Supporting Information

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SI Materials and Methods

Plasmid Constructions. pcDNA-EGFP was constructed by inserting an EGFP fragment from pEGFP-N2 (Clontech) into the EcoRI-NotI site of pcDNA3 (Invitrogen).

The $Hprt^{PB\_in2}$ targeting vector was constructed as follows. The XhoI-PstI fragment, which contains a *piggyBac* transposon carrying a CAG-*puΔtk* cassette, was cloned into the XhoI-PstI site of pARM1 (gift from H. Prosser, Sanger Institute, Cambridgeshire, UK), resulting in pML20. The 2.5-kb 5′ homology arm was excised from pCEI-3 (gift from H. Prosser) by digesting with FseI and XhoI, and cloned into pML20, resulting in pHprtTV-left. The 3′ homology arm was PCR-amplified and cloned into the PacI-AscI site of pHprtTV-left, resulting in the final targeting vector pHprtTV-PB. The targeting vector was linearized with AscI before electroporation into ES cells.

The $Hprt^{PB\_ex3}$ targeting vector was constructed using BAC recombineering. A BAC clone RP23-173F3 was used. First, a minitargeting vector, which introduces a *piggyBac* transposon carrying a *PGK-puΔtk* cassette into exon 3 of the *Hprt* gene on the BAC, was constructed as follows. Left and right minihomology arms were PCR-amplified, digested with KpnI/HindIII and HindIII/XbaI, respectively, and cloned into the KpnI-XbaI site of pBluescriptII (pBS), resulting in pBS-HprtE3R. The BsiWI-NsiI fragment of pPB-LR (1) carrying a *piggyBac* transposon with a multicloning site was cloned into the BsiWI-NsiI site of pBS-HprtE3R, resulting in pBS-HprtE3R-PB. Separately, the AscI-NotI fragment of pFlexible (2) carrying a *PGK-puΔtk* cassette was cloned into pBS-NANE that harbors NheI-AscI-NotI-EcoRI sites, and a PCR-amplified *EM7-neo* cassette was inserted into the XhoI site, resulting in pBS-PGKpuΔtk-EM7neo. Finally, the NheI-EcoRI fragment of pBS-PGKpuΔtk-EM7neo was transferred into pBS-HprtE3R-PB, resulting in pBS-HprtE3R-PB: puΔtk.neo. The KpnI-PmeI fragment of the minitargeting vector was used in BAC recombineering. A retrieving vector was constructed as follows. Left and right retrieving arms were PCR-amplified, digested with AscI/HindIII and HindIII/XhoI, respectively, and cloned into the XhoI-AscI site of pMCS-DTA (gift from J. Takeda, Osaka University, Osaka), resulting in pDTA-HprtRet. The HindIII-linearized pDTA-HprtRet was used to retrieve exon 3 together with 3.2-kb 5′ and 4.3-kb 3′ homology arms. The targeting vector was linearized with PmeI before electroporation into ES cells.

Mutant Screening in Yeast. The yeast strain, donor plasmid and transposase plasmids have been described previously (3). Random mutagenesis of the PBase gene was carried out by PCR in the presence of manganese and the resulting mutant pools were introduced into a pGALS vector (TRP+) by homologous recombination in yeast carrying the transposon donor plasmid. Colonies were grown for 2.5 d, resuspended in water, and spotted onto plates lacking uracil. Because of leakiness of the GALS promoter, ura^+ revertants appeared without galactose induction, but at a low frequency, 4.7×10^{-4}, with the WT PBase. The spontaneous reversion in a strain lacking the PBase plasmid is less than 10^{-7}. Mutant clones that gave rise to increased numbers of ura^+ revertants were further analyzed quantitatively. Yeast plasmid DNAs were isolated and the mutant PBase ORFs were sequenced.

Cell Culture and Gene Targeting. 293T and NIH 3T3 cells were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. Mouse ES cells were cultured on the pSNL76/7 feeder layers in KO-DMEM containing 15% FBS, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol. Cells (1×10^7) were electroporated with 25 μg of a linearized targeting vector in 800 μL of PBS solution by using a Gene Pulser II electroporator (230 V; 500 μF) and plated onto one 10-cm dish. The next day, puromycin selection (3 μg/mL) was initiated. Resulting colonies were picked and screened by PCR to detect targeted clones, which were further verified by Southern blot analysis. Primer sequences of PCR analysis and probe generation are shown in Table S4.

Transposition Assay in ES Cells. In the excision assay, 1×10^7 ES cells (the $Hprt^{PB\_in2}$ line) were electroporated with 10 μg of each PBase expression vector in 800 μL of PBS solution by using a Gene Pulser II (230 V; 500 μF). One fifth of the electroporated cells were then plated onto one 10-cm dish. Two days after electroporation, HAT selection was initiated. Medium was changed daily for 10 d and the resulting colonies were stained with 1% methylene blue in methanol and counted. In the integration assay, 1×10^7 AB1 ES cells were electroporated with 10 μg of a gene-trap transposon (5′-PTK-3′) (4) and 10 μg of the transposase expression vector using the same conditions as described and all cells were then plated onto one 10-cm dish. The next day, puromycin selection (3 μg/mL) was initiated and the same protocol described earlier was followed.

Assay for Excision-Induced Genomic Alteration. ES cells (1×10^7; $Hprt^{PB\_ex3}$ line) were electroporated with 40 μg of pCMV-mPBase or pCMV-hyPBase. One fifth of electroporated cells were plated in a well of a six-well plate. Three days later, cells were trypsinized and replated at 5×10^5 (mPBase) or 5×10^4 (hyPBase) cells per 10-cm dish. The next day, FIAU (0.2 μM) with or without 6TG (10 μM) were added. Medium was changed every other day for 10 d. The resulting colonies were picked and genotyped by PCR. PCR products were analyzed by sequencing. Primer sequences are shown in Table S4.

iPS Cell Reprogramming and Transposon Excision. Reprogramming by the *piggyBac* transposition, transposon removal, and footprint analysis were performed as described previously (5) with minor modifications. In the reprogramming assay, MEFs were plated onto a 12-well plate (2×10^5 cells per well) 1 d before transfection. The next day, 100 ng of pPBCAG.OSKML-puΔtk, 100 ng of the PBase expression vector, and 1.4 μg pBluescriptII were transfected using Lipofectamine 2000. The following day, transfected MEFs were plated onto one 10-cm dish. The subsequent procedures were as described previously.

CGH Analysis. Genomic DNAs were extracted by using a DNeasy Blood and Tissue kit (Qiagen). Agilent 244K Mouse Genome CGH microarrays were used according to the manufacturer's protocol. The arrays were scanned with an Agilent microarray scanner, and data were generated by Agilent Feature Extraction software. CGH calls were made with Agilent DNA analytics software by using the ADM2 algorithm (6.0 threshold) with a minimum of three probes in the region as a filter.

in Vitro Transcription and mRNA Transfection. Expression vectors were linearized with BbsI (pCMV-mPBase, pCMV-hyPBase, and pcDNA-EGFP) or XbaI (pCMV-iPBase; ref. 4), and purified by phenol-chloroform extraction and ethanol precipitation. Note that pcDNA3 vector carries the T7 promoter. In vitro transcription was performed by using the mMESSAGE mMACHINE T7 Ultra kit (Ambion) and synthesized RNA was purified by MEGAclear kit (Ambion) according to the manu-

facturer's instructions. RNA was mixed with Lipofectamine RNAiMAX (Invitrogen) in a ratio of 1 μg RNA to 5 μL at a final RNA concentration of 10 ng/μL in Opti-MEM (Invitrogen) and incubated for 15 min at room temperature. ES cells were trypsinized and resuspended in 1.7×10^6 cells/mL in Opti-MEM. ES cells (300 μL) and an appropriate volume of the RNA:RNAiMAX mixture were mixed and plated onto a well of a gelatinized 24-well plate and incubated for 90 min at 37 °C. ES cell medium supplemented with 1,000 U/mL leukemia inhibitory factor (Millipore) was then added and the cells were cultured. NIH 3T3 cells were trypsinized and resuspended in 5×10^4 cells/mL in complete medium. Cells (600 μL) and an appropriate volume of the RNA:RNAiMAX mixture were mixed and plated onto a well of a 24-well plate. Transfected cells were cultured for 16 to 20 h and subjected to flow cytometry, Western blot analysis, or immunostaining. For the excision assay, $Hprt^{PB\_ex3}$ ES cells were transfected with RNA, replated onto feeder plates 24 h after transfection, cultured overnight, and subjected to HAT selection for 7 d.

DNA Transfection and Western Blot Analysis. 293T cells or ES cells were transfected with HA-tagged transposase expression vectors by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h incubation, proteins were extracted in RIPA buffer (Pierce), separated in 4% to 15% gradient gel (Invitrogen), and transferred onto nitrocellulose membrane. The blots were analyzed with anti-HA (Roche) or anti–β-actin (Sigma) antibody. For H2AX analysis, whole-cell lysates were prepared by suspending cell pellets in 1× LDS sample buffer (Invitrogen). Anti-H2AX (R&D Systems) and anti-γH2AX (Abcam) were used.

Immunostaining. Immunostaining was performed as described previously (5). Anti-γH2AX (Cell Signaling) and Alexa 555-conjugated anti-rabbit IgG (Invitrogen) were used. DNA was counterstained with DAPI.

SCE Analysis. WT ES cells were transfected with 720 ng RNA. After 90 min incubation, ES cell medium supplemented with leukemia inhibitory factor and 3 μg/mL BrdU was added and cells were cultured for 20 h. After colcemid treatment at 0.1 μg/mL for 2 h, metaphase spreads were prepared, air-dried overnight, stained with 0.1 mg/mL acridine orange in Sorensen buffer, and observed by a fluorescent microscope with a GFP filter set.

1. Cadiñanos J, Bradley A (2007) Generation of an inducible and optimized piggyBac transposon system. *Nucleic Acids Res* 35:e87.
2. van der Weyden L, et al. (2005) Null and conditional semaphorin 3B alleles using a flexible puroDeltatk loxP/FRT vector. *Genesis* 41:171–178.
3. Mitra R, Fain-Thornton J, Craig NL (2008) piggyBac can bypass DNA synthesis during cut and paste transposition. *EMBO J* 27:1097–1109.
4. Cadiñanos J, Bradley A (2007) Generation of an inducible and optimized piggyBac transposon system. *Nucleic Acids Res* 35:e87.
5. Yusa K, Rad R, Takeda J, Bradley A (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 6:363–369.

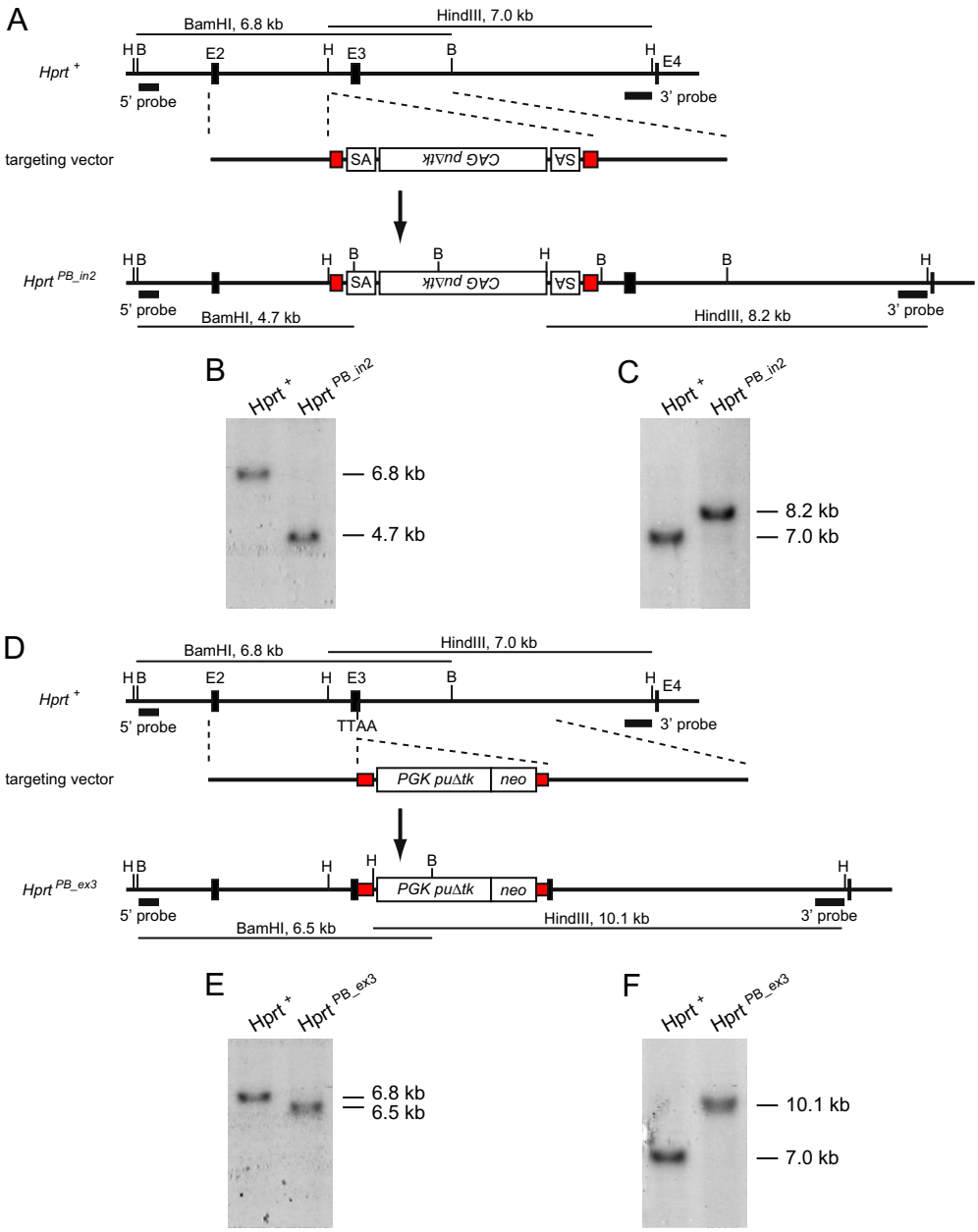

Fig. S1. Generation of the *Hprt* alleles harboring the *piggyBac* transposon. (*A*) Targeted insertion of the *piggyBac* transposon in intron 2. E, exon; H, HindIII sites; B, BamHI site; SA, splice acceptor; CAG, CAG promoter; *puΔtk*, the puromycin resistant gene fused with the herpes simplex virus *thymidine kinase* gene. (*B* and *C*) Southern blot analysis of the targeted clone for the $Hprt^{PB\_in2}$ allele with 5′ external (*B*) and 3′ external (*C*) probes. (*D*) Targeted insertion of the *piggyBac* transposon into a TTAA site in exon 3. PGK, PGK promoter; neo, EM7 promoter-driven neomycin-resistant gene for bacterial selection. (*E* and *F*) Southern blot analysis of the targeted clone for the $Hprt^{PB\_ex3}$ allele with 5′ external (*E*) and 3′ external (*F*) probes.

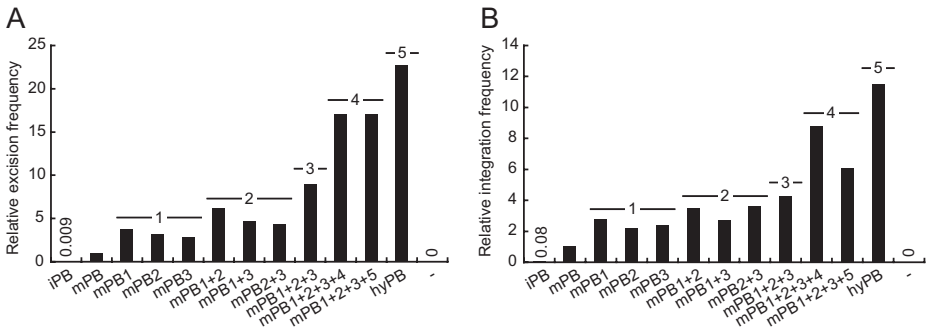

Fig. S2. Synergistic enhancement of transposition activity by hyperactive mutations. Excision (*A*) and integration (*B*) assay of combined mutants in ES cells. The assay methods are same as in Fig. 2. The frequencies are normalized to mPBase activities. The activity of iPBase is shown as a number for comparison. The number of mutants combined are indicated above the bars. iPB and mPB, *piggyBac* transposase with the insect-derived and mammalian codon-optimized sequence, respectively. hyPB, a mutant carrying all mutations. Mutant 1, M282V; 2, N538K; 3, I30V/G165S; 4, S103P; 5, S509G/N570S.

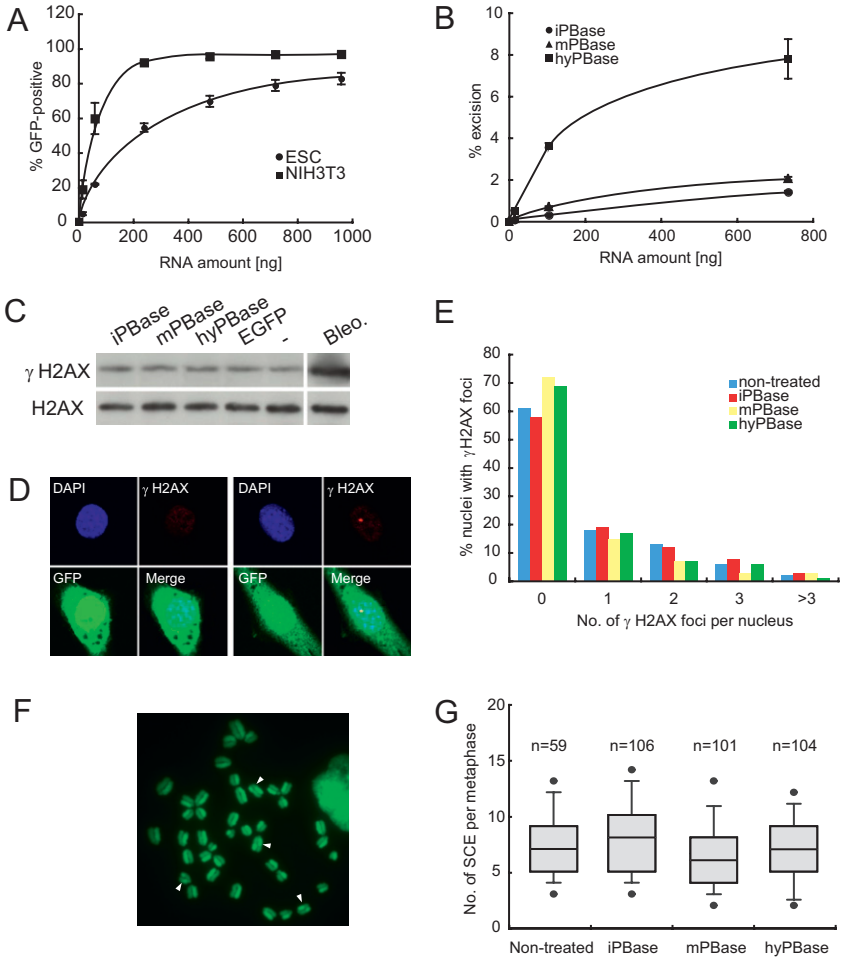

Fig. S3. Analyses of genotoxicity of PBases. (*A*) Efficiencies of mRNA transfection in ES cells and NIH 3T3 cells analyzed by flow cytometry. Data are shown as mean ± SD. (*B*) Excision assay using mRNA transfection. Data are shown as mean ± SD. (*C*) Western blot analysis of H2AX phosphorylation upon expression of PBases in ES cells. As a control, bleomycin (50 μg/mL) was added for 90 min. (*D*) Analysis of γH2AX foci. Cells were cotransfected with 60 ng of GFP mRNA and 420 ng of PBase mRNA. Representative images of cells with no (*Left*) and one γH2AX focus (*Right*) in hyPBase-transfected NIH 3T3 cells. (*E*) The numbers of γH2AX foci in nontransfected and PBase-transfected NIH 3T3 cells (100 nuclei per sample were analyzed). No statistical significances were detected ($P = 0.64$, χ^2 test). (*F* and *G*) Representative image of SCEs in an ES cell metaphase spread (*F*) and the number of SCEs in nontransfected and PBase-transfected ES cells (*G*). Arrowheads indicate sites of recombination. Bars within the box plots represent median values. The ends of bars indicate the 25th and 75th percentiles, the 10th and 90th percentiles are represented by error bars, and the 5th and 95th percentiles are shown by black circles. No significant differences were detected between nontreated cells and each PBase-transfected cells (Student *t* test).

Table S1. Analysis of footprint mutation

Type/exp.	Excision assay			Assay for excision-induced *Hprt* mutation			
	No. of cells screened*	No. of FIAU^R colonies	Excision frequency, %†	No. of cells screened*	No. of cells with excision‡	No. of FIAU/6TG^R colonies§	Frequency of *Hprt* mutation, %
mPBase							
1	38,000	105	0.27	190,000	513	4	0.78
2	38,800	98	0.25	194,000	477	3	0.63
3	46,000	107	0.23	230,000	520	3	0.58
4	304,000	701	0.22	304,000	681	4	0.59
5	252,000	657	0.25	252,000	640	1	0.16
6	262,000	665	0.25	262,000	648	11	1.70
hyPBase							
1	3,840	176	4.58	19,200	879	5	0.57
2	3,480	160	4.59	17,400	799	8	1.00
3	3,840	153	3.98	19,200	764	5	0.65

*Calculated with the number of cells plated and plating efficiency.
†Frequency of background of FIAU selection was subtracted.
‡Calculated with the number of cells screened and excision frequency.
§These colonies were genotyped and footprint mutations were analyzed.

Table S2. CGH analysis of iPS cell lines

iPS cell line	Transposase	Aberration	Aberration type	Chr.	Size, kb	Found in other lines	No. of genes
Primary iPS cell lines							
25*	—	No	—	—	—	—	—
28†	—	No	—	—	—	—	—
Transposon-free iPS cell lines							
25Δ1‡	mPBase	No					
25ΔΔ1	mPBase	Yes	1-copy deletion	8	707.8	No	1
			2-copy gain	8	26,798.1	No	355
			1-copy gain	8	8,774.3	No	108
25ΔΔ2	mPBase	No	—	—	—	—	—
25ΔΔ3	hyPBase	No	—	—	—	—	—
25ΔΔ4	hyPBase	Yes	1-copy deletion	11	108.7	No	1
28Δ6‡	mPBase	Yes	1-copy gain	16	305.4	No	0
28ΔΔ1	mPBase	No	—	—	—	—	—
28ΔΔ2	mPBase	Yes	1-copy deletion	7	31.5	No	1
28ΔΔ3	hyPBase	Yes	1-copy gain	15	162.0	No	5
28ΔΔ4	hyPBase	No	—	—	—	—	—

*A primary iPS cell line carrying two transposons that integrated in Chr. 2 and Chr. 19 (1).
†A primary iPS cell line carrying two transposons that integrated in Chr. 6 and Chr. 15 (1).
‡Transposon-free iPSC lines (1).

1. Yusa K, Rad R, Takeda J, Bradley A (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 6:363–369.

Table S3. Analyses of PBase-mediated mutation in the *Hprt* locus

Transposase type	Experiment	No. of cells analyzed*	$6TG^R$ colonies	Frequency
GFP	1	1.4×10^6	0	0
GFP	2	1.4×10^6	0	0
GFP	3	1.6×10^6	0	0
iPBase	1	1.3×10^6	0	0
iPBase	2	1.3×10^6	1	7.9×10^{-7}
iPBase	3	1.2×10^6	4	3.2×10^{-6}
mPBase	1	9.7×10^6	0	0
mPBase	2	1.3×10^6	0	0
mPBase	3	1.1×10^6	3	2.8×10^{-6}
hyPBase	1	1.7×10^6	0	0
hyPBase	2	2.0×10^6	0	0
hyPBase	3	1.5×10^6	0	0

*Calculated with the number of cells plated and plating efficiency.

Table S4. Primer sequence (5′-3′)

Direction	Sequence
3′ homology arm for $Hprt^{PB\_in2}$ targeting vector	
F	ACCTTAATTAAGATAGATGTTATAGTGTACTCTCCTCTCC
R	AAAGGCGCGCCAGGCACTCAAGATGATCCATATACT
Left homology arm for the mini targeting vector, pBS-HprtE3R	
F	GGGGTACCGGCCGGCCCTTCATAGAGACAAGGAATGTGTC
R	CCCAAGCTTATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAATGTAATCCAGCAGGTCAGCAAAG
Right homology arm for the mini targeting vector, pBS-HprtE3R	
F	CCCAAGCTTCGTACGTCACAATATGATTATCTTTCTAGGGTTAAAGCACTGAATAGAAATAGTGATAG
R	GCTCTAGAGTTTAAACTTCTACCCCAGCACAGAAAAATAA
XhoI site-flanked EM7-neo	
F	CCGCTCGAGGTTGACAATTAATCATCGGCATAG
R	CCGCTCGAGTCAGAAGAACTCGTCAAGAAGGCG
Left homology arm for the retrieving vector, pDTA-HprtRet	
F	GGCGCGCCAGACTTATTATCTAAACGTAAT
R	CCCAAGCTTATCGAGGTCTTACTAACCTGTC
Right homology arm for the retrieving vector, pDTA-HprtRet	
F	CCCAAGCTTGATATTTATTCCTTATGTCTGT
R	CCGCTCGAGGGGAGTGGAGAAGAACAAAATAA
Detection of homologous recombination for $Hprt^{PB\_in2}$	
F	CAAAATCAGTGACACTTACCGCATTGACAA
R	ACTAACAACCCTTTCTCTCAAGGTCTAGTT
Detection of homologous recombination for $Hprt^{PB\_ex3}$	
F	AAAACGGTAACATTTAACTGCTCTACATAC
R	GCGACGGATTCGCGCTATTTAGAAAG
5′ Probe for Southern blot analysis of the *Hprt* locus	
F	GCTTTAATGATTTTGCTGTACTTTTTTCTG
R	TGATCCTTTACAAACTCAAACTTAG
3′ Probe for Southern blot analysis of the *Hprt* locus	
F	CTTTTAATCATCAGTTTTCTGATTTAATAC
R	CAAATTATTAGATATAAGCTATAAG
Genotyping of the $Hprt^{PB\_ex3}$ allele	
F	CTTCATAGAGACAAGGAATGTGTC
R1	GCGACGGATTCGCGCTATTTAGAAAG
R2	TTCTACCCCAGCACAGAAAAATA