Title: Single cell detection of splicing events with fluorescent splicing reporters.

Hidehito Kuroyanagi\textsuperscript{1, 2, 3}, Akihide Takeuchi\textsuperscript{2}, Takayuki Nojima\textsuperscript{1}, and Masatoshi Hagiwara\textsuperscript{1, 2}

\textsuperscript{1}Laboratory of Gene Expression, Graduate School of Biomedical Science and \textsuperscript{2}Department of Functional Genomics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan and \textsuperscript{3}Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), Kawaguchi, Saitama, Japan.

*Address correspondence to: H. K. or M. H., Laboratory of Gene Expression, Graduate School of Biomedical Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan, FAX: +81(Japan)-3-5803-5853; E-mail: H.K. (kuroyana.end@tmd.ac.jp) and M.H. (m.hagiwara.end@mri.tmd.ac.jp).
1. Abstract

Multi-color fluorescent reporters are useful tools to visualize patterns of alternative splicing in cultured cells and in living organisms at a single cell resolution. The multi-color reporters have been utilized to search for cis-elements and trans-acting factors involved in the regulation, and to screen for chemical compounds affecting the splicing patterns. Here we describe how to construct fluorescent alternative splicing reporter mini-genes for a nematode *Caenorhabditis elegans*, cultured cells and mice. The mini-gene construction is based on site-directed recombination and various mini-genes can be easily constructed by assembling modular DNA fragments such as a promoter, tag protein cDNAs, a genomic fragment of interest, fluorescent protein cDNAs, and a 3' cassette in separate vectors. We also described points to be considered in designing fluorescent alternative splicing reporters. The splicing reporter system can theoretically be applied to any other organisms.

Kew Words: alternative splicing, fluorescent reporter, mini-gene, visualization, Gateway cloning, Entry vector, Destination vector
2. Theoretical background

2.1. Visualization of alternative splicing patterns with multiple fluorescent proteins

To clarify the regulation mechanisms of alternative splicing in living cells, reporter mini-gene constructs containing multiple exons and introns have often been used. Splicing patterns of the mini-gene-derived mRNAs have usually been quantitatively analyzed with the ratio of reverse transcription (RT)-polymerase chain reaction (PCR) products after extracting total RNAs from transfected cells. The laborious multiple steps in analyzing the splicing patterns often caused deviation in the results and prevented high-throughput analysis of alternative splicing.

Utilization of fluorescent alternative splicing reporters expressing fluorescent proteins has changed the situation. At initial stages, mono-chromatic, or single-color, fluorescent reporters were used as an indicator of splicing events in cultured cells. The mono-chromatic reporter mini-genes were designed to monitor proper splicing or skipping of alternative exons, and were utilized for isolation of mutant cell lines defective in the regulation of alternative splicing [1], functional screening for splicing regulatory elements [2], and screening for small chemical compounds that altered splicing patterns [3]. The feature of the mono-chromatic reporters is simplicity in their structure. However, the readout of the mono-chromatic reporters may be affected by influence on gene expression such as transcription and translation.

Multi-chromatic alternative splicing reporters have overcome most of these caveats of the mono-chromatic reporters. The multi-chromatic reporter mini-genes were designed so that expression of each fluorescent protein represents a certain splicing event. The advantage of multi-chromatic fluorescent reporters is that the ratio of the expressed fluorescent proteins reflects the ratio of the mRNA isoforms, or one of the fluorescent proteins acts as a control of
the expression level, in individual cells. The multi-chromatic reporters are therefore suitable for visualization of alternative splicing patterns in multi-cellular organisms.

The multi-chromatic reporter mini-genes can further be classified into two types, the multi- and the single-construct types. The former consists of multiple mini-genes each of which encodes a single fluorescent protein, while the latter contains two fluorescent protein cDNAs in a single mini-gene construct. Multi-construct reporters have been utilized to search for trans factors and cis-elements by flow cytometry of cultured cells [4], and to visualize developmentally regulated alternative splicing and further genetic analysis in *C. elegans* [5]. A remarkable feature of the single-construct bi-chromatic reporters is that the two alternative mRNA isoforms, each of which encodes a single fluorescent protein, are generated from a common pre-mRNA in a mutually exclusive manner. The single-construct reporters are therefore sensitive to subtle changes in the alternative splicing patterns. The reporters have been utilized for analyzing regulatory factors [6], high-throughput screening for chemical compounds modifying the splicing regulation [7], and for visualization of cell-type-specific alternative splicing in *C. elegans* [8] and mouse (A. T., unpublished observation).
2.2. Designing fluorescent reporter mini-genes to monitor splicing patterns

Here we show typical structures of multi-chromatic alternative splicing reporter mini-genes (Figure 1) which we constructed as described in section 3 and explain how expression of each fluorescent protein reports a specific alternative splicing event. Please notice that the reporter mini-genes described here are just a few examples of possible alternative splicing reporters. Each reporter can be flexibly designed depending on alternative splicing events of interest to be visualized. An ideal mini-gene should be designed so that expression of a specific fluorescent protein unambiguously indicates a specific mRNA isoform or a specific alternative splicing event.

As described in the previous section, the mini-genes in Figure 1 can be divided into the multi-construct type and the single-construct type. Choice of the reporter type depends on organisms and the method of mini-gene transfer. Transgenic worms generated by a standard microinjection method carry hundreds of copies of plasmid DNAs as an extra-chromosomal array [9] and, therefore, it is generally assumed that injecting a mixture of several different mini-genes with the same vector backbone results in proportional incorporation of all the constructs in the extra-chromosomal array. One of the advantages of the multi-construct reporters is that the number of co-transferred mini-genes can be increased to more than two as described in section 4. For situations where the copy number of transferred mini-genes is small or variable, single-construct reporters might be preferable.

Figure 1A shows schematic structure of a pair of reporter mini-genes for mutually exclusive exons. A genomic fragment of interest, from the upstream constitutive exon through the downstream constitutive exon, is placed downstream of a common promoter and a constitutive intron, followed by a cDNA for either of two fluorescent proteins and a 3' cassette.
An in-frame translation initiation codon is artificially introduced at the 5' end of the genomic fragment. A termination codon is artificially introduced in one of the two alternative exons in each construct. From the mini-genes shown in Figure 1A, GFP-fusion protein is produced from an mRNA isoform in which exon a alone is included and RFP-fusion protein is produced from an mRNA isoform in which exon b alone is selected.

Figure 1B shows schematic structures of a pair of reporter mini-genes to monitor inclusion and skipping of a cassette exon. Order and composition of the fragment cassettes are as those in Figure 1A. In the case shown in Figure 1B, the length of the cassette exon is not multiple of three bases and therefore inclusion of the cassette exon changes the reading frame of the downstream exon. GFP cDNA is connected in frame when the cassette exon is included and RFP cDNA is connected in frame when the cassette exon is excluded.

Figure 1C and 1D show schematic structures of single-construct bi-chromatic reporters. These constructs rely on an unusual feature of some fluorescent protein cDNAs in which an alternate reading frame lacks a termination codon [6]. In the cases shown in Figure 1C and 1D, RFP and GFP cDNAs are connected in a different reading frame so that translation of the alternate frame of RFP cDNA leads to generation of a fluorescent protein from GFP cDNA. When a fluorescent protein is generated from RFP cDNA, translation will be ceased at its own termination codon. The mini-gene shown in Figure 1C is for monitoring inclusion and skipping of a cassette exon. GFP cDNA is in frame when the cassette exon is included and RFP cDNA is in frame when the cassette exon is excluded. The mini-gene shown in Figure 1D is for monitoring selection of mutually exclusive exons. In this case, one nucleotide is inserted into exon a to cause a frame-shift when this exon is selected. GST is used as an N-terminal tag for expression of the fusion proteins. GFP-fusion protein is produced when exon a alone is included and RFP-fusion protein is produced when exon b alone is selected. Neither of the fluorescent proteins is produced in the absence of a cassette exon.
proteins is produced when both exons are included or skipped.
2.3. Constructing fluorescent reporter mini-genes

We construct fluorescent alternative splicing reporter mini-genes by site-specific recombination utilizing MultiSite Gateway system (Invitrogen). The major advantage of homologous recombination in mini-gene construction is that 'Expression' vectors with a variety of structures, as described in the previous section, can be easily and rapidly constructed by assembling modular DNA fragments cloned in ‘Entry’ and ‘Destination’ vectors. For a basic background of the Gateway system, please refer to ‘Theoretical background’ in Zhang et al. of this volume. In this section, we focus on practical use of the MultiSite Gateway system and other aspects to be considered in designing fluorescent reporter mini-genes.

2.3.1. MultiSite Gateway system

The MultiSite Gateway system uses site-specific recombinational cloning to allow simultaneous cloning of two, three or four separate DNA fragments of interest in a defined order and orientation. Figure 2 schematically illustrates construction of an ‘Expression’ clone by performing ‘2-fragment’ recombination reaction. Genomic DNA fragments of interest are cloned in ‘Entry’ vectors (Figure 2A) and the fragments are assembled between homologous recombination sites of the ‘Destination’ vectors (Figure 2B). A key feature of the MultiSite Gateway system is that five sets of modified att sites have an orientation and demonstrate the specificity of homologous recombination as in the standard Gateway system: for example, attB1 site reacts only with attP1 site, but not other attP sites, to generate attL1 and attR1 sites in ‘BP’ reaction (Figure 2A), and attL5 site reacts only with attR5 site, but not other attR sites, to generate attB5 and attP5 sites in ‘LR’ reaction (Figure 2B). For more details about the MultiSite Gateway system and ‘3-fragment’ and ‘4-fragment’ recombination reactions, please refer to the provider’s website (www.invitrogen.com).
All the *att* sites in our reporter mini-genes, or ‘Expression’ clones, are *attB* sites and reside within exons (Figure 1 and 2). The *attB* sequences (21 ~ 25 base pairs) are the shortest stretches among all *att* sites. We have not experienced cryptic splicing within the *attB* sequences in *C. elegans* or mammalian cells. It is recommended to use a fixed reading frame in the *attB* sequences and we usually do so (see section 3). As *attB1, attB5* and *attB2* sequences lack ATG and a termination codon in any frames, they can theoretically be used in any frames.

2.3.2. Other aspects to be considered in mini-gene construction

mRNAs with premature termination (nonsense) codons (PTCs) are selectively degraded by a quality-control mechanism called nonsense-mediated mRNA decay (NMD). In mammals, NMD is considered to be induced when an exon junction complex (EJC), a protein complex deposited upstream of exon-exon boundaries after RNA splicing, resides downstream of the termination codon in the first round of translation [10, 11]. It is critical to design the fluorescent reporter mini-genes so that the mRNA isoforms encoding the fluorescent proteins escape NMD. As the GFP and RFP cDNAs reside in the last exon in mini-genes shown in Figure 1, the productive isoforms from these mini-genes would escape NMD in mammals. In *C. elegans* [12, 13] and yeast [14, 15], long 3’ untranslated region (UTR) triggers NMD independent of exon-exon boundaries, and therefore the mRNA isoforms encoding RFP proteins in Figure 1C and 1D may be degraded by NMD in these organisms.

Genomic fragments utilized in the mini-gene constructs usually undergo proper splicing. However, trimming of constitutive exons and/or deletion of long intronic regions may lead to inefficient splicing or deregulation of alternative splicing. Repeated try and error may be required to establish a reporter reflecting the alternative splicing pattern of the endogenous gene. We have not experienced cryptic splicing in GFP or RFP cDNAs, but other cDNAs for
N-terminal and C-terminal tags may serve as cryptic splice sites.

Amino acid sequences derived from the gene of interest greatly affect folding, stability and/or subcellular localization of the fluorescent fusion proteins. It is therefore critical to predict the property of the fusion proteins in designing the mini-genomes. Various N-terminal tags such as glutathionine S-transferase (GST) of *E. coli* (Figure 1D) may stabilize expression of the fusion proteins and improve the result. It is also critical to force translation initiation at the designed initiation codon. ATG codons in the exonic regions and in the N-terminal tags may be the cause of aberrant translation initiation and reduce the production of the fluorescent proteins.
3. Protocol

3.1. Constructing genomic DNA fragment cassettes in ‘Entry’ vectors

We perform ‘BP’ reaction to clone genomic fragments of interest in ‘Entry’ vectors of the MultiSite Gateway system. To amplify attB-flanked genomic fragments, we usually perform a two-step PCR procedure. The first PCR is performed with primers that are template-specific and contain a part of the attB sequences at their 5’ ends. The first PCR product is then used as a template for the second PCR with attB adapter primers. The advantages of the two-step PCR procedure are that the template-specific primers to be synthesized would be shorter and that the attB adapter primers can be used repeatedly for cloning other DNA fragments in different mini-gene projects. Here we demonstrate how to construct ‘Entry’ clones for ‘2-fragment’ recombination reaction as schematically shown in Figure 2A. The genomic DNA fragment is cloned in either of the ‘Entry’ vectors depending on the design of the mini-genes to be constructed (see section 2.2). The ‘3-fragment’ and ‘4-fragment’ recombination reactions may also work in mini-gene construction, although they are less efficient and we have few experiences.

3.1.1. Primer design

The gene-specific primers (GSPs) must have 12 bases of the attB site on the 5’ end followed by 18 ~ 25 bases of template- or gene-specific sequences (Table 1). Kozak’s consensus sequence can be inserted between the attB1 and the gene-specific sequences to force translation initiation as shown in Table 1. Termination codons must be included in or excluded from the reverse GSPs, according to the design of the reporter mini-genes. If the DNA fragment is designed to be fused with N- and/or C-terminal tags, the GSPs must be carefully designed to maintain the proper reading frame in the attB sequences as indicated in Table 1.
The attB adapter primers for the second PCR consist of the following common structure: four guanine (G) residues at the 5’ end followed by a 22- or 25-base complete attB sequence (Table 1).

### Table 1. Sequences of primers used for constructing ‘Entry’ clones.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSP-attB1F</td>
<td>5’-AA AAA GCA GGC TNN -(gene-specific sequence)–3’</td>
</tr>
<tr>
<td></td>
<td>* To avoid generating a stop codon, NN cannot be AA, AG, or GA.</td>
</tr>
<tr>
<td>GSP-attB1F (with ATG)</td>
<td>5’-AA AAA GCA GGC TCC ACC ATG G -(gene-specific sequence)–3’</td>
</tr>
<tr>
<td></td>
<td>* Kozak consensus sequence allows efficient protein expression in eukaryote cells.</td>
</tr>
<tr>
<td>GSP-attB5R</td>
<td>5’-T ATA CAA AGT TGT -(gene-specific sequence)–3’</td>
</tr>
<tr>
<td>attB1adapterF</td>
<td>5’-GGGG ACAAGTTTGTAACAAAAAACAGGCT–3’</td>
</tr>
<tr>
<td>attB5adapterR</td>
<td>5’-GGGG ACAACTTTGTATAACAAAAAGTTG–3’</td>
</tr>
<tr>
<td>GSP-attB5F</td>
<td>5’-AT ACA AAA GTT G -(gene-specific sequence)–3’</td>
</tr>
<tr>
<td>GSP-attB2R</td>
<td>5’-A GAA AGC TGG GT -(gene-specific sequence)–3’</td>
</tr>
<tr>
<td>attB5adapterF</td>
<td>5’-GGGG ACAACTTTGTATAACAAAAAGTTG–3’</td>
</tr>
<tr>
<td>attB2adapterR</td>
<td>5’-GGGG ACCACTTTGTAACAAGAGCTGGGT–3’</td>
</tr>
</tbody>
</table>

Underlines indicate 12 bases of the attB sequences included in the GSPs.

#### 3.1.2. Performing PCR

The PCRs should be performed with a proofreading polymerase, such as PrimeSTAR HS DNA Polymerase (TaKaRa). The annealing temperature of the second PCR should be 45°C because the annealing sequences are just 12 base pairs.

**Protocol 1: Two-step PCR amplification of attB-DNA fragments**

1. Perform the first PCR in a 25 μl mixture containing standard reagents with 0.2 μM each of GSPs. Conditions of the PCR should be optimized depending on the
amount of the template and the size of the fragment to be amplified. Check the
PCR product by standard agarose gel electrophoresis.

2. Prepare 50 µl of the second PCR mixture containing standard reagents and 0.3 µM
each of attB adapter primers. Add the mixture to 10 µl of the first PCR reaction
mixture and perform 5 cycles of PCR with annealing at 45°C. Check by agarose gel
electrophoresis that the amount of the PCR product has increased in the second
PCR.

3. Optionally, add 1 µl Dpn I* (New England Biolabs) and incubate at 37°C for 1 hour
to destroy template DNA. *If the PCR template contains the kanamycin-resistance
gene, the PCR mixture should be treated with Dpn I before purifying the attB-PCR
products. Dpn I recognizes methylated GATC sites in bacteria-derived DNA. Dpn I
treatment greatly reduces background in the ‘BP’ recombination reaction
associated with template contamination.

4. Purify the attB-PCR product with a standard DNA purification column.

3.1.3. ‘BP’ recombination reaction and selection of ‘Entry’ clones

Perform ‘BP’ recombination reaction between each attB-flanked DNA fragment and an
appropriate attP-containing ‘Donor’ vector (Table. 2) to generate an ‘Entry’ clone.

Table 2. Selection of ‘Donor’ vectors for ‘BP’ reaction and ‘Entry’ vectors to be constructed.

<table>
<thead>
<tr>
<th>pDONR Vectors</th>
<th>DNA fragments to be cloned</th>
<th>pENTR Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR 221 P1-P5r</td>
<td>attB1F and attB5R-flanked PCR products</td>
<td>pENTR-L1-R5</td>
</tr>
<tr>
<td>pDONR 221 P5-P2</td>
<td>attB5F and attB2R-flanked PCR products</td>
<td>pENTR-L5-L2</td>
</tr>
</tbody>
</table>

Protocol 2: BP clonase II reaction and selection of appropriate ‘Entry’ clones

1. Add the following components to a 1.5-ml microcentrifuge tube at room
temperature and mix: attB-PCR product (15 ~ 150 ng), pDONR vector (supercoiled, 75 ng), and DDW or TE to 4 μl. Add 1 μl BP Clonase II enzyme mix (Invitrogen) to the components above and mix well by briefly vortexing or tapping.

2. Incubate the ‘BP’ reaction mixture at room temperature or at 25°C for 1 hour or overnight. We usually omit proteinase K digestion.

3. Transform *E. coli* strain DH5α or others* with 1 ~ 3 μl of the reaction mixture and select for kanamycin-resistant ‘Entry’ clones. Check the sequence of the insert. *E. coli* strains with F’ episome (e.g. TOP10F’) cannot be used for transformation to select ‘Entry’ clones. These strains contain the ccdA gene and will prevent negative selection with the ccdB gene in the pDONR vectors.

### 3.1.4. Modification of ‘Entry’ clones (Optional)

According to the design of the mini-genes, introduce a termination codon or a frame-shift into exons, and/or modify or disrupt putative cis-elements of the ‘Entry’ clones.

Sequences of the genomic fragment should be carefully modified to avoid disruption of putative cis-regulatory elements. We introduce termination codons or frame-shifts in less conserved stretches among related species. We utilize Quickchange II or Quickchange II XL (Stratagene) for the site-directed mutagenesis. We usually check the sequence of the entire insert after mutagenesis.
3.2. ‘LR’ recombination reaction and selection of ‘Expression’ clones

Gateway ‘Destination’ vectors usually provide a promoter and a 3’ cassette (Figure 1). A variety of ‘Destination’ vectors for expression in cultured cells are commercially available. ‘Destination’ vectors can also be constructed by ligation-based insertion of Destination vector cassettes (Invitrogen) into existing vectors containing the ampicillin-resistance gene. We performed ‘BP’ reaction to convert existing expression vectors into ‘Destination’ vectors, which were used in our previous studies [5, 8, 12]. We can convert any existing vectors containing the ampicillin-resistance gene into ‘Destination’ vectors at desired positions with desired frames. Details of the conversion method will be described elsewhere. Nucleotide sequences of the ‘Destination’ vectors we constructed for expression in C. elegans are available on the C. elegans Promoter/Marker Database (http://www.shigen.nig.ac.jp/c.elegans/promoter/index.jsp).

We have constructed a variety of ‘Entry’ clones of fluorescent protein cassettes in pENTR-L1-R5 and pENTR-L5-L2 vectors with or without initiation and/or termination codons.

Protocol 3: LR clonase II Plus reaction and selection of appropriate ‘Expression’ clones.

1. Add the following components to a 1.5-ml microcentrifuge tube at room temperature and mix: ‘Destination’ vectors (75 ng), ‘Entry’ clones (15 ~ 100 ng each), DDW or TE to 4 μl. Add 1 μl LR Clonase II Plus enzyme mix (Invitrogen) to the components above and mix well by briefly vortexing or tapping.

2. Incubate the ‘LR’ reaction mixture at 25°C or at room temperature overnight. We usually omit proteinase K digestion.

3. Transform E. coli strain DH5α or others* with 1 ~ 3 μl of the reaction mixture and select for ampicillin/carbenicillin-resistant ‘Expression’ clones. We routinely check resistance of the ampicillin/carbenicillin-resistant colonies to ampicillin/carbenicillin,
chloramphenicol and kanamycin. *E. coli* strains with F’ episome cannot be used to select ‘Expression’ clones.

4. Select clones that are resistant only to ampicillin/carbenicillin, check restriction enzyme digestion patterns of the mini-prep plasmid DNAs and sequence the boundaries of the DNA fragments.
3.3. Transfection of cultured cells and generation of transgenic animals.

Transient transfection of cultured cells with the fluorescent reporter mini-gene(s) is performed using standard transfection reagents. Expression of the fluorescent proteins can be analyzed by utilizing standard compound microscopes, flow cytometry and other instruments.

Transgenic animals can be generated by standard methods. Expression of the fluorescent proteins in mice can be analyzed by observing sections under compound microscopes or confocal microscopes. We usually utilized confocal microscopes to analyze cell- and tissue-specific expression of the fluorescent reporter proteins in transgenic worms.
3.4. Checking splicing pattern of the mini-gene-derived mRNAs.

We strongly recommend analyzing splicing patterns of the mini-gene-derived mRNAs to confirm that the reporter mini-genes are expressed and properly spliced and that the alternative splicing pattern is as expected from that of the endogenous gene and is consistent with the expression pattern of the fluorescent reporter proteins.

Protocol 4: RT-PCR analysis of mini-gene-derived mRNAs

1. Total RNA is extracted from cells, tissues or organisms by utilizing RNeasy Mini (QIAGEN) or equivalents and DNase I following manufacturer's instructions.

2. In RT, 1 ~ 2 μg of the total RNA is reverse transcribed with PrimeScript (TaKaRa) or Superscript II (Invitrogen) and oligo(dT) as a primer following manufacturers' instructions.

3. For PCR, we usually use non-proofreading polymerases such as Ex Taq (TaKaRa) and BIOTAQ (BIOLINE), and a mini-gene-specific primer set. As GFP-specific reverse primers, we use 5'-TGTGGCCGTTTACGTCG-3' or 5'-TTTACTTGTACAGCTCGT-3'. As mRFP-specific reverse primers, we use 5'-GGAGCCGTACTGGAACTGAG-3' or 5'-TTAGGCGCCGGTGAGTG-3'. attB adapter primers can also be used.

4. To analyze RT-PCR products, directly sequence the purified products, or clone the products in TA-vectors such as pGEM-T Easy (Promega) and sequence them.
4. Examples of experiments

Figure 3A and 3B show an example of visualizing tissue-specific alternative splicing of mutually exclusive exons in \textit{C. elegans} (H. K., unpublished observation). Figure 3C and 3D show an example of visualizing viral infection-induced intron retention in HeLa cells [16]. Figure 3E and 3F show an example of visualizing mutually exclusive alternative splicing in mice (A. T., unpublished observation).
### 5. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason + Solution</th>
</tr>
</thead>
</table>
| Few or no colonies obtained from BP and LR homologous recombination reaction. | > PCR primers incorrectly designed. Make sure that each *att*B or *att*Br PCR primer is properly designed and/or selected.  
> Poor purity of plasmid DNAs. Mini-prep plasmid DNAs should be purified with a standard DNA purification column. |
| Little or no expression of fluorescent proteins.                       | > Very low amount of mRNAs from the mini-gene(s).  
> The pre-mRNAs were aberrantly spliced. Check the splicing pattern and reconstruct the mini-genes.  
> The mRNAs were aberrantly translated. Optimize the preferred ATG and remove unnecessary ATGs out of frame.  
> The peptide sequence derived from the gene-specific region affected folding or stability of the fluorescent proteins. Change the N-terminal tag. |
| Very low amount of mRNAs from the mini-gene(s).                        | > Aberrant splicing. Check the splicing pattern and reconstruct mini-genes.  
> Low expression from the vector. Change |
vectors/promoters.
Figure legends

Figure 1: Schematic structure of fluorescent reporter mini-genes and expected mRNAs.

(A, B) Two-construct fluorescent alternative splicing Cloning reporter mini-genes for mutually exclusive exons (A) and a cassette exon (B). (C, D) Single-construct fluorescent alternative splicing Cloning reporter mini-genes for a cassette exon (C) and mutually exclusive exons (D). Boxes indicate exons. CE, cassette exon.

Genomic DNA fragments to be analyzed are colored in orange. GFP, RFP and GST cDNAs are in green, magenta and purple, respectively. Green circles, red diamonds and red arrowheads indicate artificially introduced translation initiation codons, termination codons and frame-shifts, respectively. attB sites are indicated as blue lines. Open reading frames of expected mRNAs are colored; GFP-fusion proteins, RFP-fusion proteins and others are in green, magenta and cyan, respectively.

Figure 2: Construction of an ‘Expression’ clone by ‘2-fragment’ recombination reaction utilizing MultiSite Gateway system.

(A) Cloning DNA fragments of interest in ‘Entry’ vectors by ‘BP’ reaction. attB-flanked PCR products and two MultiSite Gateway ‘Donor’ vectors are used in separate ‘BP’ recombination reactions to generate two ‘Entry’ clones, one with attL1 and attR5 sites, and the other with attL5 and attL2 sites. att sites are not palindromic and have an orientation. The direction of the arrowhead designates the orientation of each att site in relation to the insert; the attP or attB site is designated with “r” when the arrowhead does not point towards the insert.

(B) Construction of an ‘Expression’ clone by ‘LR’ reaction. The two ‘Entry’ clones and a ‘Destination’ vector are used together in ‘LR’ recombination reaction to create an
‘Expression’ clone containing the two DNA fragments.

**Figure 3: Examples of the analysis.**

(A, B) Visualization of mutually exclusive alternative splicing in *C. elegans*. (A) Schematic structure of a trio of reporter mini-genes to monitor selection profiles of three mutually exclusive exons (a, b and c). The promoter, the constitutive first intron and the 3’ cassette were provided by a ‘Destination’ vector. Two termination codons (red diamonds) are introduced into two of the alternative exons in each construct. attB sites are indicated as blue lines. (B) A microphotograph of a fluorescent alternative splicing reporter worm carrying the mini-genes in (A). Expression of Venus (green), mRFP (red) and ECFP (blue) shows tissue-specificity.

(C, D) Visualization of virus-induced intron retention. (C) Schematic structure of a reporter mini-gene to monitor splicing of an alternatively retained intron, and mRNAs derived from it. RFP protein is produced only when the alternative intron is properly spliced. (D) Microphotographs of uninfected (left) and Venus-HSV-2-infected (right) HeLa cells. All uninfected cells express RFP (magenta), while cells infected with Venus-HSV-2 (green) shut off RFP expression.

(E, F) Visualization of tissue-specific alternative splicing in mice. (E) Schematic structure of a reporter mini-gene to monitor selection of mutually exclusive alternative exons, and mRNAs derived from it. GFP protein is produced when exon a alone is selected and RFP is produced when exon b alone is selected. (F) Microphotographs of a mouse embryo at E14.5. (Left) Expression of GFP is detected in epidermis (arrowheads). (Right) Expression of RFP is detected in the nervous system and mesenchymal tissues.
Abbreviations

EJC, exon junction complex;
GFP, green fluorescent protein;
GSP, gene-specific primer;
GST, glutathionine S-transferase;
mRNA, messenger RNA;
NMD, nonsense-mediated mRNA decay;
PCR, polymerase chain reaction;
PTC, premature termination codon;
RFP, red fluorescent protein;
RT, reverse transcription;
UTR, untranslated region.

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