaction is subsequently introduced into bacterial strains that do not propagate the ccdB expressing vectors, such as TOP10 and screened for positive colonies. The recombined plasmid excises the ccdB element during recombination and hence survives in TOP10 cells. Cells containing parental pSpliceExpress are negatively selected due to toxic ccdB gene. ccdB harboring plasmids are therefore propagated in ccdB resistant cells like DB3.1 Using these systems, we generated more than 19 minigenes and observed differences in the recombination frequency. Using the pSpliceExpress system, we saw a sharp decline of recombination positives when the PCR products are longer than 4000 nts (Figure 3A). Similarly, it is possible to clone PCR products up to 4000 nts with high recombination

efficiency into pDONR vector (Figure 3B), which can be recombined with high frequency with pDESTsplice (Figure 3C).

3.5 Comparison between conventional and recombination based minigenes

Reporter minigenes generated by recombination and conventional cloning are identical with the exception of the restriction sites and recombination sites introduced by the different cloning procedures. The recombination sites do not harbor any known splicing regulatory sequences. In addition, they are located in the intron in the final construct, usually more than 500 nt away from the splice sites, and consequently they are not expected to influence splice site selection. To confirm this assertion, we compared conventionally cloned minigenes with minigenes cloned by recombination. It has been previously shown that exon 4 of cdc2-like kinase clk2 is autoregulated by an increase of the clk2 concentration and is also regulated by an increase of the tra2-beta1 concentration (Glatz et al., 2006). As shown in Figure 4 (C–F), both minigenes behave identically in cotransfection assays, in response to an increasing amount of tra2-beta1 or clk2.

In addition, minigenes generated by conventional cloning and by recombination that exhibit other splicing patterns showed a similar regulation. As an example, we show in Figure 4 G–H the alternative 5’ usage of the serotonin receptor pre-mRNA in response to SF2/ASF overexpression. Addition of ASF/SF2 promoted Exon Vb skipping in both the reporter constructs (Figure 4, G–I).

4. Discussion

The development of pSpliceExpress allows the fast generation of splicing reporter constructs using direct recombination between PCR products and a modified Exontrap vector. The Exontrap vector contains two constitutively spliced insulin exons that serve as an internal control. The system allows fast cloning of DNA fragments containing an alternative exon. If the fragments are shorter than 4000 nts, we observe more than a 50% recombination frequency and routinely obtain more than 100 clones per reaction. This frequency drops when the fragments are longer. We also devised a second system that is compatible with DNA fragments that are flanked by attL sites. PCR fragments up to 4000 nts length can be cloned into the commercially available donor vectors with high efficiency.

The constructs made with these vectors show the same splicing regulation as similar constructs made by conventional restriction cloning when used in transfection-based splicing assays.

The major advantages of the system are its speed and the lack of any need for restriction enzymes. Since no restriction sites are used, any fragment can be cloned and analyzed for splicing regulation.

The speed of the system allows its usage in medically relevant application. An increasing number of synonymous or intronic mutations appear to be associated with human diseases. The rapid cloning from PCR products, as described here, will greatly facilitate investigations aimed to determine whether such changes have an effect on pre-mRNA splicing.

Abbreviations

CFTR	cystic fibrosis transmembrane conductance regulator
MCAD	Medium-chain specific acyl-CoA dehydrogenase
acyl-CoA	acyl coenzyme A

SNPs	single nucleotide polymorphisms
att	attachment
LB	Luria Bertani
ccd	coupled cell division
RT-PCR	reverse transcription-polymerase chain reaction
PCR	polymerase chain reaction
CmR	chloramphenicol resistance gene
AmpR	Ampicillin resistance gene
SV40	simian virus 40
RSV LTR	Rous sarcoma virus long terminal repeat
nt	nucleotide
kb	kilobase

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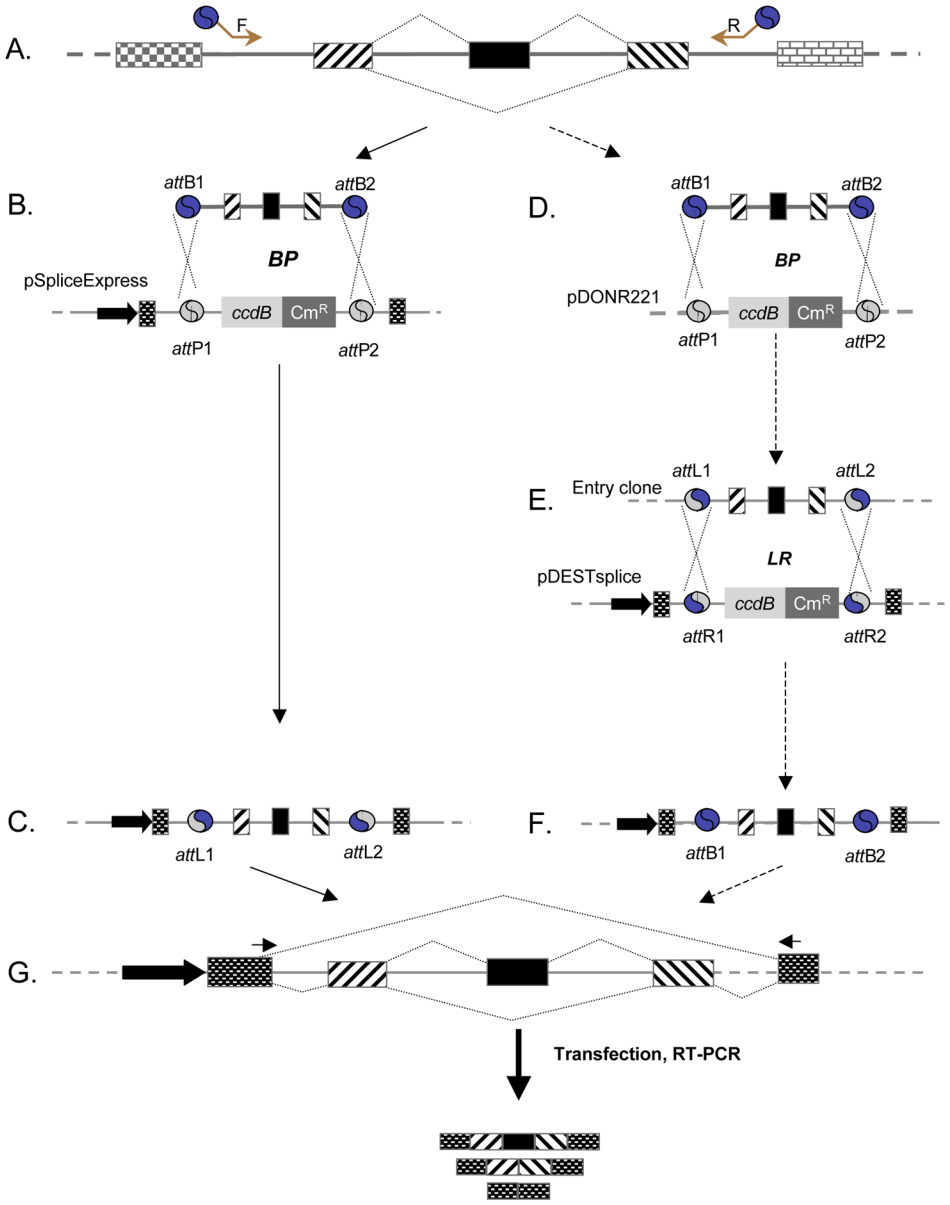


Figure 1. Overview of the method

The minigene of interest can be cloned by two methods. Either a PCR product is directly cloned into pSpliceExpress (A, B, C) or it is first cloned into a gateway entry clone (D), which is then recombined with pDESTsplice (steps E–F) to generate the final construct (G).

A. Amplification of the region of interest. Two primers F and R are used to amplify a part of the genomic DNA that harbors the alternative exon (black, splicing patterns are indicated). The primers have recombination sites that are indicated by circles.

B. Construction of the splicing reporter using pSpliceExpress. The PCR fragment is recombined *in vitro* with pSpliceExpress vector. The vector contains Cm and ccdB selection markers that are used to isolate recombined clones.

C. Structure of the final construct using pSpliceExpress. The inserted DNA is flanked by two constitutive rat insulin exons, indicated by doted pattern. The transcript is driven by a RSV LTR promoter (arrow) and the subcloned genomic fragment is flanked by attL sites, generated by the recombination of attB and attP sites.

D. Subcloning of the genomic fragment for use with pDESTsplice. The genomic fragment of interest is generated with attB sites by PCR, which are recombined using any Gateway entry clone that has the ccdB-CmR selection cassette flanked by attL sites.
E. Construction of the reporter gene using pDESTsplice. The attR1 sites of pDESTsplice are recombined *in vitro* with the attL1 sites of the entry clone.
F. Structure of the final construct using pDESTsplice. The subcloned genomic fragment is flanked by attB1 sites. Except for the recombination sites, the structures of pDESTsplice minigenes are identical to those generated with pSpliceExpress (C).
G. Analysis of the splicing reporter. The splicing reporter construct is transfected into a cell line of choice. The RNA generated is determined by RT-PCR, using the primers indicated (small arrows). The mRNA structures, indicated below the gene structure are expected to be generated by the construct.

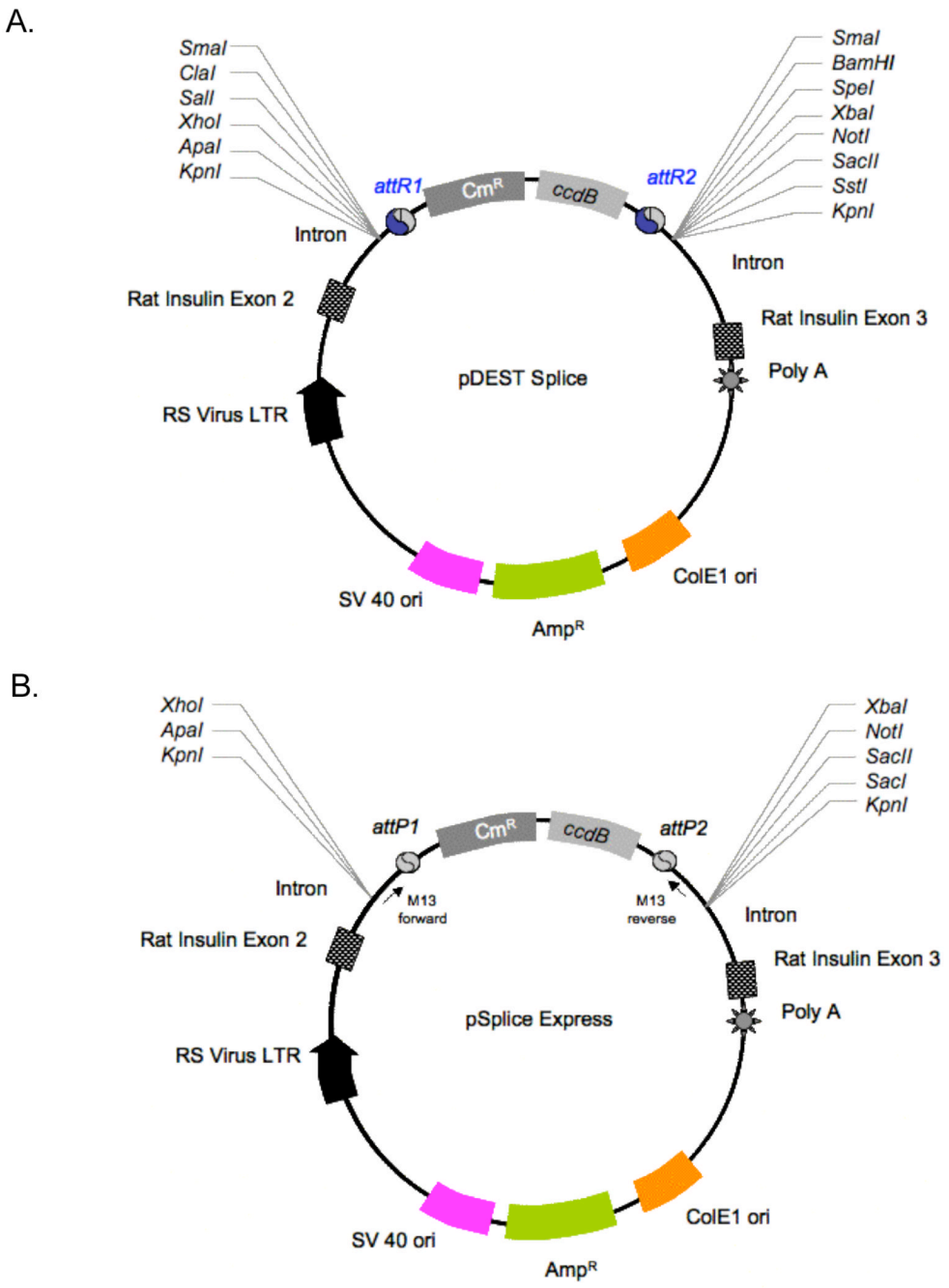


Figure 2. Maps of the cloning vectors pDESTsplice and pSpliceExpress

A. pSpliceExpress. The vector contains the CmR, ccdB, colE1 ori, AmpR, SV40 ori and RS virus LTR that are indicated. Restriction sites of the multiple cloning sites are indicated. Two m13 sites can be used for sequencing. The selection cassette is flanked by attP sites.

B. pDESTsplice. The vector has similar features as pSpliceExpress. The only difference is the attR attachment sites that are used for recombination.

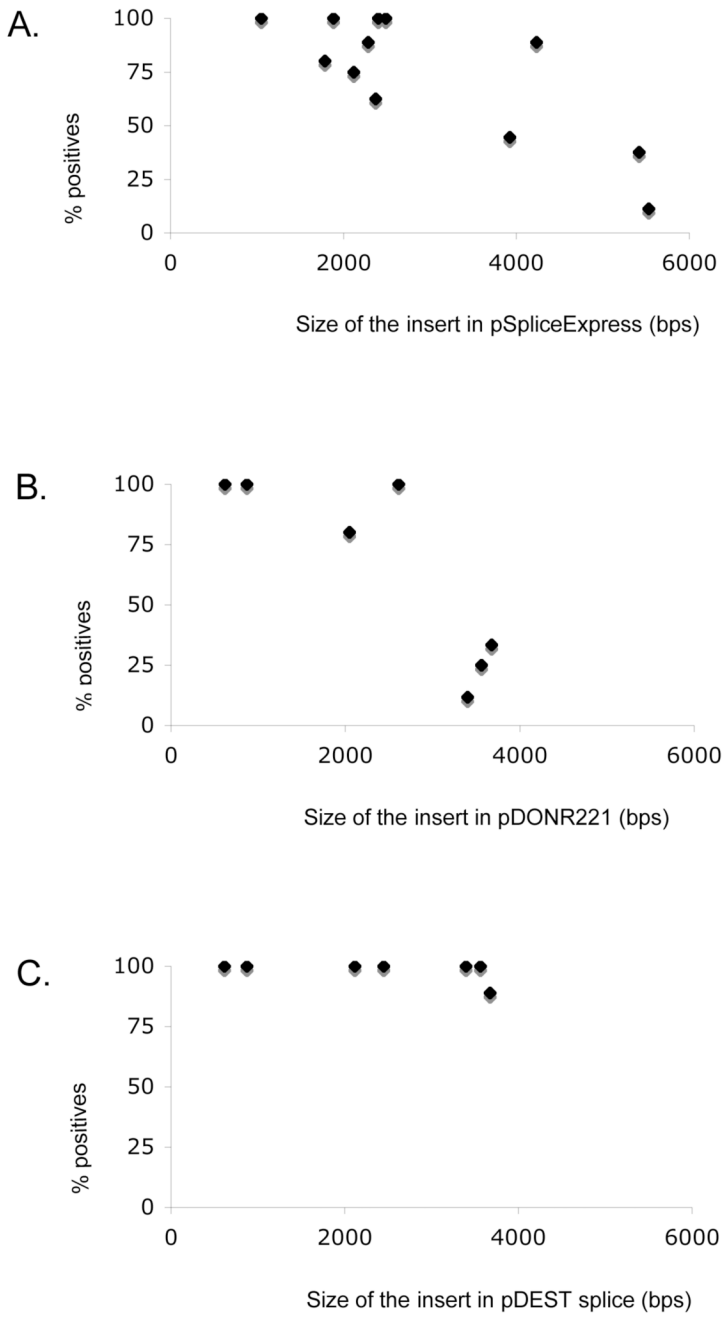


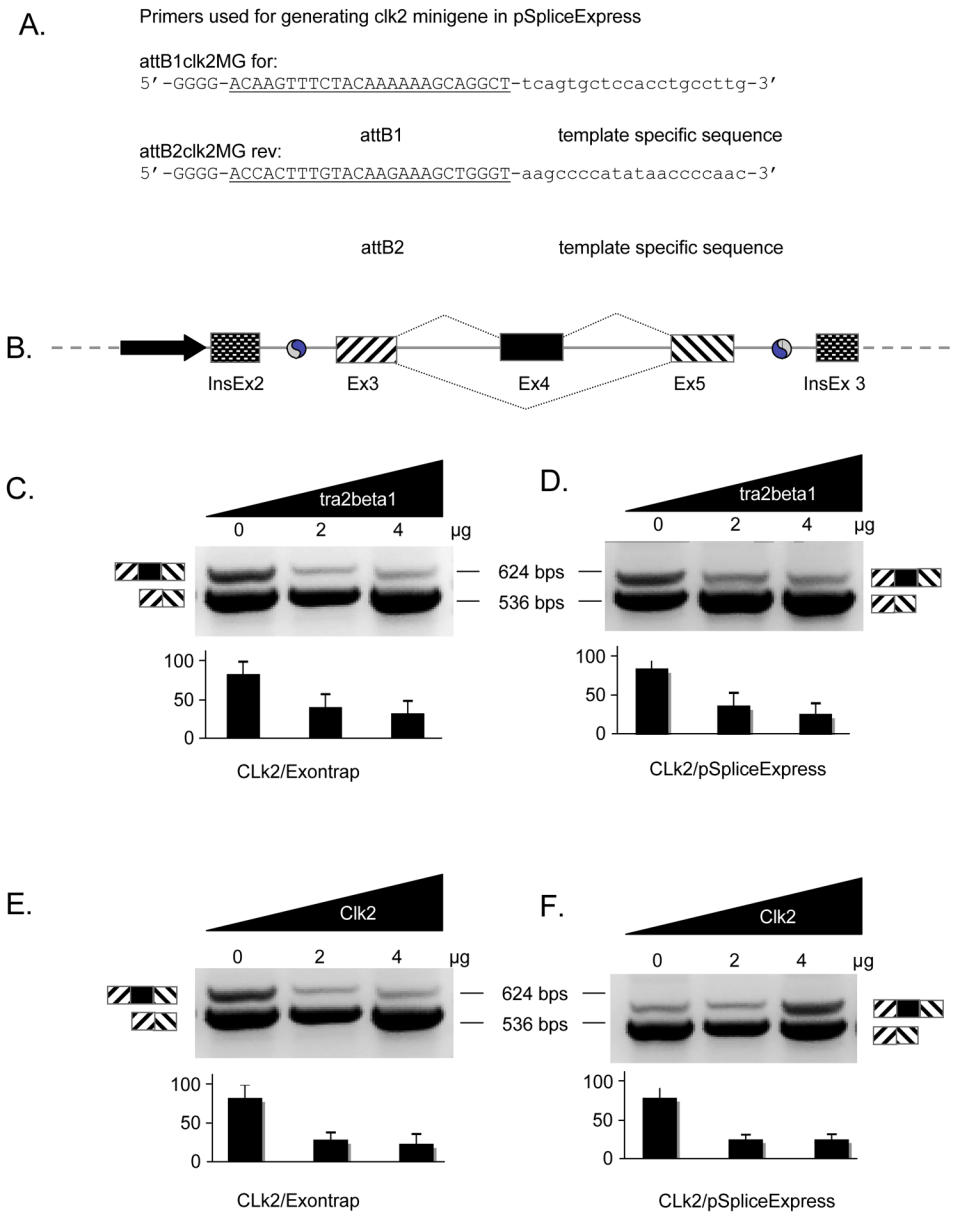
Figure 3. Cloning efficiency of vectors used

The *in vitro* recombination typically generates more than 50 clones. The graphs show the percent of clones with an insert of the expected size, determined by PCR and restriction digest. Each point represents the percent of successful recombinations as a function of the insert length.

A: Cloning efficiency of PCR products into pSpliceExpress

B: Cloning efficiency between PCR fragments and pDONR221

C: Cloning efficiency between pDONR221 inserts and pDESTSplice.



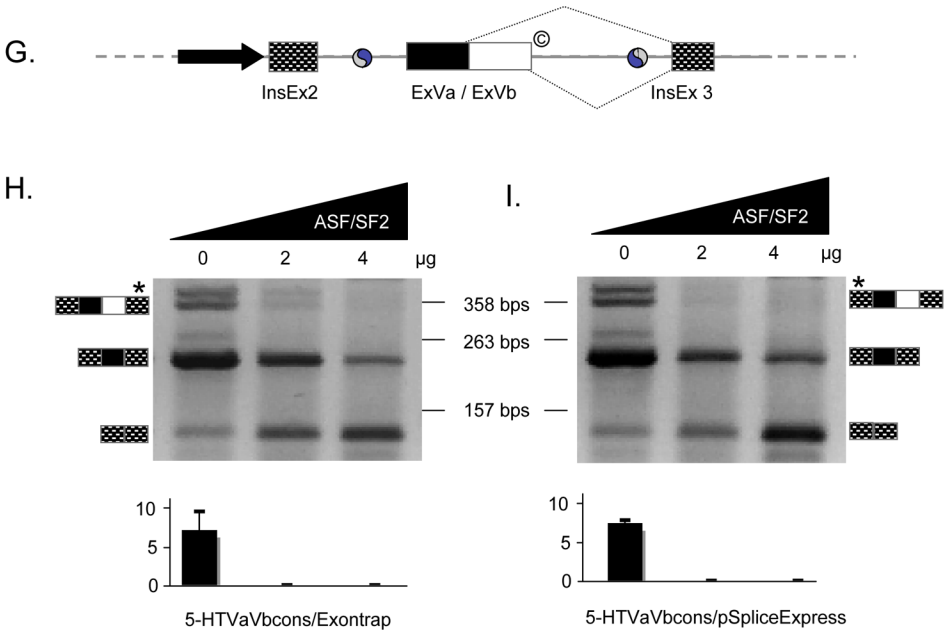


Figure 4. Comparison between splicing reporters generated by pSpliceExpress and conventional cloning

A. Sequences for the primers used to generate the clk2 minigene.

B. Structure of the pSpliceExpress generated clk2 reporter gene. The structure of the conventional cloned pclk2-Exontrap minigene is identical, except that *BamHI* and *NotI* restriction sites are present instead of the recombination sites.

C. Cotransfection of tra2-beta1 with the conventionally cloned reporter gene. Numbers indicate µg transfected expression plasmid. A representative ethidium stained gels are shown. The structures of the RT-PCR products are indicated. A statistical evaluation of four independent experiments is shown below the gels.

D. Cotransfection of tra2-beta1 with the clk2 reporter gene cloned by pSpliceExpress

E. Cotransfection of CLK2 with the conventionally cloned reporter gene.

F. Cotransfection of CLK2 with the clk2 reporter gene cloned by pSpliceExpress

G. Structure of the pSpliceExpress generated 5-HTVaVbcons reporter gene. The structure of the conventional cloned 5HTVaVbcons-Exontrap minigene is identical, except that *XhoI* and *BamHI* restriction sites are present instead of the recombination sites.

H. Cotransfection of ASF/SF2 with the conventionally cloned 5HTVaVbcons reporter gene. © denotes that the distal 5' splice site of Exon V has been mutated to U1 binding consensus, which is described in (Kishore and Stamm, 2006). The star (*) indicates a heterodimer band. The statistical evaluation underneath the ethidium bromide stained gel represent the % exon Vb inclusion from three experiments.

I. Cotransfection of ASF/SF2 with the 5HTVaVbcons reporter gene cloned by pSpliceExpress.

Table 1

Sequences of the attB1 forward and attB2 reverse primers.

attB1F	5’-GGGG-ACAAGTTTCTACAAAAAAGCAGGCT—(template specific sequence)-3’
attB2R	5’-GGGG-ACCACTTTGTACAAGAAAGCTGGGT—(template specific sequence)-3’
attB1nestedF	5’-AAAAAGCAGGCT-template-specific sequences-3’
attB2nestedR	5’-AGAAAGCTGGGT-template-specific sequences-3’
attB1adapterF	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3’
attB2adapterR	5’-GGGGACCACTTTGTACAAGAAAGCTGGGT-3’
5HT attB1F	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGGATGACGTGTTCTTGTC-3’
5HT attB2R	5’- GGGGACCACTTTGTACAAGAAAGCTGGGTTGATCTCAGCTCACTGCAGA-3’