Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62181R1
Full Title:	Using the E1A Minigene Tool to Study mRNA Splicing Changes
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Additional Information:	
Question	**Response**
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TITLE:

Using the E1A Minigene Tool to Study mRNA Splicing Changes

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KEYWORDS:

mRNA alternative splicing, minigene E1A, Nek4, chemotherapy response, isoforms expression, HEK293 stable cells.

SUMMARY:

This protocol presents a rapid and useful tool for evaluating the role of a protein with uncharacterized function in alternative splicing regulation after chemotherapeutic treatment.

ABSTRACT:

mRNA processing involves multiple simultaneous steps to prepare mRNA for translation, such as 5´capping, poly-A addition and splicing. Besides constitutive splicing, alternative mRNA splicing allows the expression of multifunctional proteins from one gene. As interactome studies are generally the first analysis for new or unknown proteins, the association of the bait protein with splicing factors is an indication that it can participate in mRNA splicing process, but to determine in what context or what genes are regulated is an empirical process. A good starting point to evaluate this function is using the classical minigene tool. Here we present the adenoviral E1A minigene usage for evaluating the alternative splicing changes after different cellular stress stimuli. We evaluated the splicing of E1A minigene in HEK293 stably overexpressing Nek4 protein after different stressing treatments. The protocol includes E1A minigene transfection, cell treatment, RNA extraction and cDNA synthesis, followed by PCR and gel analysis and quantification of the E1A spliced variants. The use of this simple and well-established method combined with specific treatments is a reliable starting point to shed light on cellular processes or what genes can be regulated by mRNA splicing.

INTRODUCTION:

Splicing is among the most important steps in eukaryotic mRNA processing that occurs simultaneously to 5´mRNA capping and 3´mRNA polyadenylation, comprising of intron removal

followed by exon junction. The recognition of the splicing sites (SS) by the spliceosome, a ribonucleoprotein complex containing small ribonucleoproteins (snRNP U1, U2, U4 and U6), small RNAs (snRNAs) and several regulatory proteins[1] is necessary for splicing.

Besides intron removal (constitutive splicing), in eukaryotes, introns can be retained and exons can be excluded, configuring the process called mRNA alternative splicing (AS). The alternative pre-mRNA splicing expands the coding capacity of eukaryotic genomes allowing the production of a large and diverse number of proteins from a relatively small number of genes. It is estimated that 95-100% of human mRNAs that contain more than one exon can undergo alternative splicing[2,3]. This is fundamental for biological processes like neuronal development, apoptosis activation and cellular stress response[4], providing the organism alternatives to regulate cell functioning using the same repertoire of genes.

The machinery necessary for alternative splicing is the same used for constitutive splicing and the usage of the SS is the main determinant for alternative splicing occurrence. Constitutive splicing is related to the use of strong splicing sites, which are usually more similar to consensus motifs for spliceosome recognition[5].

Alternative exons are typically recognized less efficiently than constitutive exons once its *cis*-regulatory elements, the sequences in 5´SS and 3´SS flanking these exons, show an inferior binding capacity to the spliceosome. mRNA also contains regions named enhancers or silencers located in exons (exonic splicing enhancers (ESEs) and exonic splicing silencers (ESSs)) and introns (intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs)) that enhance or repress exon usage, respectively[5]. These sequences are recognized by trans-regulatory elements, or splicing factors (SF). SFs are represented mainly by two families of proteins, the serine/arginine rich splicing factors (SRSFs) which bind to ESEs and the family of heterogeneous nuclear ribonucleoproteins (hnRNPs) which bind to ESSs sequences[5].

Alternative splicing can be modulated by phosphorylation/ dephosphorylation of trans- factors modifying the interactions partners and cellular localization of splicing factors[6–8]. Identifying new regulators of splicing factors can provide new tools to regulate splicing and, consequently, some cancer treatments.

Anufrieva et al.[9], in a mRNA microarray gene expression profile, observed consistent changes in levels of spliceosomal components in 101 cell lines and after different stress conditions (platinum-based drugs, gamma irradiation, topoisomerase inhibitors, tyrosine kinase inhibitors and taxanes). The relationship among splicing pattern and chemotherapy efficacy has already been demonstrated in lung cancer cells, which are chemotherapy resistant, showing changes in caspase-9 variants rate[10]. HEK293 cells treated with chemotherapeutics panel show changes in splicing with an increase in pro-apoptotic variants. Gabriel et al.[11] observed changes in at least 700 events of splicing after cisplatin treatment in different cell lines, pointing out that splicing pathways are cisplatin-affected. Splicing modulators have already demonstrated anti-tumoral activity, showing that splicing is important to tumoral development and, mainly, chemotherapy

response[12]. Hence, characterizing new proteins that regulate splicing after cellular stressors agents, like chemotherapeutics, is very important to discover new strategies of treatment.

The clues of alternative splicing regulation from interactome studies, particularly important to characterize functions of new or uncharacterized proteins, can demand a more general and simple approach to verify the real role of the protein in AS. Minigenes are important tools for analysis of the general role of a protein affecting splicing regulation. They contain segments from a gene of interest containing alternatively spliced and flanking genomic regions[13]. Using a minigene tool allows the analysis of splicing in vivo with several advantages such as the length of the minigene which is minor and therefore is not a limitation to the amplification reaction; the same minigene can be evaluated in different cell lines; all cellular components, mainly their regulating post-translational modification (phosphorylation and changes in cell compartments) are present and can be addressed[13, 14]. Moreover, changes in alternative splicing pattern can be observed after cellular stress and, the use of a minigene system, allow to identify the pathway being modulated by different stimuli.

There are several minigene systems already described which are specific for different kinds of splicing events[13, 14], however, as a preliminary assay, the minigene E1A[15] is a very well established alternative splicing reporter system for the study of 5´SS selection in vivo. From only one gene, E1A, five mRNAs are produced by alternative splicing based on selection of three different 5' splice sites and of one major or one minor 3' splice site[16–18]. The expression of E1A variants changes according to the period of Adenoviral infection[19,20].

We have shown previously both Nek4 isoforms interacts with splicing factors such as SRSF1 and hnRNPA1 and while isoform 2 changes minigene E1A alternative splicing, isoform 1 has no effect in that[21]. Because isoform 1 is the most abundant isoform and changes chemotherapy resistance and DNA damage response, we evaluate if it could change minigene E1A alternative splicing in a stress condition.

Minigene assay is a simple, low-cost and rapid method, since it only needs RNA extraction, cDNA synthesis, amplification and agarose gel analyses, and can be a useful tool to evaluate since a possible effect on alternative splicing by a protein of interests until the effect of different treatments on cellular alternative splicing pattern.

PROTOCOL:

1. Plating cells

NOTE: In this described protocol, HEK293 stable cell lines, previously generated for stable inducible expression of Nek4 were used[21], however, the same protocol is suitable to many other cell lines, such as HEK293[22], HeLa[23–26], U-2 OS[27], COS7[28]; SH-SY5Y[29]. The pattern of expression of minigene E1A isoforms under basal conditions varies between these cells and should be characterized for each condition. This protocol is not limited to stable cell lines. The most

common approach in evaluation of candidate protein is by transient co-transfection of increasing its amount with fixed amount of the minigene. The same protocol is suitable for knockout cells.

1.1. Plate cells considering vehicle, untransfected and GFP controls.

1.2. Culture HEK293 cells with stable expression of the gene of interest in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of Fetal Bovine Serum (FBS), 4.5 g/L Glucose, 4 mM L-Glutamine and maintain with 100 µg/mL of hygromycin B, on tissue culture-treated plates at 37 °C in 5% CO_2 and a humidified atmosphere containing 90% air.

1.3. Split cells using 0.25% trypsin-EDTA. Plate 3 x 10^5 cells in 6-well plates and incubate them for 24 h at 37 °C in 5% CO_2.

NOTE: For transfection, cells must be 70-80% confluent to decrease cell death after the transfection.

2. Cell transfection

NOTE: 1-2 µg of pMTE1A minigene plasmid was used here, however the DNA amount, as well as the time of expression, must be kept at minimal to avoid toxicity. For example, high toxicity was observed in HeLa cells after 30 h of transfection with 1 µg of pMTE1A DNA. For the transfection described here, a lipid-based transfection reagent was used

2.1. Check cell confluence 24 h after plating and transfect HEK293 stable cells only when 70-80% confluent.

2.2. Remove the cell culture medium carefully with a pipette, instead of using a vacuum pump system. Then carefully add 2 mL of complete DMEM medium without antibiotics and put the plate back in the incubator.

2.3. Prepare a tube with the transfection buffer (200 µL/well), add 2 µg of pMTE1A DNA, vortex and then add 2 µL of transfection reagent. Vortex again and incubate for 10 min at room temperature.

2.4. Remove plates from the incubator and carefully add the transfection mixture dropwise.

2.5. To HEK293 stable cells, add tetracycline (0.5 µg/mL) for Nek4 expression induction 6 h after the transfection. Medium change is not necessary.

NOTE: Volumes/amounts described in this section are for one well. Prepare the mixture for all wells used in the experiment in the same tube.

2.6. Prepare one well to transfect with EGFP, or another fluorophore expressing plasmid to estimate the transfection efficiency. Better results can be observed with transfection efficiency

of at least 40%, but good performances with lower transfection efficiency were previously observed.

2.7. When using co-transfection (interest protein and minigene) keep a well with non-transfected cells to avoid obtaining results from endogenous mRNA. In the case of E1A, HEK293 cells already express the E1A gene[30].

3. Preparing the drugs

NOTE: The time and concentration of treatment were chosen based on literature results, which point out changes in alternative splicing for some genes.

3.1. Perform a dose-response curve before starting the assay to determine the minimal concentration to alternative splicing induction with no effect in cell viability.

3.2. Prepare a Paclitaxel stock solution at 5 mM concentration in ethanol. The final concentration is 1 µM. Keep at -20 °C. Use 0.02% ethanol as vehicle control.

3.3. Prepare a cisplatin solution by diluting in 0.9% NaCl at around 0.5 mg/mL (1.66 mM). Protect from light, vortex and incubate in a thermal bath, 37 °C for 30 min. The final concentration is 30 µM. Prepare fresh or store at 2-10 °C for until one month.

NOTE: All drugs must be protected from the light.

4. Cell treatment and collection

NOTE: HEK293 stable cells were collected 48 h after the transfection and for this were treated 24 h after the transfection because the highest Nek4 expression level is achieved within 48 h. However, high levels of 13S isoform expression (until 90%) were observed at this time. To decrease the proportion of 13S isoform, try to treat and collect cells 30 h after transfection maximum.

4.1. Use RNA/DNase free tips and tubes. Perform total RNA extraction with phenol-chloroform reagent following manufacturer's recommendation.

4.2. 24 h after the transfection, verify cell morphology and transfection efficiency using a fluorescent microscope.

4.3. Remove the cell culture medium, preferentially using a pipette instead of a vacuum pump system. Then add cell culture medium with the chemotherapeutics in the previously described final concentration.

4.4. Incubate at 37 °C, 5% CO_2 for 18 - 24 h.

4.5. Collect RNA by discarding the cell culture medium in a container and adding 0.5 – 1 mL of RNA extraction reagent directly to the well. If wells are very confluent, use 1 mL of RNA extraction reagent to improve RNA quality.

4.6. Homogenize with the pipette and transfer to a 1.5 mL centrifuge tube. At this point, the protocol can be paused by storing samples at -80 °C or proceed immediately to the RNA extraction.

WARNING: The phenol-based RNA extraction reagent is toxic and all procedures should be performed in a chemical fume hood and the residues disposed properly.

5. RNA extraction and cDNA synthesis

5.1. In a fume hood thaw the samples and incubate for 5 min at room temperature. Add 0.1 - 0.2 mL of chloroform and agitate vigorously.

5.2. Incubate for 3 min at room temperature.

5.3. Centrifuge for 15 min at 12,000 x *g* and 4 °C.

5.4. Collect the upper aqueous phase and transfer to a new 1.5 mL centrifuge tube. Collect around 60% of the total volume; however, do not collect the DNA or the organic (lower) phase.

5.5. Add 0.25 – 0.5 mL of isopropanol and agitate by inversion 4 times. Incubate for 10 min at room temperature.

5.6. Centrifuge at 12,000 x *g* for 10 min, at 4 °C and discard the supernatant.

5.7. Wash the RNA pellet twice with ethanol (75% in diethylpyrocarbonate (DEPC)-treated water). Centrifuge at 7,500 x *g* for 5 min and discard the ethanol.

5.8. Remove excess ethanol by inverting the tube on a towel paper and then leave the tube open inside a fume hood to partially dry the pellet for 5 - 10 min.

5.9. Resuspend the RNA pellet in 15 µL of DEPC-treated water.

5.10. Quantify total RNA using absorbance at 230 nm, 260 nm, and 280 nm to verify RNA quality.

5.11. To verify total RNA quality, run a 1% agarose gel pre-treated with 1.2% (v/v) of a 2.5% sodium hypochlorite solution for 30 min[31].

5.12. Perform cDNA synthesis using 1-2 µg of total RNA.

5.12.1. Pipette RNA, 1 µL of oligo-dT (50 µM), 1 µL of dNTP (10 mM) and make up the volume to 12 µL with nuclease free water. Incubate in the thermocycler for 5 min at 65 °C.

5.12.2. Remove samples from the thermocycler to cool down and prepare the reaction mixture: 4 µL of reverse transcriptase buffer, 2 µL of dithiothreitol (DTT), and 1 µL of ribonuclease inhibitor. Incubate at 37 °C for 2 min.

5.12.3. Add 1 µL of thermo-stable reverse transcriptase. Incubate at 37 °C for 50 min and inactivate the enzyme at 70 °C for 15 min.

NOTE: The cDNA can be stored at -20 °C for several weeks. Perform a non-reverse transcriptase (NRT) control for genomic contamination from putative intron-retention events discrimination.

6. pMTE1A minigene PCR

6.1. Perform PCR with the reaction composition (1.5 mM of $MgCl_2$, 0.3 mM of dNTP mix, 0.5 µM of each primer, 2.5 U of a hot-start Taq Polymerase and 150 ng of cDNA) with the following conditions:

94 °C for 2 min
29 cycles of: 94 °C for 1 min, 50 °C for 2 min, 72 °C for 2 min
72 °C for 10 min

6.2. Load 20-25 µL of the PCR product in a 3% agarose gel containing nucleic acid stain and run at 100 V for approximately 1 h.

7. Analysis of the gel using an image processing and analysis software[32]

7.1. After the run, photograph the gel (using a gel imaging acquisition system) avoiding any band saturation and quantify the bands using and image processing software.

7.2. For quantification consider the bands at ~631 bp, ~493 bp, and ~156 bp to correspond to the 13S, 12S and 9S isoforms, respectively.

7.3. From the software's **File** menu, open the image file obtained from the imaging acquisition system. Convert to greyscale, adjust brightness and contrast and remove outlier noise if necessary.

7.4. Draw a rectangle around the first lane with the **Rectangle Selection** tool and select it through the **Analyze | Gels | Select First Lane** command, or by pressing the keyboard shortcut for it.

7.5. Use the mouse to click and hold in the middle of the rectangle on the first lane and drag it over to the next lane. Go to **Analyze | Gels | Select Next Lane**, or press the available shortcut.

Repeat this step to all remaining lanes.

7.6. After all the lanes are highlighted and numbered, go to **Analyze | Gels | Plot Lanes** to draw a profile plot of each lane.

7.7. With the **Straight-line selection** tool, draw a line across the base of each peak corresponding to each band, leaving out the background noise. After all the peaks, from every lane, has been closed off, select the **Wand** tool and click inside each peak. For each peak that highlighted, measurements should pop up in the **Results** window that appears.

7.8. Sum the intensity from all the 3 bands for each sample and calculate the percentage for each isoform relative to the total.

7.9. Plot the percentages of each isoform or the differences in the percentage relative to untreated samples.

NOTE: Ensure that the sum of three E1A variants must be equal to 100%

REPRESENTATIVE RESULTS:

A 5´SS assay using E1A minigene was performed to evaluate changes in splicing profile in cells after chemotherapy exposition. The role of Nek4 – isoform 1 in AS regulation in HEK293 stable cells after paclitaxel or cisplatin treatment was evaluated.

Adenoviral E1A region is responsible for the production of three main mRNAs from one RNA precursor because of the use of different splice donors. They share common 5' and 3' termini but differ in the size of their excised introns. Adenoviral E1A mRNAs are named according to their sedimentation coefficients, 13S, 12S and 9S. During the early phase of adenovirus infection (around 7 h), proteins important to prepare the infected cell for viral DNA replication are produced (13S – 723 aa and 12S – 586 aa) and, in the late phase (around 18 h) besides those, a small protein (9S -249 aa) is produced[20]. Using a plasmid containing the minigene from E1A, the effect on alternative splicing can be observed in cells after the transfection evaluating the proportion of mRNA from each isoform produced: 13S: 631 bp, 12S: 493 bp and 9S 156 bp (**Figure 1A and B**).

Basal expression of E1A isoforms variants depends on cell line and time of E1A expression. It was observed that HEK293-stable cell line (HEK293-Flag) or HEK293 recombinase containing site (HEK293-FRT - the original cell line) shows a higher expression of 13S in comparison to HeLa cells (HeLa-PLKO) that shows similar levels of 13S and 12S isoforms after 48 h of E1A expression **(Figure 1C and D)**.

The high level of 13S expression observed in HEK293 stable cells is considerably decreased under shorter times of E1A expression (around 30 h). The proportion (%) of 13S:12S:9S at 30 h and 48 h is 60:33:7 and 80:15:5, respectively (unpresented data). For this reason, it is important to characterize the basal cellular minigene E1A splicing profile before starting the experiments.

Cells exposed to cisplatin showed a shift in 5´SS splicing selection favoring 12S expression (an increase of around 15% compared to untreated cells). This effect was observed in HEK293 stably expressing Flag empty vector as well as isoform 1 of Nek4. When major changes are observed in the percentage of expression, a plot with percentages clearly represents the results (**Figure 2**).

When comparing two conditions (Flag and Nek4 overexpression) responding to a treatment, usually the best way to represent the data is plotting the differences on a graph, because the basal level of expression can be different, and the percentages will not reflect the real effect of the treatment. This can be observed in **Figure 3**. Changes in AS after paclitaxel treatment were very discrete, but the directions of the changes were the opposite in Flag and Nek4 expressing cells.

Despite small changes after the treatment, the results were consistent, indicating that the paclitaxel treatment leads to a decrease in 13S isoform, with an increase in 12S and 9S in Flag expressing cells, while, on the other hand, in Nek4 expressing cells, the opposite effect is observed.

FIGURE AND TABLE LEGENDS:

Figure 1: Minigene E1A splicing pattern depends on cell line. A) Schematic representation of minigene E1A splicing sites. The arrows indicate the primer annealing region for minigene E1A isoforms amplification. **B**) Isoforms generated from alternative splicing of minigene E1A. **C**) HEK293 stably expressing Flag empty vector (HEK293 -Flag), HeLa transfected with PLKO vector (HeLa – PLKO) or, HEK293 recombinase-containing sites (from what HEK293 stably expressing Flag or Nek4.1 were generated – HEK293-FRT) were transfected with pMTE1A plasmid. 48h after the transfection total RNA was isolated and E1A isoforms were separated in agarose gel (**D**). Graph comparing the percentage of 13S, 12S and 9S isoform in HEK293-Flag and HeLa-PLKO cells under basal conditions. Data from three independent experiments.

Figure 2: Effect of Cisplatin treatment in minigene E1A splicing pattern. HEK293 stably expressing Flag empty vector (Flag) or Nek4.1 Flag tag fused, were transfected with pMTE1A plasmid. Six hours after the transfection tetracycline was added to proteins expression induction. 24 h to 48 h, the cell culture medium was replaced for medium containing 30 µM of Cisplatin. After 24 h of incubation, total RNA was extracted and the products of PCR were separated at 3% agarose gel. **A**) Predominant minigene E1A isoforms are depicted. Graphs B-D represent the % of each isoform relative to the sum of three variants (13S, 12S and 9S). In E, the difference in the percentage of expression to each isoform is presented relative to vehicle (medium) control. Graphs are presented as the mean and SEM of three independent experiments. * $p< 0.05$, ** $p<0.01$ in unpaired t test.

Figure 3: Effect of Paclitaxel treatment in minigene E1A splicing pattern. HEK293 stably expressing Flag empty vector (Flag) or Nek4.1 Flag fused, were transfected with pMTE1A plasmid. Six hours after the transfection tetracycline was added to proteins expression induction. 24 h to 48 h, the cell culture medium was replaced by medium containing 1 µM of Paclitaxel or ethanol

(0.02%) used as vehicle control. After 24 h of incubation, total RNA was extracted and the products of PCR were separated at 3% agarose gel. **A**) Predominant isoforms are depicted. Graphs B-D represent the % of each isoform relative to the sum of three variants (13S, 12S and 9S). In E, the difference in the percentage of expression to each isoform is presented relative to vehicle (ethanol) control. Graphs are presented as the mean and SEM of three independent experiments. * $p < 0.05$ in unpaired t test.

DISCUSSION:

Minigenes are important tools to determine the effects in global alternative splicing in vivo. The adenoviral minigene E1A has been used successfully for decades to evaluate the role of proteins by increasing the amount of these in the cell[13,14]. Here, we propose the minigene E1A use for evaluating alternative splicing after chemotherapeutic exposure. A stable cell line expressing Nek4 isoform 1 was used, avoiding the artifacts of overexpression caused by transient transfection. The isoform 1 of Nek4 did not show effect in the minigene E1A alternative splicing in basal conditions[21], but have many splicing related interactors, therefore, allowing us to evaluate the specific effect of the chemotherapeutic treatment in E1A alternative splicing in these cells.

Despite its low sensitivity, mainly compared to radioactive approaches, the method described here is simple and does not require special reagents or laboratory conditions. However, it is important to note that the minigene E1A is a global reporter of 5´SS selections, although 3´SS selection can be evaluated with this protocol the specific minigene reporter must be used[14,33,34]. Moreover, the results can be influenced by the cell line and should be carefully evaluated to avoid misinterpretation because of the basal alternative splicing profile.

Usually, great differences in minigene E1A splicing pattern are observed only when changing the expression of splicing factors. Other changes are less obvious because of the large number of proteins modulating the activity of these factors. For this reason, when starting studies for an indirect candidate, the classical approach, based on increasing amounts of this candidate protein should be preferred. When some effect is observed, the treatments can be performed to explore if the regulation can be specific to a particular cellular stimulus.

After a preliminary positive result, the standardization of time and drug concentration can be performed to optimize the experiment.

This simple protocol is a preliminary assay, a start-point which can answer whether the protein of interest shows an effect in alternative splicing and also, when some effect in alternative splicing regulation is already known, can direct the studies to the more consistent pathway where the protein plays a role regulating alternative splicing in chemotherapy response.

ACKNOWLEDGMENTS:

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, through Grant Temático 2017/03489-1 to JK and fellowship to FLB 2018/05350-3) and the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) for funding this research. We would like to

thank Dr Adrian Krainer for providing the pMTE1A plasmid and Zerler and colleagues for their work in E1A cloning. We also thank Prof. Dr. Patrícia Moriel, Prof. Dr. Wanda Pereira Almeida, Prof. Dr. Marcelo Lancellotti and Prof. Dr. Karina Kogo Cogo Müller to allow us to use their laboratory space and equipment.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

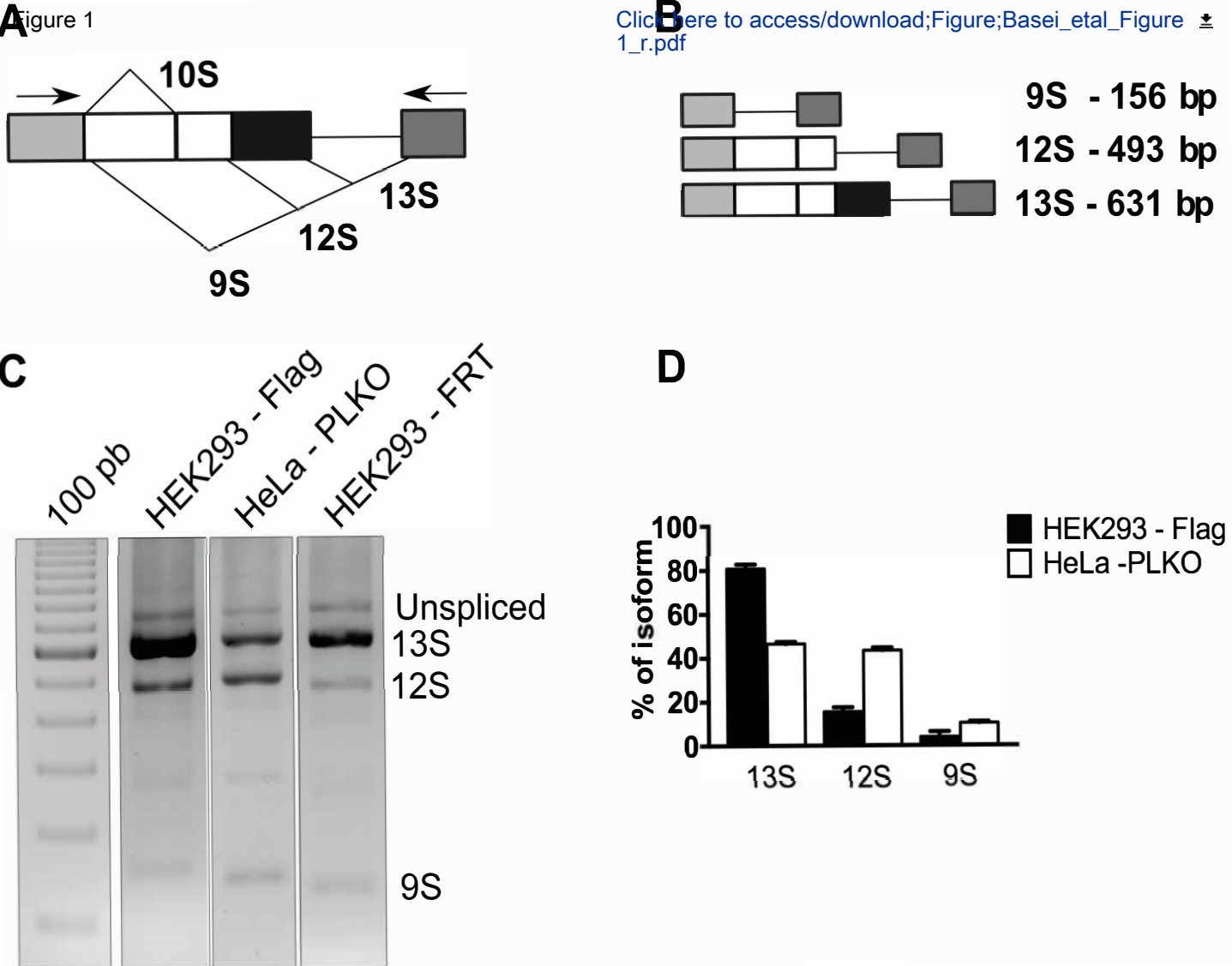

Figure_2

Click here to access/download;Figure;Basei_etal_Figure 2_r.png

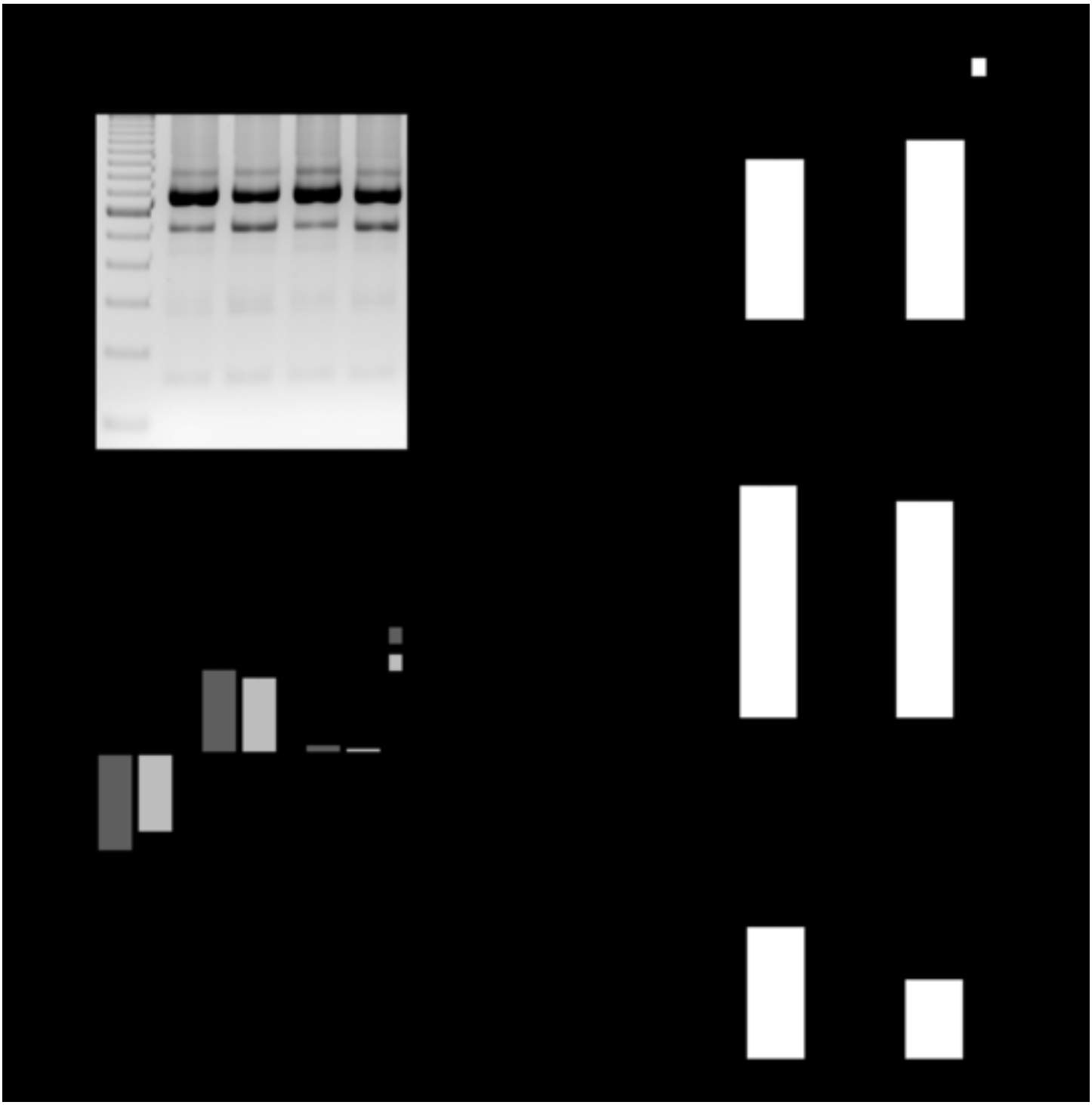

Figure_3

Click here to access/download;Figure;Basei_etal_Figure 3_r.png

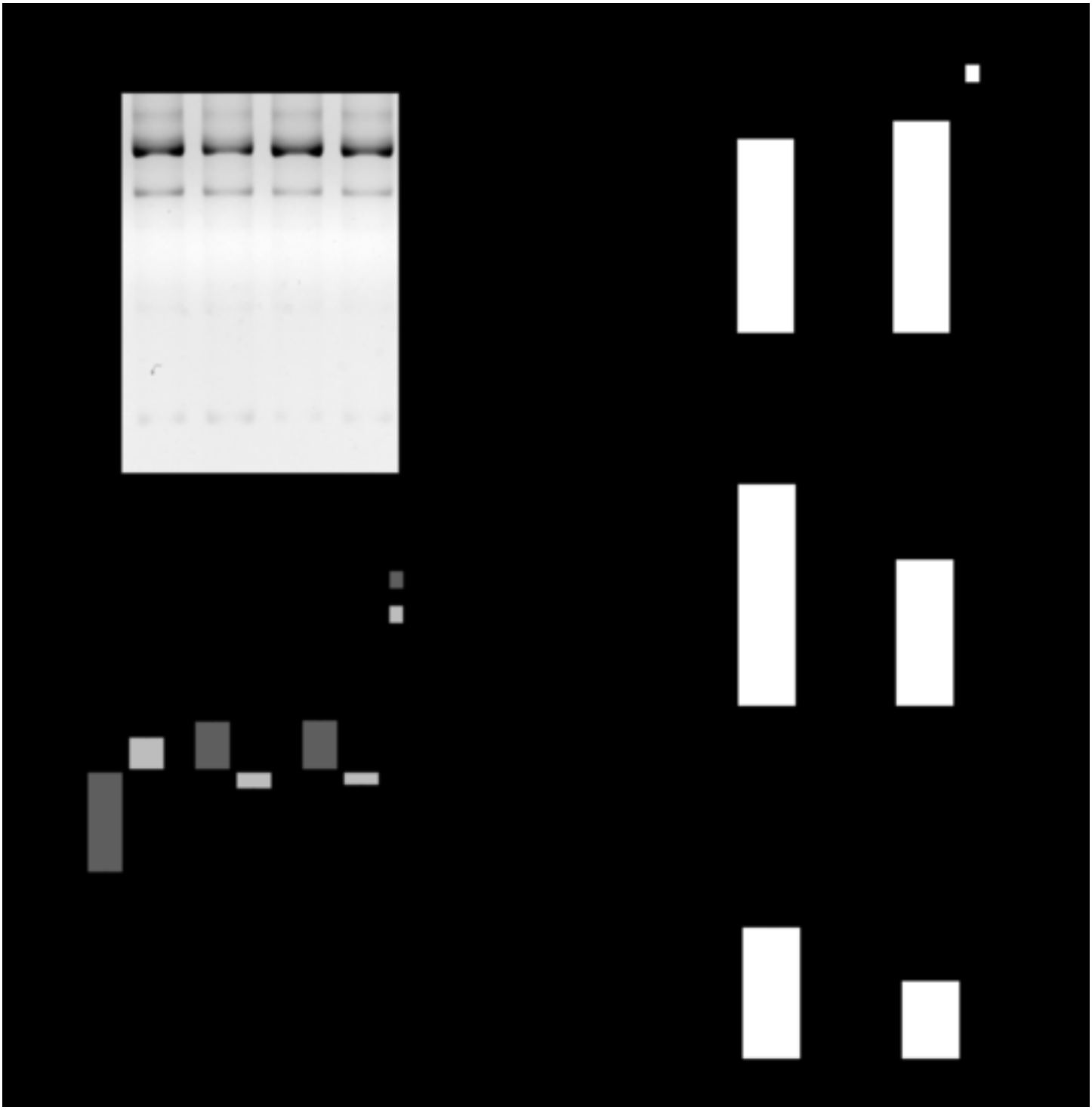

Table of Materials

Click here to access/download;Table of Materials;Basei_etal_mRNA_splicing_Methods_Collection_Materials_r.xlsx

Name of Material/ Equipment	Company
100 pb DNA Ladder	Invitrogen
6 wells plate	Sarstedt
Agarose	Sigma
Cisplatin	Sigma
DEPC water	ThermoFisher
DMEM	ThermoFisher
dNTP mix	ThermoFisher
Fetal Bovine Serum - FBS	ThermoFisher
Fluorescent Microscope	Leica
Gel imaging acquisition system - ChemiDoc Gel Imagin System	Bio-Rad
GFP - pEGFPC3	Clontech
HEK293 stable cells - HEK293 Flp-In	
Hygromycin B	ThermoFisher
Image processing and analysis software - FIJI software	
Lipid- based transfection reagent - jetOPTIMUS Polyplus Reagen	Polyplus
Oligo DT	ThermoFisher
Paclitxel	Invitrogen
Plate Reader/ UV absorbance	Biotech
pMTE1A plasmid	
pMTE1A F	Invitrogen
pMTE1A R	Invitrogen
Refrigerated centrifuge	Eppendorf
Reverse Transcriptase - M-MLV	ThermoFisher
Reverse transcriptase - Superscript IV	ThermoFisher
Ribunuclease inhibitor RNAse OUT	ThermoFisher
RNA extraction phenol-chloroform based reagent - Trizol	ThermoFisher
SybrSafe DNA gel stain	ThermoFisher
Taq Platinum	Thermo
Tetracyclin	Sigma
Thermocycler Bio-Rad	Bio-Rad

Trypsin	Sigma

	Catalog Number	Comments/Description
	15628-050	
	833920	
	A9539-250G	
	P4394	
	AM9920	
	11965118	
	10297-018	
	12657029	
	DMIL LED FLUO	
		Generated from Flp-In™ T-REx™ 293 - Invitrogen and described in ref 2
	10687010	Used for Flp-In cells maintenemant
		ref. 32
	117-07	
	18418020	
	P3456	
		Epoch Biotek/ Take3 adapter
		Provided by Dr. Adrian Krainer
5’ -ATTATCTGCCACGGAGGTGT-3		
5’ -GGATAGCAGGCGCCATTTTA-3’		
F5810R		
	28025013	
	18090050	
	10777-019	
	15596018	
	S33102	
	10966026	
	T3383	Used for Flag empty or Nek4- Flag expression induction
	T100	

T4799

21

JoVE62181 "A Simple Method as Starting Point to Study mRNA Splicing Changes

Fernanda L Basei, Livia AR Moura, Jörg Kobarg

Dear Dr. Bajaj,

We gratefully acknowledge your attention and the opportunity to improve our manuscript to be considered for publication in *JoVE Methods Collection*.

We carefully considered each of the concerns and revised our manuscript accordingly, and we believe that yours and reviewers suggestions greatly improved the overall quality of our manuscript.

We agree with reviewers that this protocol can be useful for initial studies in splicing regulation or even molecular biology/alternative splicing practical teaching, and not limited to cellular treatment, and for this reason we added details to the protocol.

A detailed response to each of the reviewer's concerns is listed below. We hope that the manuscript now warrants publication.

We thank you in advance for your time and consideration.

Sincerely Yours,

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Please note we have performed those corrections.

2. Please revise the title to "Using the E1A Minigene Tool to Study mRNA Splicing Changes"

We agree the new suggested title fits very well to the proposed protocol.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: TRIzol, RNAseOUT, SuperScript, ImageJ or FIJI software (although these are open-source, please add these to the Table of Materials and remove from the text) etc

Please note we have performed those corrections.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Please note we have corrected the text.

5. Please use periods for decimal, not comma.

Please note we have replaced all commas for periods.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Please note we have corrected the protocol text.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added more details to the protocol when it was possible.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have paid attention on that and revised the discussion

9. Please sort the Materials Table alphabetically by the name of the material.

Please note we have performed that.

Reviewer #1:

Manuscript Summary:

This manuscript describes in detail a protocol employing the well-established minigene E1A to assess if a candidate molecule overexpressed in a eukaryotic cell system has effects on the spliceosome choice among three 5' splice sites on the minigene pre-mRNA. The protocol is well presented, and I believe it will be useful for research groups that plan to initially assess the role of a molecule as an upstream splicing regulator. As the trans-acting factors that directly bind the pre-mRNA show some sequence specificity and E1A is a minigene reporter, this protocol is more suitable if the candidate molecule acts indirectly and upstream. There are points that could have been included or need clarification (see below).

This is a straightforward and low-cost protocol, well suited for research labs as well as for a molecular biology practice lecture. Although the therapy chemicals are toxic, the manuscript could discuss the application of the protocol without the chemical testing under the practical (wet lab) teaching context. Moreover, the video reproducing the whole protocol will be a valuable tool for distance education.

Major Concerns:

The authors used a cell line stably expressing the candidate factor, Nek4. If other research groups do not have this resource and need to transiently express both, the cis and trans factors, is there an appropriate vector that could express both cistrons (eg. EA1 and trans factor)? Would the research group need to express the trans factor in fusion with GFP and sort the cells out?

The goal of using a stable cell line overexpressing Nek4 (in a FlpIn system tetracycline-induced) is to circumvent the variability in transfection efficiency and also the high level in transient transfection. However, the most common approach using minigene described in the literature, when not performed with a direct AS modulator, is using co-transfection of the minigene vector and crescent amount of the candidate protein (Bressan et al., 2009, Naro et al., 2014).

Besides overexpression conditions, this protocol can also be used with knockdown or knockout stable cells. However, in these conditions as well as in stable expression, if the candidate is not a direct splicing modulator the changes in splicing profile can be very small. We have addressed this point in the text (note in section 1 – Protocol -lines 125-127 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

We have no knowledge about a bi-cistronic vector to cis and trans elements simultaneous expression, but it could be a very interesting tool if both were under strong promotors control. There are bi-cistronic vector for studying alternative splicing – fluorescent splicing reporter minigenes – which allow the insertion of fluorescent codifying sequences, antibiotic resistance for cell selection or also regulatory elements (Newman et al., 2006).

The transfection efficiency is one of the most limiting point in minigene assay, but, efficiency of 40% have already shown sufficient amount of mRNA to quantify splicing variants.

Usually pEGFP or other fluorophore expressing vector is used for equalization of transfected DNA amount (mainly in that assays using crescent amount of trans element) or in parallel to evaluate transfection efficiency.

For hard to transfect cell lines, such as HeLa or primary culture, maybe it worth using an GFP-tagged expression protein and sort it to increase the efficiency of the assay.

It is important to note that cells normally do not express E1A proteins and, in this case, E1A variants amplified probably came from the same cell which was transfected with a trans element. Some cell lines, like HEK293, indeed, express E1A protein (Russel et al., 1977) and in case of low transfection efficiency, the effect of candidate protein can be underestimated because of "endogenous" E1A expression. In our model we do not have this bias because our cell line expressing Nek4 is from a monoclonal selection and the effect of Nek4 expression in E1A splicing is occurring in all cells.

Minor Concerns:

1. The same protocol may be applied to assess 3' SS choice using a different minigene. The plasmid for this alternative could be cited.

We have added this information at the Discussion section and cited Stoss and col. (1999) paper which describe several minigenes for different proposals and also the minigene 3´SS reporters, the pDC20 or CT/CGRP (Lines 405-408 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

2. Line 81: Clarify ambiguity: The HEK293 cell line or two hundred ninety-three cells?

We have corrected that (Line 81 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

3. On line 103 a reference needs to be provided for the minigene E1A, and a brief description that it contains three 5'SS and one 3'SS. This is mentioned only after the protocol has been presented, under 'results'.

Please note we have corrected that and mentioned minigene E1A information also in Introduction section (eighth paragraph in Introduction - current lines 101-106 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

4. On line 104, the authors could have introduced the aim to test if Nek4 affects 5' SS choice and could have justified the choice of Nek4. It is unclear if it is the trans-acting RNA-binding protein or if it induces the expression of a trans-acting protein or RNA factor.

We have observed previously both Nek4 isoforms interacts to splicing factors (hnRNPA1, hNRNPF, hnRNPM, hnRNPC, hnRNPQ, hnRNPL hnRNPD) and isoform 2 interacts to SRSF1, SRSF2 and SRPK1) Moreover, Nek4 isoform 2 changes E1A alternative splicing to proximal sites favoring 13S formation (Basei et al., 2015). Although Nek4 isoform 1 does not change minigene E1A splicing site selection it is the canonical isoform and shows higher levels of expression than isoform 2. Moreover, isoform 1 has already been related to chemotherapy resistance and DNA damage response (Doles et al., 2010; Neguyen et al., 2012). Because of that, we think isoform 1 could interact with splicing factors under stress conditions and change the splicing site selection to induce particular cellular response.

The choice of a 5´SS minigene reporter was a starting point and because is largely available and used. Deeply investigation about the specific type of splicing that Nek4 regulates should be performed further. Because JoVE is a method Journal our focus was

not on Nek4 function. However, since Nek4 is explored as a model case we added more general information regarding Nek4 and its role in splicing (ninth paragraph in Introduction - Current Lines 107-111 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

5. In Figure 2, the legend (open or closed boxes) refers to medium X cisplatin in B to D and also to flag X neK4 in E. If E refers to the gel in A, it could have been explained in the legend, E and B switched or still the E legend changed. The same applies for figure 3.

In B - D from figure 2 and 3 is presented the percentage of each isoform comparing treated (open bars) to control or vehicle (close bars) for Flag and Nek4 expressing cells, separately, while in E is presented the percentage of each isoform normalized by control or vehicle and comparing Flag versus Nek4 expressing cells. We have changed the colors of the bars to differentiate both comparisons.

6. The manuscript needs minor English revision.

We carefully reviewed the English.

Reviewer #2:

Manuscript Summary:

In this manuscript Basei and colleagues describe a well-known method in the field of mRNA processing for the evaluation of alternative splicing changes elicited by select protein overexpression either alone or in combination with chemotherapy treatment. This method is based on the transfection of a reporter minigene in recipients cells and subsequent semi-quantitative PCR analysis of the mRNA transcribed and processed from this vector, following exposition to different treatments or genetic manipulation.

The method described is appropriated to the biological question, but some steps should be revised and some additional steps at least suggested to improve analysis accuracy; some critical points as indication of proper controls should also be more thoroughly explained and discussed.

Major Concerns:

- In addition to a well transfected with EGFP for probing transfection efficiency, plating a well for a mock transfection should be advised. Analysis from such samples are especially recommended when using "de-novo" made minigene, to make sure that RT-PCR analysis specifically amplifies mRNAs arising from minigene-transcription/processing.

We absolutely agree with the comments, specifically in HEK293 cells what already expresses E1A gene (Russel et al., 1977). In this case, when the candidate protein is co-transfected with minigene the untransfected control must be done to avoid seeing the result of splicing from endogenous E1A. In our particular assay, as we use a stable cell line all cells show candidate protein expression and for this reason the E1A splicing will be influenced by Nek4 expression. Please note we have added this comment in the text (note in 2.5 section - Current lines 170-172 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

- Treatment of RNA samples with DNAse for removal of contaminating genomic DNA and RT-PCR on minus RT samples should at least be strongly advised, as in PCR analysis for alternative splicing events is crucial to be able to discriminate genomic contamination from putative intron-retention events, especially when evaluating effects of treatment such chemotherapy that might induce a general reduction of splicing efficiency as authors themselves referenced (Anufrieva et al. 2018).

We usually perform NRT (no reverse transcriptase) control and unfortunately, we forgot to mention that in the text. We completely agree with the importance of this control. We highlight this information in the text (Note 5.12 - Current lines 264 - 266 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

- In the selection of timing and dose of drug treatments authors should advised readers to perform dose-response assays in order to identify the minimal dose able to induce the biological effect and splicing change, without inducing massive cell death. Authors should recommend to perform such test preliminarily to analysis of how variation of the expression levels of a putative protein regulator modifies this response, otherwise effects from distinct phenomenon could lead to confusing results.

We agree that the effect of the drugs in cell viability should be emphasized and we have included this advice as a note (Note in section 3 Current lines 178-179 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed). Indeed, we have omitted the information about the Nek4 isoform 1 effect on minigene E1A alternative splicing because that already was published (Basei et al., 2015). Moreover, changes observed in vehicle control already are indicative of some effect in minigene E1A splicing per se, not related to the treatment. Besides, we believe that the analyses of treatment effect in splicing will be performed when the researcher already have indicative of splicing changes.

- Authors should pay more attention and invite their reader to do the same in the densitometric analysis of their PCR. When calculating percentage of different splice-variants relative to the total transcript, is important that the sum of these percentages equals to 100%. In both figure 2 and 3 the sum of the three isoform percentages does not seem to equal 100% for several conditions (i.e. Fig 2 both Flag and Nek4.1 medium treated samples; Fig 3 none of the samples).
- More information about how to perform densitometric analysis should be provided, such as stressing the importance of subtracting correct background, especially when quantifying band of weak intensity.

We have verified the results and, indeed, 13S isoforms from figure 2 are not correctly represented. We apologize for that, and we have corrected the values in the graph. The mistake was in the use of the values from other treatments and not in the percentage calculation. We agree with the reviewer that this is an important point and the sum of all isoforms must be 100 because it is set as total. In figure 3 we verify all values and they are correct and the sum of the percentage of three isoforms is equal to 100%. Maybe because the mean standard error is difficult to visualize but the average of percentage from the three experiments are show as follow:

	Isoform (Mean +/- SEM)		
Sample	13S	12S	9S
Flag Ethanol	81,83 (1,697)	16,37 (1,405)	1,767 (1,477)
Flag Paclitaxel	79,03 (2,245)	17,77 (1,068)	3,2 (1,724)
Nek4.1 Ethanol	85,27 (1,676)	12,37 (0,9939)	2,367 (0,9939)
Nek4.1 Paclitaxel	86,23 (1,586)	11,83 (1,014)	1,933 (0,6173)

We have emphasized that the sum of isoforms percentages must be equals to 100% (Note in section 7, current line 318 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed) and also, we added more information about isoforms quantification.

Minor Concerns:

- Definition of the experimental points to be plated, including all controls, should be anticipated to the Plating cell section

Please note we added this information at line 129, in “plating” section at Basei_etal_mRNA_splicing_Methods_Collection_reviewed file.

- In the abstract authors state that the method herein described is "a reliable starting point to shed light on cellular processes or what genes can be regulated by mRNA splicing". This sentence is inaccurate. By default, reporter minigenes and in particular the E1A minigene, does not give information about "what genes can be regulated by mRNA splicing", but they allow to test if a specific condition, either a drug treatment or expression modulation of a putative regulator, might generally affect alternative splicing.

This mention is related more to the treatment, once many genes which are alternatively spliced because of specific treatments are already described. According to that, if the tested treatment changes splicing pattern, the splicing of the genes which are modulated by this treatment can be investigated. For example, Bcl-xL, clusterin, PLD2 splicing changes are observed after cisplatin, radiotherapy and paclitaxel treatment, respectively (Shkreta et al., 2016; Zellweger et al., 2003; Zhu et al.2018). But we agree, the sentence can induce other kind of interpretation and for this reason we changed it.

- Reference 6 at line 69 is not appropriate. It should be acknowledged lately when describing the relevance of minigene systems in the study of alternative splicing (line 101-104), but here a more general reference to common mechanisms of alternative splicing regulation should be preferred.

We agree and probably it was a citation mistake. We intended to cite the revision of Kornblihtt and col., (2013) (fourth paragraph in Introduction - Current line 63-71 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed). Cooper revision about minigene systems was cited when we describe the minigene principles (seventh

paragraph in Introduction - Current line 91-100 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed)

- Sentence at line 73-74 should be rephrased as it now seems confusing. It is not clear what does "changes in its cellular localization" refer to.

We have re-written the sentence to clarify that phosphorylation changes splicing factors cellular localization and properly referenced it (fifth paragraph - Current lines 72-75 at Basei_etal_mRNA_splicing_Methods_Collection_reviewed).

- References for data discussed at line 79-82 are missing. Please indicate appropriate references.

Please note we have added the reference.

- Being all the manuscript based on usage of the E1A minigene, paper describing from Zerler and colleague describing its cloning should be acknowledged (PMID: 3022137).

We completely agree and apologize for that. We have added Zerler and col. at the acknowledgement section.

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