Chapter 18

Cell-Based Splicing of Minigenes

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Abstract

Cell-based splicing of minigenes is used extensively in the analysis of alternative splicing events. In particular, such assays are critical for identifying or confirming the in vivo relevance of *cis*- and *trans*-acting factors in the regulation of particular splicing patterns. Here we provide detailed information on the methods specific to the cell-based analysis of minigene splicing. In addition, we discuss some of the theoretical considerations that must be given to the design of the minigene and subsequent experimental conditions.

Key words Minigene, RNA isolation, RT-PCR, Transfection, Stable cell lines, Alternative splicing, Exon, Intron

1 Introduction

The use of minigenes has long been a central tool in the characterization of splicing regulation and mechanisms. As the name implies, a "minigene" is a simplified version of an endogenous pre-mRNA. Most pre-mRNAs are vastly too long for ready manipulation, and mutation of endogenous genes in living cells is both inefficient and potential toxic. Therefore, use of a simplified model of a pre-mRNA, or "minigene," opens the door to lines of investigation not otherwise feasible. Indeed, the vast majority of known sequence elements that control pre-mRNA splicing were identified and/or characterized through minigene studies (e.g., [1–3]). Minigenes are also widely used to report on splicing patterns in cell-based screens for trans-acting proteins and regulatory pathways (e.g., [4–6]).

At the point at which one has identified a splicing event of interest—perhaps a change in the alternative splicing pattern of a particular gene in normal and diseased cells—the next step in understanding how the splicing pattern is regulated is most typically the development and characterization of a minigene. In vitro analysis of minigenes, as described in Chapter 11, can answer many questions. However, splicing competent extracts have only been generated from a handful of cell types and thus cannot recapitulate

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many biologically important splicing events. Moreover, even in cases in which in vitro splicing is possible, analysis of splicing in living cells represents a powerful complementary approach.

Importantly, cell-based analysis of minigene splicing is relatively straightforward and widely applicable to almost any cell type and splicing event of interest. In brief, such assays involve (1) appropriate design of the minigene, (2) transfection and expression of the minigene in a suitable cell line, (3) harvest of RNA, and (4) analysis of splicing pattern by RT-PCR. This chapter covers each of these aspects in turn, discussing both the theoretic considerations and providing protocols for those aspects of the assay that are most unique. Common techniques such as PCR and subcloning, as well as cell-type specific methods for cell transfection and maintenance, are not covered here but references to other resources are provided.

2 Materials

2.1 Design and Construction of the Minigene

1. Genomic DNA.
2. Primers with appropriate restriction sites and complementarity to the gene region of interest.
3. Standard reagents for high-fidelity PCR, electrophoresis, subcloning, and plasmid preparation.
4. Expression vector for cloning sequence of interest.

2.2 Transfection and Expression of Minigene

1. Tissue culture cell line.
2. Tissue culture media, serum, and antibiotics.
3. Transfection reagent, such as Lipofectamine 2000 (Invitrogen) or 0.4 cm electroporation cuvettes (USA Scientific).
4. Purified minigene vector DNA (10 μg in 10 μl DI (distilled and deionized) H_2O) (*see* **Note 1**).
5. Incubator with CO_2.
6. Tissue culture plastic ware including sterile flasks, plates, and pipettes.
7. Low-speed centrifuge with capacity for 15 ml conical tubes (*see* **Note 2**).

2.3 Harvest of Total RNA

1. Low-speed centrifuge with capacity for 15 ml conical tubes (*see* **Note 2**).
2. RNase-free microcentrifuge tubes and pipette tips.
3. Refrigerated microcentrifuge or microcentrifuge in 4 °C room.
4. RNA-Bee (TelTest, Inc., *see* **Note 3**).
5. Chloroform.

6. Ice-cold 70 % ethanol.
7. RNase-free DI (distilled and deionized) H_2O.

2.4 RT-PCR Reagents for Analysis of Splicing Pattern

1. Vector-specific forward and reverse primers (*see* **Note 4**).
2. ^{32}P-gamma-ATP.
3. Polynucleotide kinase (PNK) and accompanying buffer.
4. Phenol:chloroform:isoamylalcohol.
5. 100 % Ethanol and ice-cold 70 % ethanol.
6. 5× Hyb buffer (1.5 M NaCl, 50 mM Tris–HCl pH 7.5, 10 mM EDTA).
7. 1.25× RT-mix (1.25 mM each dNTPs, 12.5 mM DTT, 12.5 mM Tris–HCl pH 8.0, 7.5 mM $MgCl_2$) (*see* **Note 5**).
8. MMLV reverse transcriptase (*see* **Note 6**).
9. RT-PCR buffer (0.5 M KCl, 0.1 M Tris–HCl pH 8.3, 15 mM $MgCl_2$, 0.01 % gelatin).
10. Taq polymerase.
11. Formamide buffer (45 ml formamide, 2.5 ml 0.5 M EDTA, 0.01 g bromophenol blue, 0.01 g xylene cyanol).
12. Thermocycler.
13. Mineral oil (*see* **Note 7**).

3 Methods

3.1 Design and Construction of the Minigene

Minigene design can vary depending on the question to be addressed. Typically, one aims to create a minigene that mimics the endogenous splicing pattern of a given exon or intron. The following protocol gives several examples of how this is done.

1. Using standard PCR methods [7–9] isolate a fragment of genomic DNA encompassing the sequencing event of interest (*see* Fig. 1). Include in the PCR primers restriction sites as needed for **step 2**.
2. Digest the PCR product with restriction enzymes and clone into a suitable expression vector such as shown in Fig. 2. *See* [8, 9] if unfamiliar with standard subcloning methods.
3. Confirm minigene sequence and prepare DNA by a method that generates sufficiently pure and concentrated DNA for cell transfection.

3.2 Transfection and Expression of Minigene

The methods for transfection and cell grown/minigene expression are highly dependent on the choice of cell line, which in turn, is highly dependent on the splicing event one wishes to study. Here, we provide a protocol for transient transfection of the commonly

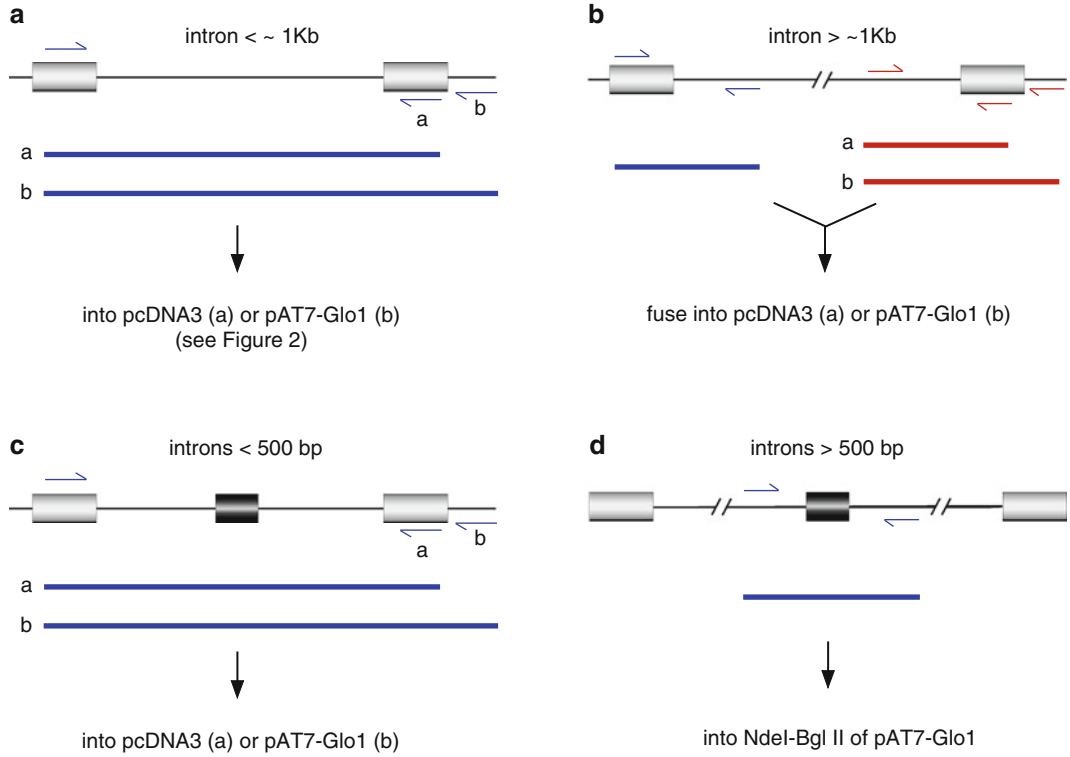

Fig. 1 Genomic segments used to generate minigenes. Typical range of genomic sequence isolated to generate minigenes to test splicing of a single intron (**a**, **b**) or inclusion of a cassette exon (**c**, **d**). Exons are indicated by *boxes*, introns by *black lines*, PCR primers by *blue* and *red single arrowhead*, PCR products by *blue* and *red lines*. Examples are given for strategies typical for large (**b**, **d**) or small (**a**, **c**) introns. In scenarios (**a**–**c**) the minigene could include only sequences from the endogenous gene (*a*) cloned into an empty mammalian expression construct such as pcDNA3, or the downstream exon could be followed by its intron in the PCR fragment (*b*) and then fused to a test intron/exon such as that from β-globin as found in pAT7-Glo1 (*see* Fig. 2). The latter construction has the advantage that splicing of the final exon to the β-globin exon functions as an internal positive control in the ultimate cell-based assay and abrogates concerns of false results from contaminating DNA (*see* **Note 30**)

used HEK293 cell line (Subheading 3.2.1), and a protocol for transfection and establishment of stable minigene expressing lines (Subheading 3.2.2), which is suitable for hard-to-transfect cell lines. Discussion of considerations for choosing an appropriate cell line and method is given in **Notes 8** and **9**.

3.2.1 Transient Transfection of HEK293 Cells

1. For each transfection, plate 3×10^5 HEK293 cells in one well of a 6-well plate in a volume of 2 ml DMEM plus serum.
2. Let attach for 24 h.
3. Carefully remove DMEM and overlay cells with 1.5 ml prewarmed Opti-Mem (Invitrogen).
4. Mix 1 μg DNA (1 μg/μl) with 250 μl Opti-Mem and let sit 5 min at room temperature.

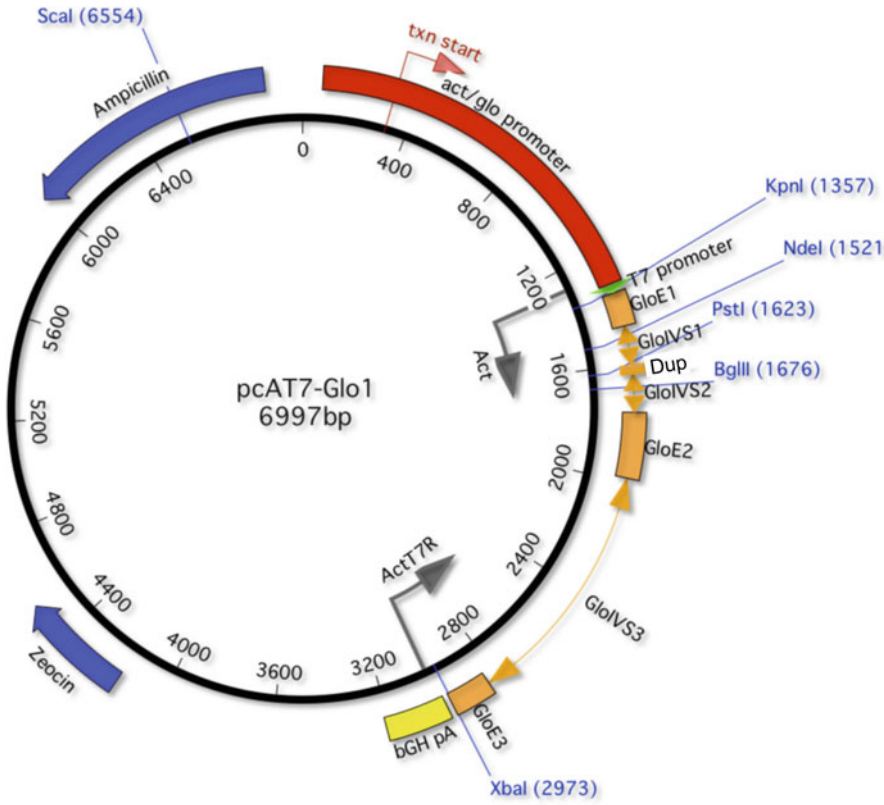

Fig. 2 pAT7-Glo1: A convenient backbone for minigene analysis. Plasmid map of pAT7-Glo1, which contains a modified version of the β-globin gene. The human β-globin gene is one of two splicing modules that has been extensively characterized and is often used as a background for minigenes (the other is the Adenovirus Major Late gene or AdML; [10]). pAT7-Glo1 contains the three exons endogenous to β-globin (*gold boxes*) plus a small test exon (*Dup, gold box*) that contains duplicated splice sites from the first and second endogenous exons [1]. Additional restriction sites have been engineered to facilitate subcloning of appropriate fragments of genomic DNA as described in Fig. 1. Other salient features are selectable markers (*blue*) for bacterial (Amp) and mammalian (Zeocin) selection, a eukaryotic promoter (act/glo, *red*) that functions in all mammalian cell lines tested, and a T7 promoter (*green*). Location of primers used for RT-PCR (Act, ActT7R) are also indicated

5. Mix 5 μl Lipofectamine 2000 with 250 μl Opti-Mem and let sit 5 min at room temperature (*see* **Note 10**).
6. Combine mixtures from **steps 4** and **5**, flick with finger to mix and let sit 20 min at room temperature for precipitate to form (*see* **Note 11**).
7. Add mixture from **step 6** in a drop-wise manner overtop of cells.
8. Harvest cells after 48 h to analyze splicing as described below (Subheadings 3.3 and 3.4).

3.2.2 Generating Stable Clonal Transfectants of Jurkat Cells

1. Split 10 million Jurkat cells per transfection to 0.5 million per ml. Let cells grow for about 24 h so that they are in mid-log phase for transfection (0.8–1.2 million per ml).
2. The next day, spin down 10–20 million Jurkat cells for each construct to be transfected.
3. Wash cells twice in serum-free antibiotic-free medium.
4. Resuspend cells in 400 μl serum-free antibiotic-free medium per transfection. Transfer 400 μl cells to an electroporation cuvette (*see* **Note 12**).
5. Add 10 μl plasmid DNA (1 μg/μl). Flick cuvette to mix well. Let stand up to 5 min.
6. Set electroporator for 250 mV, capacitance = 960 and time to constant. Place each cuvette into holder and electroporate sample (*see* **Note 13**).
7. Flick cuvette vigorously to mix pH gradient that has been formed and let stand for 5 min.
8. Remove cells from cuvette, being careful to avoid transferring the clump of dead cells and debris. Add cells to a well of a 6-well plate to which 6 ml of medium plus 10 % serum has been added.
9. After 48–72 h, serially dilute cells to achieve 20 ml each of cells diluted to 1×10^5, 3×10^4, and 1×10^4 per ml into medium containing serum and antibiotics for selection (*see* **Note 14**).
10. Aliquot each dilution into a full 96-well plate, using 200 μl diluted cells per well.
11. Allow 14–21 days for colonies to appear. Slowly expand wells that contain a single colony eventually to a 6-well plate (*see* **Note 15**).
12. Harvest a 5 ml sample of cells for each clone using Subheading 3.3 below (leaving sufficient cells continuing to grow to maintain clone) and perform RT-PCR (Subheading 3.4) to screen for minigene expression and splicing (*see* **Note 16**).

3.3 Harvest of RNA

1. Harvest up to 20 million cells into an appropriate conical or microcentrifuge tube and collect by centrifugation (*see* **Note 2**).
2. Remove and discard supernatant.
3. Resuspend cell pellet with 1 ml PBS and transfer to microcentrifuge tube.
4. Spin in a microcentrifuge for 1 min at 900 × *g* (revolutions per minute) (*see* **Note 2**).
5. Remove and discard supernatant (aspiration works well here).
6. Resuspend cell pellet in 800 μl RNA-Bee (*see* **Note 17**) and place tubes on ice (*see* **Note 18**).

7. Add 200 µl chloroform. Invert 2–3 times and vortex for 5–10 s (*see* **Note 19**).
8. Hold on ice for 10 min inverting occasionally.
9. Spin in a refrigerated (4 °C) microcentrifuge for 12 min at 17,000 ×*g* (*see* above).
10. Meanwhile, label fresh RNase-free tubes and add 600 µl isopropanol. Place tubes on ice to chill alcohol.
11. Remove tubes from microcentrifuge and place at room temperature (*see* **Note 20**). Transfer clear supernatant to tubes with isopropanol. Do not carry over white interface that contains DNA.
12. Vortex tubes thoroughly and spin in a refrigerated (4 °C) microcentrifuge for 12 min at 17,000 ×*g* (*see* above).
13. Decant supernatant and add 800 µl ice-cold 70 % ethanol (*see* **Note 21**).
14. Spin in a refrigerated (4 °C) microcentrifuge for 2 min at 17,000×*g* (see above).
15. Decant supernatant being careful not to lose pellet and repeat **steps 13** and **14** twice more (*see* **Note 22**).
16. Remove all liquid with a pipet tip (*see* **Note 23**)
17. Resuspend in 12 µl RNase-free DI H_2O.
18. Check concentration by OD_{260} and adjust to 0.5 mg/ml in RNase-free DI H_2O.

3.4 Analysis of Splicing Pattern

The most widely used and robust assay to analyze splicing patterns in cells is low-cycle RT-PCR. The following protocol provides a highly reproducible assay to quantify changes in isoform expression between two conditions (i.e., cell growth conditions, presence or absence of a trans-acting factor, or between wild-type and mutant minigenes) (*see* **Note 24**).

3.4.1 Making 5′ ^{32}P-End Labeled PCR Primer

1. Mix together 77 µl dH_2O, 10 µl ^{32}P-gamma-ATP, 10 µl 10× PNK buffer, 2 µl (100 ng/µl) downstream primer, and 1 µl PNK enzyme.
2. Incubate at 37 °C for 30 min.
3. PCA extract, precipitate with ethanol and wash in 70 % ice-cold ethanol (*see* **Note 25**).
4. Resuspend in 80 µl RNase-free DI H_2O for a final concentration of ~2.5 ng/µl.

3.4.2 RT-PCR

1. To a PCR tube on ice, add 2 µl RNA at 0.5 µg/µl, 1 µl 5× Hyb buffer, and 1 µl downstream primer (1 ng/µl) (*see* **Note 4**).
2. Using a thermocycler, heat RNA/primer/Hyb buffer mix to 90 °C for 20 s then cool slowly to 43 °C by decreasing

temperature in 1 °C increments every 20 s then holding at 43 °C. This denatures the RNA and anneals the primer.

3. Add 19.5 μl RT-mix plus 0.5 μl MMLV, which have been premixed and warmed to 43 °C (*see* **Note 26**).
4. Continue incubation at 43 °C for 30 min followed by 94 °C for 5 min, and rapid cool to 4 °C. Hold at 4 °C until next step.
5. Set up hot PCR reaction by adding 1 μl downstream primer (5 ng/μl), 1 μl upstream primer (2.5 ng/μl), 1 μl ^{32}P-labeled upstream primer (from Subheading 3.4.1. **step 4**), 1.5 μl RT-PCR buffer, 10.3 μl dH_2O, and 0.2 μl Taq DNA polymerase. Mix well. Add a drop of mineral oil onto sample (*see* **Note 7**).
6. Set Thermocycler to run a program such as the following: 94 °C 2 min; *X* cycles of (94 °C, 1 min; *Y*°C, 1 min; 72 °C, *Z*min); 72 °C, 7 min, 4 °C and hold. Annealing temperature (*Y*) and extension time (*Z*) should be determined by your primers and length of predicted product. Cycle number (*X*) must be determined empirically for each transcript to provide signal that is linear with respect to input RNA and is dependent on expression level of the RNA, but is typically 20–25 cycles.
7. Add 15 μl formamide buffer. Store samples at −20 °C.

3.4.3 Denaturing Gel Electrophoresis

1. Prepare and pre-run a 5 % denaturing TBE–urea–polyacrylamide gel.
2. Boil reactions from Subheading 3.4.2, **step 8** for 5 min and place immediately on ice.
3. Turn off power to gel. Load 5 μl of each reaction per lane plus an appropriate marker in an additional lane (*see* **Note 27**). Restore power to gel (*see* **Note 28**).
4. Run gel an appropriate length of time to resolve bands.
5. Disassemble gel plates, submerge gel on one plate in 10 % acetic acid + 10 % methanol for 15 min to fix.
6. Transfer gel to Whatmann paper. Use an additional sheet of Whatmann underneath and overlay with saran-wrap (don't wrap saran-wrap underneath Whatmann paper).
7. Dry on gel dryer, then expose to phosphorimage screen (*see* **Notes 29** and **30**).

4 Notes

1. For stable transfections, it is optimal to linearize the minigene expression plasmid prior to transfection. This provides free ends that increase the efficiency of chromosomal integration. Moreover, if the plasmid is linearized by restriction digest prior to transfection, one can ensure that the cut is made *outside* of

the minigene sequence and its promoter, thereby decreasing the number of drug-resistant clones that have lost the ability to express the minigene due to random nicking. Typically, one uses a restriction site that is unique to the bacterial resistance gene (i.e. Amp^r) as the integrity of this gene is not relevant for growth in mammalian cells. After linearization in a standard restriction digest, the DNA should be repurified by PCA extraction/ethanol precipitation (*see* **Note 25**) and resuspended in DI H_2O.

2. Mammalian cultured cells are collected by low-speed centrifugation (<1,000 × *g*). This typically is done by spinning at 3K rpm for 1 min in a microcentrifuge or 5 min in a typical tabletop centrifuge at 1K rpm (~200 × *g*). The choice of centrifuge will depend on the volume of media from which cells are being collected. Centrifugation at a force higher than 1,000 × *g* will cause cells to lyse.
3. The use of RNA-Bee and the above protocol for RNA harvest is easy, fast and yields high-quality RNA that supports reproducible results in subsequent applications. RNA-Bee is a monophase solution containing phenol and guanidine thiocyanate. Many similar reagents are also available commercially, such as Trizol (Invitrogen), while some labs chose to make their own solutions. Other methods for RNA isolation include column-based kits from companies such as Qiagen and Ambion.
4. Appropriate design of primers is essential for the RT-PCR to be robust and quantitative. First, the primers need to be specific to the minigene expression construct and not cross-reactive to the endogenous gene. Typically, we use a forward primer that is complementary to the first 20–30 transcribed nucleotides of the minigene, which includes significant sequence from the vector cloning sites. Similarly, the reverse primer is optimally complementary to some constitutive portion of the minigene, such as the final exon in the sample vector shown in Fig. 2 (Act and ActT7R). Use of a poly-dT reverse primer should be avoided, as this does not have a sufficiently high Tm to allow for stringent RT-PCR conditions such that the results obtained with such a primer can be highly variable. The minimal Tm of the primer for stringent RT-PCR is 60 °C. Whenever possible, we design primers to anneal at 70 °C with the 3′ terminal 2–3 nucleotides consisting of a G or C.
5. The 1.25× RT-mix should be stored in single or double-use aliquots at −80 °C to avoid repeated freeze–thaw cycles that decrease the stability of the dNTPs.
6. Several RT (reverse transcriptase) enzymes are commercially available. In our hands MMLV is the most robust and is most heat-stable such that the RT reaction can be done at a temperature that limits the existence of RNA secondary structure.

However, for one sequence we have found AMV to be more robust. By contrast, the "Superscript" family of RT enzymes yield highly variable results and their use in quantitative assays such as those outlined here should be avoided.

7. Oil should be used even with "hot-lid" machines, as ^{32}P can be volatile. Also in our experience "hot-lids" can alter the accuracy of the temperature of the reaction, occasionally causing problems with reproducibility in the RT-PCR reaction.
8. Any standard method of transfection and gene expression can be used for the cell-based analysis of splicing. HeLa, HEK293, and COS cells have been widely used for the analysis of general splicing events due to the ease of transfection. However, understanding tissue-specific, disease-specific or pathway-specific splicing events often requires use of more specialized cell lines. Thus, researchers should use the cell-line that best fits the biology of the system, and chose a method for transfection and gene expression that is most optimal for the specified cell type.
9. Transient transfection/expression of a minigene allows analysis of splicing within 2–4 days, versus the 3–4 weeks required for the creation of a stable cell line. However, establishing stable, clonal cell lines is advantageous in the instances where transfection efficiency is lower, or when the minigene is to be used for cell-based screening. In our experience transient transfections also at times yield highly variable results due to the unstable nature of the expression of the minigene. Therefore, while transient transfection is a good "quick and dirty" method, the total time required to obtain statistically significant results is often similar for stable versus transient transfection.
10. The exact ratio of DNA to Lipofectamine can have significant impact on the efficiency of transfection. The optimal ratio must be determined empirically for each cell type. See the Invitrogen protocol for more detail (http://www.invitrogen.com).
11. Note that the precipitate is often not visible to the naked eye. This does not alter the efficiency of transfection.
12. The efficiency of electroporation is highly sensitive to volume but not to total cell number. It is important to use exactly 400 μl of cells in each cuvette even if this means using less than the optimal 10–20 million cells.
13. Settings may vary for different machines and different strains of Jurkats or other suspension cells.
14. The given plating densities are appropriate for Jurkat transfections selected with Zeocin (250 μg/ml) or G418 (2 mg active compound/ml). Optimal plating densities for other cell lines or drugs should be determined empirically. The goal is to obtain at least one 96 well plate in which ~ 30 % of wells have colonies,

as statistically most of these will be single clones and this will provide enough clones for further expansion and analysis.

15. Expand by first diluting the 200 µl from the 96 well plate into 2 ml, then eventually 6–10 ml. Overly diluting cells at this stage often causes cell death and loss of clone.
16. If the minigene DNA has been linearized prior to transfection (*see* **Note 1**) generally 80 % or greater of drug-resistant clones will express the minigene. This success rate typically drops to 25 % if a circularized vector is used. The transfection may yield many more single clones than one wishes to pursue for further study. It is prudent to screen 2–4 times more clones than one wants to ensure that a sufficient number is obtained to ensure statistical significance of the final data (3–4 clones if the minigene is spliced consistently in all clones).
17. The final quantity and quality of the RNA is highly dependent on efficient cell lysis at this step. Solubilize the cell pellet in RNA-Bee by repeated pipeting (~10×). A sign that the cells have lysed will be that the solution will become increasingly viscous, air bubbles will not dissipate as quickly and one may notice a "schlurpping" sound.
18. If it is not convenient to do the entire RNA harvest in 1 day, the process can be stopped after the cells are solublized in RNA-Bee and the tubes stored at −80 °C. Similarly, the RNA can be stored at −80 °C after isopropanol is added in **step 10**.
19. Do not vortex too aggressively at this stage. The component should be well mixed, but if vortexed too long an emulsion will form between the chloroform and RNA-Bee that cannot be separated by subsequent centrifugation. If this happens no RNA will be able to be obtained from the sample.
20. At this point there should be a clear liquid phase (containing RNA) over a clear blue phase (containing protein) with a white solid interface (containing DNA). Putting tubes on ice at this point will cause the clear phases to become turbid, making discrimination of RNA phase from DNA phase more difficult.
21. Following centrifugation a small, white pellet of RNA should be visible at the bottom on the tube. Be careful not to dislodge while decanting supernatant. Use a pipet to remove the liquid if this is a problem.
22. Thorough washing of the RNA pellet is required to ensure no carry over of contaminants that can hinder the subsequent RT-PCR reaction.
23. If over-dried the RNA pellet will become resistant to being solublized in H_2O. Do not use a speed-vac to dry. Simply remove any visible liquid with a pipet, allow to air-dry for no more than 5 min and immediately add 12 µl RNase-free DI H_2O.

24. Radiolabeled RT-PCR (as in the protocol here) is superior to Northern blots, real-time RT-PCR or non-labeled RT-PCR for the analysis of splicing for several reasons. Ethidium bromide staining of RT-PCR (in the absence of radiolabel) is much less sensitive and quantitative and can only be used to roughly judge qualitative differences in splicing. Real-time RT-PCR requires use of distinct primers to measure alternate isoforms, adding variability to the results. In addition this method can only measure predicted isoforms and does not report on the presence of unanticipated or cryptic products. Northern blots are highly quantitative, but cumbersome and often don't have the size resolution needed.
25. PCA extraction and ethanol precipitation are standard techniques in any molecular biology lab. PCA is a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol that is used at equal volume to the aqueous reaction to extract protein. RNA/DNA is then precipitated using 0.1 volumes of 3 M NaOAC and 2.5 volumes of 100 % ethanol. *See* [9] for more details.
26. Pre-warm the RT + MMLV mix to 43 °C for 2 min before adding to the RNA/primer mix. This prevents the RNA from cooling below 43 °C at any point and thus limits the formation of RNA secondary structure, which can inhibit the reverse transcription reaction. We have seen significantly greater reproducibility in the RT-PCR results when the RNA is kept at 43 °C or greater versus conditions that cool the reaction to 37 °C.
27. Any radiolabeled markers can be used here. We typically use the pBR322-MspI digest markers from NEB. These cover a size range that is typically appropriate (622 bp and smaller) and can be easily radiolabeled by filling in the fragment ends with Klenow polymerase fragment and ^{32}P-dCTP (*see* www.neb.com).
28. Never handle or load a gel with the power supply still attached and/or current still running as this can lead to electrocution.
29. Quantification of splicing is done by using a phosphorimager to quantify the intensity of the spliced products compared to the background lane. Splicing is then quantified as either % variable exon inclusion (intensity of product including the variable exon/total intensity of all products), or alternatively, when comparing the splicing of minigenes grown under different cellular conditions it is often advantageous to calculate a fold difference in isoform ratio by $([\text{included}/\text{excluded}]_{\text{condition 1}}/[\text{included}/\text{excluded}]_{\text{condition 2}}$ as described in [1].
30. Designing the experiment to quantify two alternative spliced products (i.e. variable exon inclusion versus exclusion between two constitutive exons) and/or including at least

one constitutively spliced intron in the minigene (i.e. β-globin exon 2-exon 3; *see* Fig. 2) has the significant advantage that one does not have to differentiate between unspliced message and vector DNA. Therefore DNase treatment and extensive "no-RT" controls are not necessary.

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