

Post-integration stabilization of a transposon vector by terminal sequence deletion in *Drosophila melanogaster*

Alfred M Handler¹, Grazyna J Zimowska¹ & Carsten Horn²

Germline transformation systems for nearly 20 insect species have been derived from transposable elements, allowing the development of transgenic insects for basic and applied studies^{1–3}. These systems use a defective nonautonomous vector that results in stable vector integrations after the disappearance of transiently provided transposase helper plasmid⁴, which is essential to maintain true breeding lines and consistent transgene expression that would otherwise be lost after vector remobilization. The risk of remobilization by an unintended transposase source has so far not been a concern for laboratory studies, but the prospective use of millions of transgenic insects in biocontrol programs will likely increase the risk, therefore making this a critical issue for the ecological safety of field release programs^{5,6}. Here we describe an efficient method that deletes a terminal repeat sequence of a transposon vector after genomic integration. This procedure prevents transposase-mediated remobilization of the other terminal sequence and associated genes, ensuring their genomic stability.

Class II transposable elements transpose via an internally encoded transposase acting on the flanking 5' and 3' terminal inverted repeat (TIR) sequences and adjacent DNA that may include subterminal inverted repeat sequences⁷. In principle, stabilization of transposon-based vectors could be achieved by rearrangement or deletion of one or both of the TIRs after genomic integration. Rearrangement strategies have been proposed based on site-specific recombination within a single vector, or between two vectors, that would result in deletion or inversion of the terminal sequences⁶. However, these strategies are not straightforward because they rely on either the positioning of recombination sites within the terminal region that could compromise vector integration efficiency, or the genomic integration of vectors at closely linked loci that would occur rarely. We considered an alternative strategy that allows terminal sequence deletion after germline transformation, by introducing a head-to-tail tandem duplication of one of the termini, with a marker gene and genes of interest inserted in between the duplicated sequences. After genomic integration of the vector construct, transposase-mediated excision of the internal duplicated terminus with the flanking nonduplicated terminus should result in stabilization of the remaining terminus and associated transgene sequences.

To test this method for vector stabilization, we created the pBac{L1-PUBDsRed1-L2-3xP3-ECFP-R1} vector having a duplicated 5' terminal *piggyBac* sequence (L2) placed internal to the flanking 5' (L1) and 3' (R1) termini (Fig. 1). To allow discrimination of each set of terminal sequences, dominant visible fluorescent markers distinguishable by epifluorescence were inserted in between each pair of termini. The red fluorescing protein under control of the ubiquitously active polyubiquitin promoter, PUBDsRed1⁸, was placed between L1 and L2, and the cyan fluorescing protein under control of the eye-specific 3xP3 promoter, 3xP3-ECFP⁹, placed between L2 and R1. Both transformation markers have been shown to be applicable to a wide host range of insects from three different orders¹⁰. This vector was integrated into the *Drosophila melanogaster* *w*[m] host strain by *piggyBac*-mediated germline transformation¹¹. Of eight putative transformant G1 founder individuals selected, seven expressed only the 3xP3-ECFP marker, which is consistent with integration of only the embedded L2-3xP3-ECFP-R1 vector. One G1 founder male, designated F34, exhibited both thoracic DsRed fluorescence and ECFP eye expression (phenotype shown in Fig. 1), consistent with integration of the entire L1-PUBDsRed1-L2-3xP3-ECFP-R1 vector. The 7:1 transformation ratio of the shorter embedded vector relative to the longer complete vector is consistent with more efficient transposition of shorter vectors¹². Backcrossing of F34 males and females independently for two additional generations indicated X-chromosomal localization of the transgene in line F34.

To remobilize the embedded vector from the F34 strain, we mated F34 flies as transgene heterozygotes to a *piggyBac*-transposase expressing 'jumpstarter' strain, with *w*⁺; F34 (DsRed1/ECFP) progeny outcrossed to the *w*[m] strain. Progeny expressing only DsRed1 fluorescence, consistent with loss of the L2-3xP3-ECFP-R1 sequence, were detected at an approximate frequency of 2%–3% of all flies screened. A single white-eye male (lacking the transposase gene) expressing DsRed1, and not ECFP (phenotype shown in Fig. 1), was outcrossed to *w*[m] females with the resultant line designated as F34-1M.

The pBac{L1-PUBDsRed1-L2-3xP3-ECFP-R1} integration within the F34 *D. melanogaster* genome was initially identified by phenotypic expression of the PUBDsRed1 and 3xP3-ECFP marker genes and verified by PCR amplification of transformant DNA using primers internal to the vector sequence (data not shown). Genomic DNA

¹Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, US Department of Agriculture, 1700 SW 23rd Drive, Gainesville, Florida 32608, USA. ²Max-Planck-Institut für molekulare Genetik, Center for Cardiovascular Research, Hessische Strasse 3-4, D-10115 Berlin, Germany. Correspondence should be addressed to A.M.H. (handler@nersp.nerdc.ufl.edu).

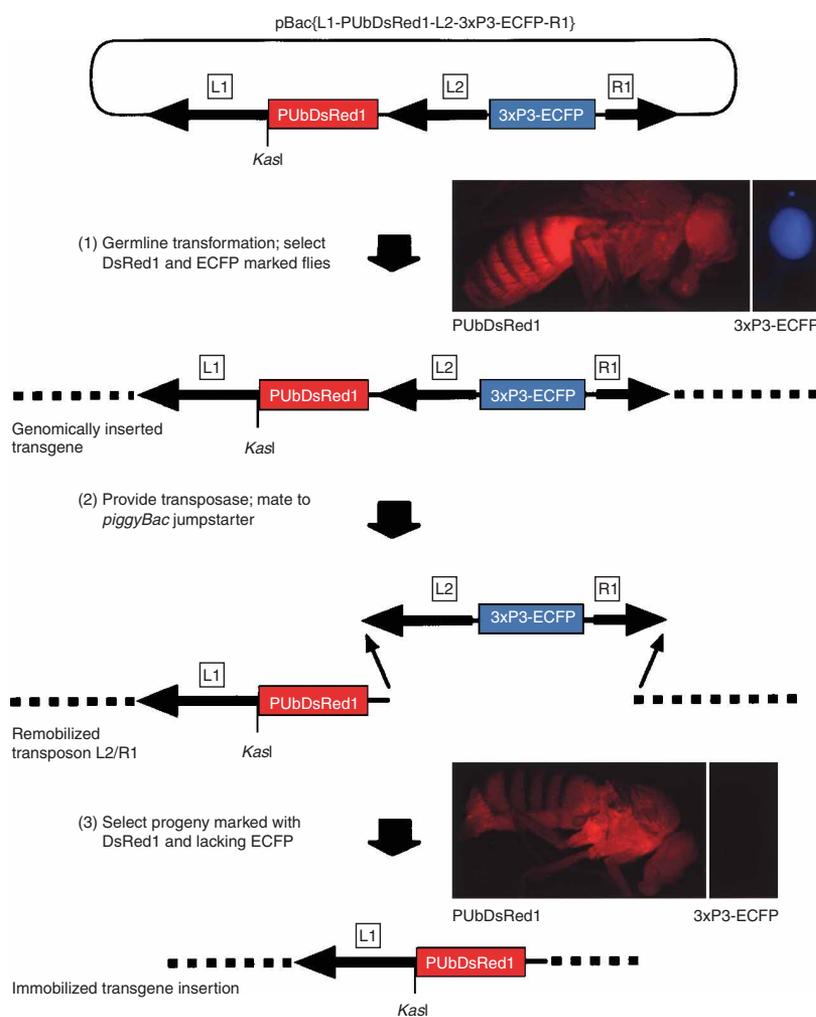


Figure 1 Methodology for achieving transgene stabilization using the pBac(L1-PUBDsRed1-L2-3xP3-ECFP-R1) vector. Diagram (not to scale) shows relative positions of the L1, L2 and R1 *piggyBac* terminal sequences, the PUBDsRed1 and 3xP3-ECFP markers, and a unique *KasI* site that can be used for insertion of genes of interest. Image inserts show the DsRed and ECFP phenotypes of F34 (1) and F34-1M (3) flies under epifluorescence optics. See Methods for details on vector structure, transformation and remobilization.

(typically 100 bp or more) are necessary for efficient transposition^{15–17}. Thus, on a theoretical basis the nonexcised portion of the integrated vector in F34-1M, including a sole 5'-terminus and PUBDsRed1 marker, should be refractory to remobilization by a source of transposase. To analyze the stability of the F34-1M integration, we mated the original F34 transformant line and the stabilized F34-1M line to the *piggyBac* jumpstarter strain. Because the vector integration is X-linked, transposase-mediated vector excision (scored by loss of fluorescence markers) was assessed in female offspring of males carrying the vector and genomic transposase, and vector transpositions (scored by presence of fluorescence markers) in male offspring (Table 2). Vector transpositions could not be detected in females because the phenotype would not differ from a nonmobilized vector (unless a strong position effect resulted). In male progeny not carrying the paternal X chromosome, only interchromosomal transpositions to the Y or autosomes in the paternal

sequence flanking the *piggyBac*L1 5'-end insertion site was obtained by inverse PCR¹³ and compared by BLAST analysis¹⁴ to the *Drosophila* Genome Sequence Database (<http://www.flybase.net/blast/>). Consistent with segregation analysis, the insertion site was found to be homologous to the sequence found on the X-chromosome at locus 9B4. The sequence was used to derive the *piggyBac*R1 3'-end insertion site, and primers were created to the 5'- and 3'-end genomic (DmX) flanking sequences (see Methods). The genomic primers, 197F and 196R, were used to amplify the 7-kb sequence that spans the pBac(L1-PUBDsRed1-L2-3xP3-ECFP-R1) integration in F34, and sequencing of approximately 800 bp from each primer further verified it as the primary *piggyBac* vector integration. Similarly, the genomic primers allowed PCR and partial sequencing of the vector insertion in F34-1M genomic DNA, which confirmed the deletion of the embedded L2-3xP3-ECFP-R1 sequence. For both strains, which were heterozygous for the transgene insertion, the 124-bp nonintegrated genomic insertion site was also isolated and sequenced. Further confirmation for vector structure in F34 and F34-1M was achieved by PCR analysis with primers to genomic and internal vector DNA that yielded product sizes consistent with integration of pBac(L1-PUBDsRed1-L2-3xP3-ECFP-R1) in F34, and the deletion of the L2-3xP3-ECFP-R1 sequence in F34-1M flies (Table 1 and Fig. 2). PCR products were not obtained in F34-1M flies using primers to L2-3xP3-ECFP-R1. Studies on several insect and other eukaryotic transposons are consistent in finding that both the 5' and 3' TIRs and associated proximal DNA

line would be detected. In the F34 matings a total frequency of 5.34% marker excisions of both ECFP, and ECFP with DsRed from 3,467 female progeny was detected, whereas a total frequency of 1.77% marker transpositions were detected in 3,613 F34 males. In the F34-1M matings, no DsRed excisions were detected from 3,532 females scored, whereas 4 out of 3,646 males exhibited a DsRed phenotype. Although this might suggest transpositions of the L1-PUBDsRed1 sequence to the Y chromosome or autosomes segregating to F34-1M males, PCR analysis of these males showed that the partially deleted vector retained the original 5' and 3' genomic insertion site sequence (using primer sets 197F/140R and 193F/196R; data not shown), and thus vector movement did not result from transposase-mediated transpositions. It is most likely that X/Y recombinations occurred in the paternal germ line linking the transgene to the Y chromosome, which is consistent with the four males being sterile. Phenotypic males resulting from rare X/Y recombinations in *D. melanogaster* are often sterile owing to loss of Y-linked fertility factors¹⁸. A similar number of ECFP/DsRed males from the F34 matings may have also resulted from X/Y recombinations and not transpositions. In summary, the results of this stability analysis strongly support the conclusion that the non-excised L1-PUBDsRed1 vector sequence in the F34-1M genome is stabilized, or refractory to the vector transposase, relative to the original unmodified vector.

The observation that shorter vectors are more transpositionally efficient¹² was demonstrated in both the initial vector integration,

Table 1 Predicted and obtained PCR products from F34 and F34-1M genomic DNA using indicated primer pairs (see Fig. 2)

Primer pairs	Line F34		Line F34-1M	
	Predicted	Obtained	Predicted	Obtained
1-pBR/196	237	0.2	–	–
2-193/196	2,630	2.6	624	0.6
3-192/197	4,897	4.0	–	–
4-140/197	713	0.7	713	0.7
5-pBL/197	278	0.3	278	0.3
	4,063	4.0	–	–
6-94/196	2,084	2.1	3,952	4.0
	5,958	–	–	–
7-196/197	6,003	6.0	3,997	4.0
	124	0.12	124	0.12

and in the stability tests where the entire vector and the shorter embedded vector were both remobilized. The shorter vector inserted or excised 7 and 5.9 times more frequently, respectively, than the entire larger vector. Thus, a limitation of this system is that it may be increasingly difficult to integrate an entire vector as the sequences to be stabilized, external to the embedded vector, become larger for particular gene(s) of interest. A potential method to address this limitation is to have the internal TIR in opposite orientation to its flanking duplicate, and surrounded by site-specific recombination sites (e.g., FRT or loxP)¹⁹ in opposite orientation to one another. Assuming that a duplicated TIR pair in opposite orientation is not mobilizable^{15,16,20}, only the external nonduplicated TIRs could be used for the initial transformation. The internal TIR could then be inverted by introducing the corresponding recombinase putting it in

Table 2 Stability of F34 and F34-1M transgene vectors in the presence of genomic transposase as indicated by absence or presence of fluorescent protein marker phenotypes

	F34		F34-1M	
	<i>n</i>	Freq	<i>n</i>	Freq
Females	3,467	–	3,532	–
–ECFP ^a	158	.0456	Na ^b	–
–ECFP/DsRed	27	.0078	Na	–
–DsRed	0	0	0	0
Total excisions	185	.0534	0	0
Males	3,613	–	3,646	–
+ ECFP	56	.0155	Na	–
+ ECFP/DsRed	8	.0022	Na	–
+ DsRed	0	0	4 ^c	.0011
Total transpositions	64	.0177	0	0

^aAbsence (–) or presence (+) of indicated phenotypes.

^bNa, not applicable because ECFP was not in parental flies.

^cPhenotype did not result from transposase-mediated transposition (see text).

proper orientation for terminal sequence deletion by transposase-mediated remobilization.

Transposon-based plasmid vectors have proven to be efficient tools for producing genetically modified insects for research purposes on, thus far, a small laboratory scale. Yet, the mobile nature of DNA transposable elements and the ability of nonautonomous vectors to be mobilized by the same or related systems may prove to be disadvantageous during the mass rearing of transgenic insects for applied use²¹. Owing to potential remobilization, the stability of genomic transgene integrations cannot be assured, raising significant concerns relating to strain stability and the ecological safety of releasing such genetically modified insects into the environment. Transgene integrations that negatively affect host strain fitness and reproduction also confer a selective disadvantage to the transformed organism in a population, relative to wild-type organisms. Thus, a selective advantage is provided to nontransformed organisms or transformants that have lost or relocated the transgene because of a remobilization event. Vector loss due to remobilization can occur directly by simple excision but may also occur after transposition to another locus with loss occurring with chromosomal segregation. Although the transposase required for such remobilization is not, typically, expected to be encoded by the host species' genome, transposase introduction by symbiotic or infectious agents is conceivable, and cross-reactivity to related transposase enzymes that are genomically encoded cannot be excluded. Such cross-reactivities were demonstrated by the genomic excision of a *Musca domestica* *Hermes* transposon vector in *hobo*-containing *D. melanogaster* strains^{22,23}. Well-characterized families of transposable elements contain multiple members and the cross-reactivity among them is largely unknown²⁴.

Remobilization that results in vector loss will eliminate the desired transgene expression, and even if the presence of vector is maintained after genomic transposition, transgene expression may be altered owing to varying chromosomal position effects. Thus desired characteristics for applied use may become variable and unreliable eliminating strain effectiveness. A more serious concern for vector instability, however, is the potential for lateral transmission of the transgene into unintended host strains or species. Many commercial applications of insect transgene technology will include the field release of genetically modified insects for biological control, and

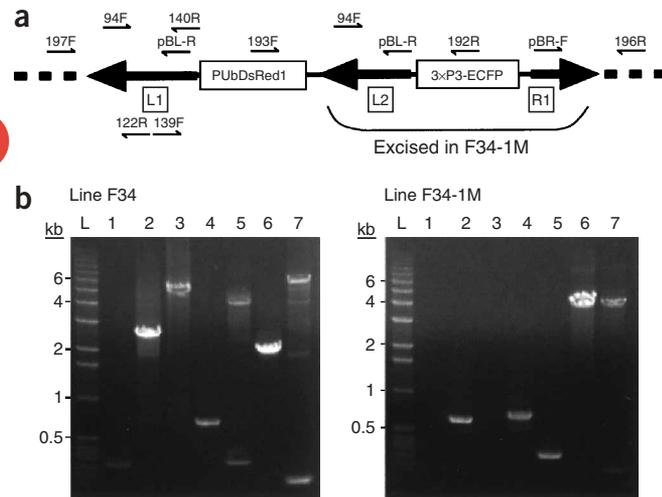


Figure 2 PCR analysis of the vector sequences in lines F34 and F34-1M. (See Methods for primer sequences and PCR protocols.) (a) The relative primer positions are indicated above and below the diagram of the integrated pBac(L1-PUBDsRed1-L2-3xP3-ECFP-R1) vector (not to scale) in the F34 line with the excised sequence indicated for line F34-1M. (b) An ethidium bromide-stained agarose gel showing PCR products using indicated primer pairs on genomic DNA obtained from individuals heterozygous for the transgene. The 0.12 kb product in lane 7 for both lines (faint band in F34-1M) represents the nonintegrated genomic insertion site. Primer pairs: lane 1, pBR/196; lane 2, 193/196; lane 3, 192/197; lane 4, 140/197; lane 5, pBL/197; lane 6, 94/196; lane 7, 196/197.

consideration of biosafety and ecological risk assessment will be of fundamental importance⁵. A primary goal for biosafety is minimizing the risk of unintended transgene transmission from the host to other prokaryotic or eukaryotic species during rearing and after release into the field. Horizontal gene transfer is apparently a natural phenomenon for many transposons^{25,26} and indeed, nearly identical *piggyBac* transposons exist in phylogenetically and geographically distinct moths and flies²⁷. Cross-mobilization of defective vectors by the corresponding transposase or a related system is also a viable possibility, and minimizing, if not eliminating the potential for unintended vector transmission to nontarget organisms will be highly desirable. Given the relative simplicity and effectiveness of vector immobilization by terminal sequence deletion, it is likely that the improved environmental safety allowed by such stabilization vectors will make their use a high priority for transgenic organisms intended for release. This potential application in economically important insects is supported by the recent integration of a similar *piggyBac* stabilization vector system in the Caribbean fruit fly, *Anastrepha suspensa*. The embedded vector was remobilized by injected transposase helper plasmid and tests for stability are currently underway (A.M.H., R.A. Harrell and G.J.Z., unpublished data).

A caveat to the use of this stabilization system in species that have or may have genomic sequence corresponding to the transposon vector is the potential for vector integration proximal to a sequence that restores the deleted terminus. This may be addressed by use of a stabilization vector known not to exist in the species, sequencing of the genomic insertion site, or performing stability tests as described. For any transgenic strain intended for release, an assessment for potential instability by all these methods would be prudent.

METHODS

Insect strains. The *w*[m] strain is a *D. melanogaster white* mutant strain with an M cytotype that was obtained from the Bloomington Stock Center. The *piggyBac* 'jumpstarter' strain, having a *w*/Y; *P*[*w*⁺, *hsp*-pBac]/ *P*[*w*⁺, *hsp*-pBac]; +/+ genotype, was kindly provided by M. Fraser (University of Notre Dame). This is a *white* mutant host strain having a homozygous 2nd chromosome *P* element vector integration, marked with the wild type *white*⁺ allele, that includes an *hsp70*-regulated *piggyBac* transposase gene.

Plasmids. The pBac{L1-PUBDsRed1-L2-3xP3-ECFP-R1} vector was created by isolating a 3.7-kb *Af*III-*Af*III fragment from pB{PUBDsRed1}⁸ that contains a 676-bp *piggyBac* 5' terminal sequence and the polyubiquitin-regulated gene that encodes DsRed1. After blunting by Klenow-mediated nucleotide fill-in, it was ligated into the *MscI* site of pXL-BacII-3xP3-ECFP (kindly provided by M. Fraser, Univ. of Notre Dame; see: <http://piggybac.bio.nd.edu/>) with plasmids having the 3xP3-ECFP⁹ and PUBDsRed1 reading frames in opposite orientation selected. The final plasmid contains a 308-bp internal *piggyBac* 5' terminal sequence and a 200-bp 3' terminal sequence. The *phspBac* transposase helper plasmid was previously described¹¹.

Germline transformation and marker detection. Germline transformation of *D. melanogaster* with a *piggyBac* vector was done as previously described¹¹, with 553 *w*[m] strain G0 embryos coinjected with the pBac{L1-PUBDsRed1-L2-3xP3-ECFP-R1} vector and *phspBac* helper at a concentration of 500:200 ng/μl, respectively, in injection buffer. Surviving G0 adults were backcrossed to *w*[m] flies in 120 mating groups having either one G0 male or three G0 females. G1 progeny were screened by epifluorescence optics for expression of the PUBDsRed1 and 3xP3-ECFP fluorescent proteins. A single G1 male, designated F34, expressing both markers was backcrossed to *w*[m] females.

Fluorescent protein expression was detected under a Leica MZ FLIII fluorescence stereozoom microscope using the HQ Texas Red filter set for DsRed detection (exciter HQ560/55 ×; emission HQ645/75m; Chroma Technology) and the Cyan GFP filter set for ECFP detection (exciter D436/20 ×; emitter D480/40m).

Vector linkage and remobilization. F34 males and females were separately backcrossed to *w*[m] flies for two generations. The presence of the transgene markers solely in female progeny of F34 males indicated X-chromosome linkage for the vector. Remobilization of the embedded vector was done by crossing F34 flies to the homozygous jumpstarter strain. Larval and pupal offspring were heat shocked at 37 °C for 60 min every second day until adult emergence to promote transposase gene expression in the germ line. Adult progeny carrying the transposase gene (pigmented eyes) and expressing the fluorescent protein markers, PUBDsRed1 and 3xP3-ECFP, were outcrossed to *w*[m] individuals in small groups. Progeny were screened for expression of PUBDsRed1 and the absence of 3xP3-ECFP, which would be consistent with deletion of the *piggyBac*L2 and *piggyBac*R1 termini and the internal 3xP3-ECFP marker DNA. A single male, designated F34-1M, expressing only DsRed1 and a white eye phenotype (lacking genomic transposase) was outcrossed to *w*[m] females.

Stability analysis. The relative stability of the vector in the F34 line and the partially deleted vector in the F34-1M line was tested by crossing these strains to the homozygous jumpstarter flies and subjecting their larval and pupal progeny to heat shock as described above. For each vector strain eight group matings were set up having 10 transgenic males outcrossed to 30 *w*[m] females for 4 d. Male and female progeny from these matings were screened daily for the presence or absence of fluorescent protein markers. F34 progeny were scored for both PUBDsRed1 and 3xP3-ECFP expression, whereas F34-1M progeny were scored for PUBDsRed1 expression.

PCR analysis. Direct PCR was done on genomic DNA prepared with DNAzol (Molecular Research Center) under the following cycling conditions: 94 °C for 2 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 5 min for 35 cycles with a final extension at 72 °C for 10 min. For inverse PCR, genomic DNA from F34 adults was digested with *MspI* for 4 h and ligated overnight at 12 °C. Outward facing primers for the *piggyBac* L1 terminus, 122R and 139F, were used for inverse PCR using the same cycling conditions as for direct PCR. Amplified DNA was separated and visualized on 0.8% agarose gels, and for some sequences, isolated products were subcloned into TOPO TA cloning vectors (Invitrogen) and sequenced using M13 forward and reverse primers.

The primers (and locations) used were as follows:

94F (pB1-20): 5'-CCCTAGAAAGATAGTCTGCG-3'
 122R (pB159): 5'-ATCAGTGACACTTACCGCATTGACA-3'
 139F (pB445): 5'-CCAGAGCGATACAGAAGAAGC-3'
 140R (pB668): 5'-TGTTTCAGTGCAGAGACTCGG-3'
 pBL-R (pB234): 5'-TATGAGTTAAATCTTAAAAGTCACG-3'
 pBR-F (pB2315): 5'-GTTGAATTTATTATTAGTATGTAAGTG-3'
 192R (ECFP): 5'-AGAAGAACGGCATCAAGGC-3'
 193F (DsRed): 5'-ACTCCAAGCTGGACATCACC-3'
 196R (DmX-3'): 5'-CGCAGACGAAGAACAACAGTA-3'
 197F (DmX-5'): 5'-GCTGTTTGCTTTGTTGTTGTCAT-3'

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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