

Figure S1. Construction and structure of vectors. (A) Steps to pGTnanos3. (B) Map of pGTnanos3. Positions and extension directions of PCR primers are depicted by thin arrows. Important restriction sites used for cloning are indicated. CMV, human cytomegalovirus early enhancer/promoter; SV, enhancer element of the Simian virus 40; TK, herpes simplex virus (HSV) thymidine kinase promoter; tk, gene encoding the HSV thymidine kinase; gfp, gene encoding green fluorescent protein; neo, neomycin acetyltransferase.

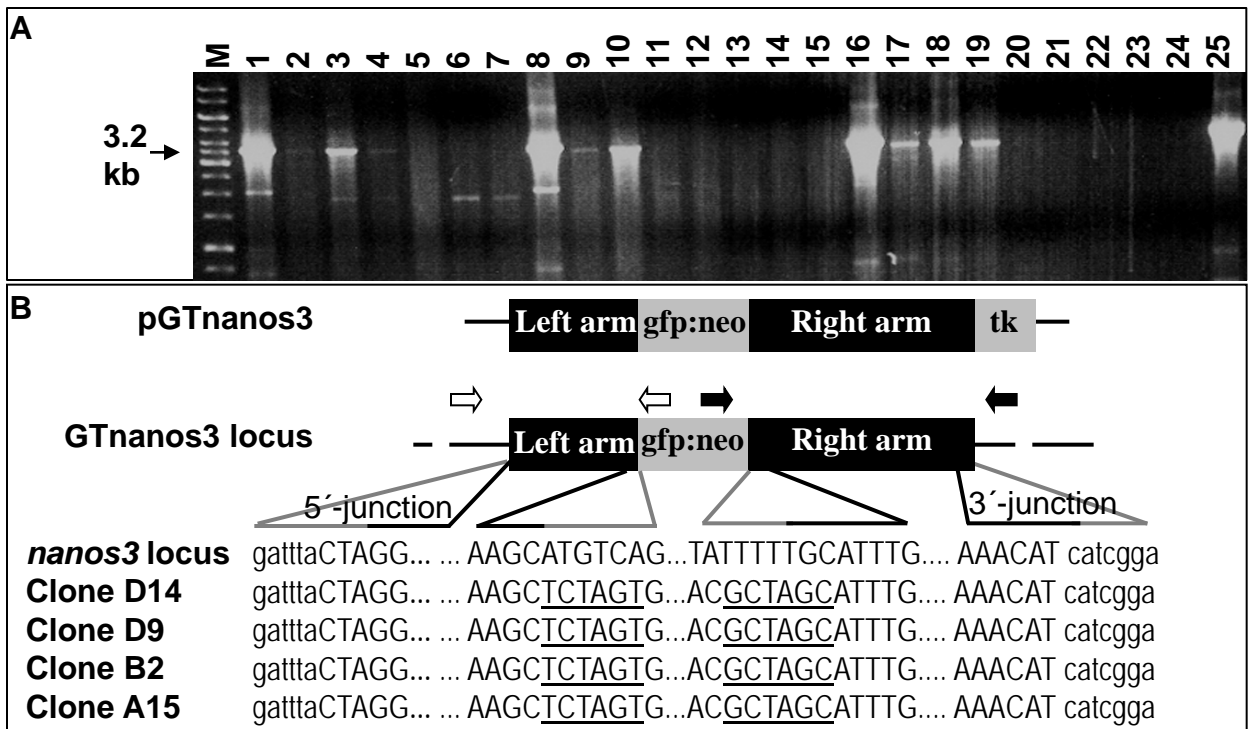


Figure S2. Genotyping of PNS-resistant colonies. A. The PCR product of the predicted size for the targeted *nanos3* locus (arrows) is seen in 9 out of 25 representative colonies (numerals on lanes). B. The predicted sequence was validated.

A allele a

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1 TTCTTTAGCCTCAAACCTACCGTGGGAGCTAGCATAACCTGCTTTCGGAAACACCCCCAGATGTCACCTTGTGTTTTGAACAGTTGGGCGTGCATTCTCC
101 TAGAAATAACGAAATGAACCCGAAACATTTCCCAACCAGCGTCAGTTCATTGCATCAACGTCAGTTCATTGCCCTTTACCATGGCTCCTTGTAAAAAATGT
201 ACTGTGGTTTTCGGGATCATTAAACTCGATTTTTTCTGAATAAAAACAATACACTGAGCACATTGTGTATAAAAAAATACTTCAGCAAATCTATAATGGC
301 AGGCTGAATTAGAGTCGGTAGAGTAAGCTCAGTGGAAACAGTCAGTGGGCTGTGTATCCAGAGGTTCTAAGTTCAAGACCAGTGGTTTATTACTTCTTTTT
401 TTTTGTCTTTGTGTTTTTTTTCTTCGTGTGATGTGATTTTGTCTGTGTCAAGCACCTCTCACAGAAAACGTCACCTTGTGGTGTGTTTTACATTGTTTTT
501 TTAATCATCATTGACAACATGTTTTGTTTTACATTTTTTCATCATCATTTCCAAATTGAATTGTTACAAGGAGCTTAGTTGAAACATATATCGATATAC
601 GTCCAGTCCGCCACCAGATGCCGCTGTTCGCTGTTTTGTGTGGTTTCAAGCCCTGAATGGGCGGTTTCATTTTTCCGGCACAATTACATGAACCCCAAGG
701 GAAGCTTCATGGGGCTTCTATTCCCATCCCTAGCAGAAGGTAAGTTAGACGAGAGCAAAAACACATGAACTAGATTTACTAGGCTGACCAGACTAAGCAC
801 CATCAGGGGCAACGAACTGGCGATAAATACTGGTACTGGATCTCCTTAAGTAGGCTCGGAGGCATGATGAGTAGACTGGA

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allele b

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1 TTCTTTAGCCTCAAACCTACCGTGGGAGCTAGCATAACCTGCTTTCGGAAACACCCCCAGATGTCACCTTGTGTTTTGAACAGTTGGGCGTGCATTCC
101 TAGAAATAACGAAATGAACCCGAAACATTTCCCAACCAGCGTCAGTTCATTGCATCAACGTCAGTTCATTGCCCTTTACCATGGCTCCTTGTAAAAAATGT
201 ACTGTGGTTTTCGGGATCATTAAACTCGATTTTTTCTGAATAAAAACAATACACTGAGCACATTGTGTATAAAAAAAGATACTTCAGCAAATCTATAATGGC
301 AGGCTGAATTAGAGTCGGTAGAGTAAGCTCAGTGGAAACAGTCAGTGGGCTGTGTATCCAGAGGTTCTAAGTTCAAGACCAGTGGTTTATTACTTCTTTTT
401 TTTTGTCTTTGTGTTTTTTTTCTTCGTGTGATGTGATTTTGTCTGTGTCAAGCACCTCTCACAGAAAACGTCACCTTGTGGTGTGTTTTACATTGTTTTT
501 TTAATCATCATTGACAACATGTTTTGTTTTACATTTTTTCATCATCATTTCCAAATTGAATTGTTACAAGGAGCTTAGTTGAAACATATATCGATATAC
601 GTCCAGTCCGCCACCAGATGCCGCTGTTCGCTGTTTTGTGTGGTTTCAAGCCCTGAATGGGCGGTTTCATTTCCGGCACAATTACATGAACCCCAAGG
701 GAAGCTTCATGGGGCTTCTATTCCCATCCCTAGCAGAAGGTAAGTTAGACGAGAGCAAAAACACATGAACTAGATTTACTAGGCTGACCAGACTAAGCAC
801 CATCAGGGGCAACGAACTGGCGATAAATACTGGTACTGGATCTCCTTAAGTAGGCTCGGAGGCATGATGAGTAGACTGGA

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B Identity 99.43%

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TTCTTTAGCCTCAAACCTACCGTGGGAGCTAGCATAACCTGCTTTCGGAAACACCCCCAGATGTCACCTTGTGTTTTGAACAGTTGGGCGTGCATTCTCC
*****C*****C**
TAGAAATACGAAATGAACCCGAAACATTTCCCAACCAGCGTCAGTTCATTGCATCAACGTCAGTTCATTGCCCTTTACCATGGCTCCTTGTAAAAAATGT
*****
ACTGTGGTTTTCGGGATCATTAAACTCGATTTTTTCTGAATAAAAACAATACACTGAGCACATTGTGTATAAAAAAATACTTCAGCAAATCTATAATGGC
*****C*****G*****
AGGCTGAATTAGAGTCGGTAGAGTAAGCTCAGTGGAAACAGTCAGTGGGCTGTGTATCCAGAGGTTCTAAGTTCAAGACCAGTGGTTTATTACTTCTTTTT
*****
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*****
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*****
GTCCAGTCCGCCACCAGATGCCGCTGTTCGCTGTTTTGTGTGGTTTCAAGCCCTGAATGGGCGGTTTCATTTTTCCGGCACAATTACATGAACCCCAAGG
*****C*****
GAAGCTTCATGGGGCTTCTATTCCCATCCCTAGCAGAAGGTAAGTTAGACGAGAGCAAAAACACATGAACTAGATTTACTAGGCTGACCAGACTAAGCAC
*****
CATCAGGGGCAACGAACTGGCGATAAATACTGGTACTGGATCTCCTTAAGTAGGCTCGGAGGCATGATGAGTAGACTGGA
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Figure S3. nanos3 sequences surrounding polymorphic restriction site XbaI. (A) Sequences spanning the polymorphic XbaI site are highlighted in color (allele a in blue; b in red) shown in Fig. 2, or underlined in (B), the sequence alignment of a and b. broken underline, absence of the site; solid underline, presence of the site.

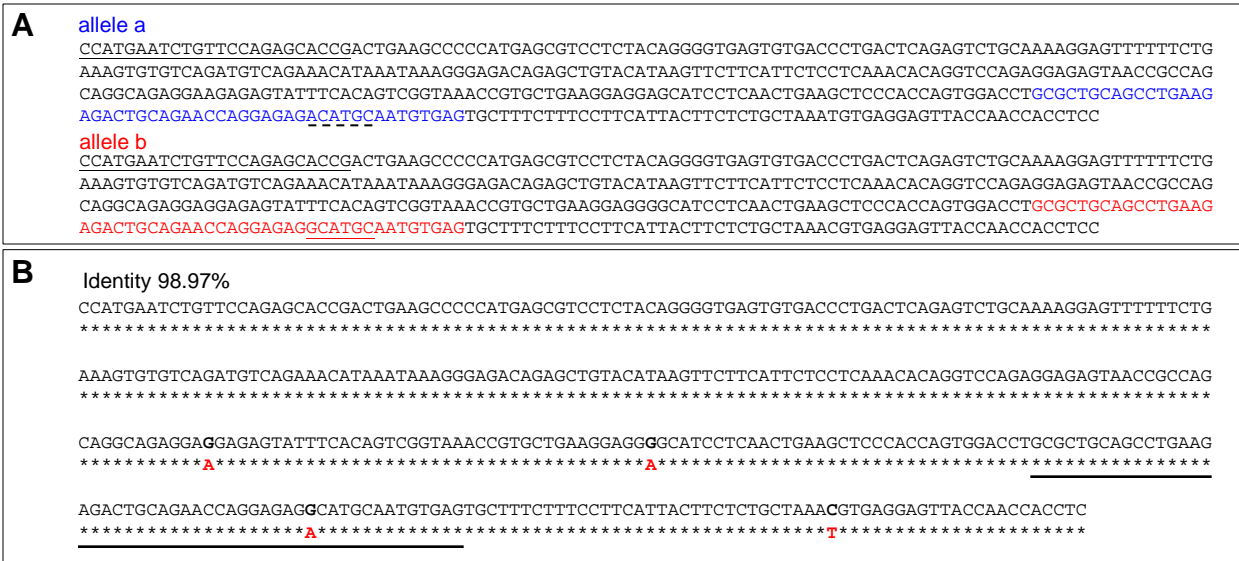


Figure S4. nanos3 sequences surrounding polymorphic restriction site SphI. (A) Sequences spanning the polymorphic SphI site are highlighted in color (allele a in blue; b in red) shown in Fig. 2, or underlined in (B), the sequence alignment of a and b. broken underline, absence of the site; solid underline, presence of the site.

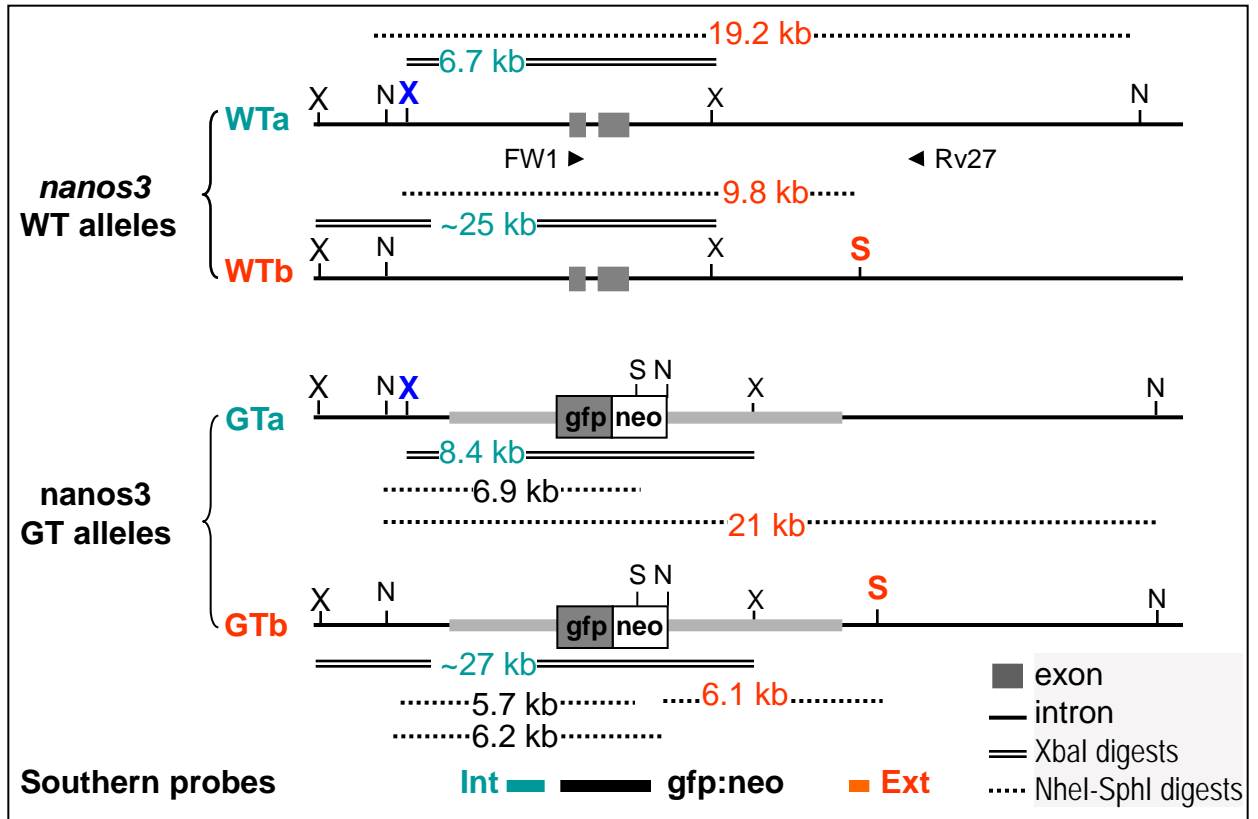


Figure S5. Strategic Southern analyses of *nanos3* gene targeting in medaka ES cells. (Top panel) WTa and WTb alleles in MES1 cells, showing polymorphic XbaI and SphI sites (bold). (Middle panel). GTa and GTb alleles from WTa and WTb via cointegration of the *gfp:neo* cassette by GT. (Bottom panel). Southern blot probes. Also shown are predicted fragments and their sizes of genomic digests by XbaI or NheI-SphI.

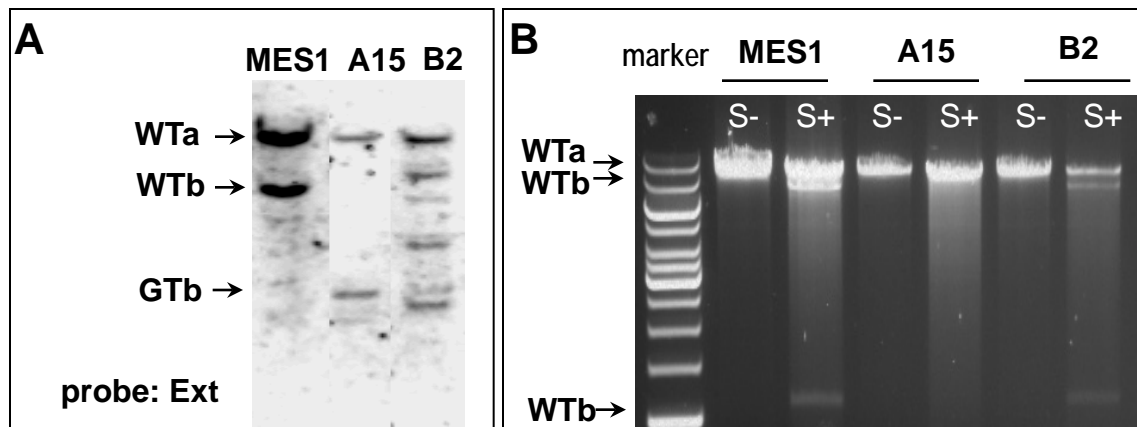


Figure S6. Allelic analysis. (A) Southern analysis of NheI-SphI digests, showing allele GTb in clones A15 and B2. (B) Agarose gel of PCR products before (S-) and after SphI digestion (S+), highlighting the loss of allele WTb but not WTa in clone A15, and presence of both WTa and WTb in MES1 and clone B2. PCR was run by using primers Fw1 and Rv27 (see Fig. 1), and the products were digested with *SphI* and separated on 1% agarose gel.

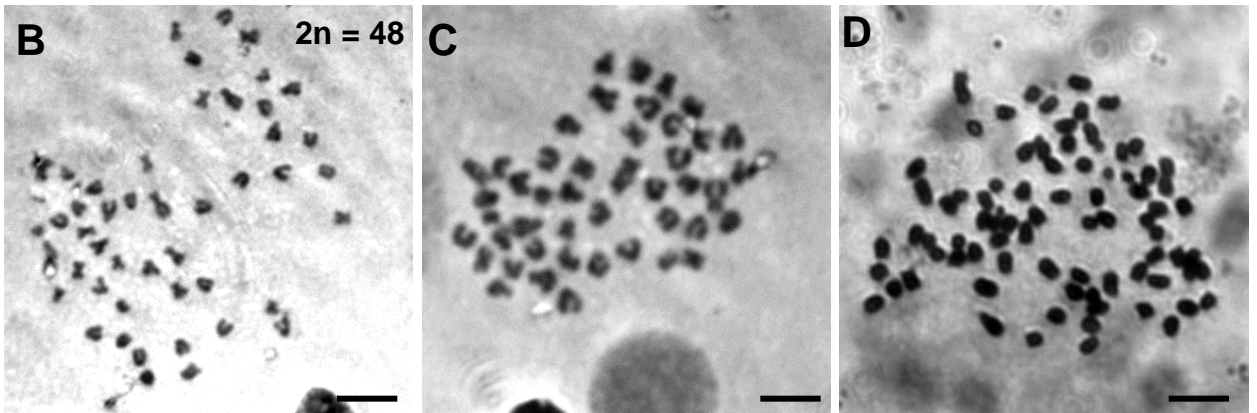
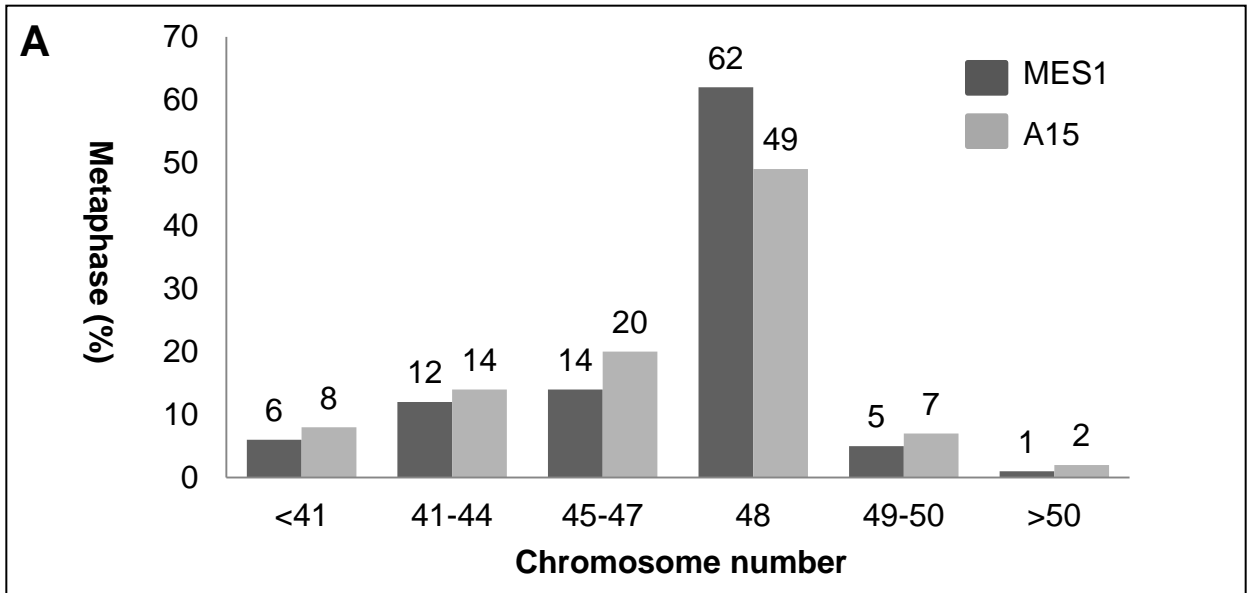


Figure S7. Chromosome analysis. (A) Distribution of chromosome number. A total of 100 metaphases were counted for MES1 and A15 each. (B-D) Representative A15 metaphases with 48 chromosomes (B), 45 chromosomes (C) or more chromosomes (D).

Table S1 PCR primers

Items	Gene (accession)	Primer	Primer sequence	Size (bp)
Targeted locus	nanos3	Fw1	atgtcagacgtggagtctggact	603
		Rv2	tcaacgcctagacttcacataaac	
Vector arms	nanos3 5'- arm	Fw3	ctaggctgaccagactaagc	2791
		Rv11	ccctcgagcttagcgcacctctgaggtccgc	
	nanos3 3'- arm	Fw6	gggctagcatttgacacctttggtagtc	5205
		Rv7	atgtttccgactcccagctcaacacc	
Stem-ness	oct4 (AY639946)	Oc1	atgtctgacaggccgcacagc	321
		Oc2	agtcgcgccgggtacctggcc	
	nanog (NP_001153902)	Ng1	atggcggagtggaaaactcag	400
		Ng2	atgaactccaagaatccgggtg	
	klf4 (ENSORL00000005643)	Klf4a	cagagggcgtgatgatctctag	347
		Klf4b	taagtgcctctcatgtggag	
ronin (ENSORL00000008903)	Ronin1	aactgagaagcgacgagtactc	302	
	Ronin2	cattttctttctgaaaccaac		
ectoderm	nf200 (ENSG00000100285)	Nf200a Nf200b	aacctgccgagccaaagaacc gatttaggcacaggcttctctg	283
mesoderm	brachyury (ntl, no tail) (ENSORL00000011262)	T1 T2	atgagcgcgtcgaaccgggac agacgggcgctttcatccagt	403
endoderm	sox17 (ENSORL00000011542)	17a 17b	atgagtagtcccgatgcccggg gccggagtcacgcctcttaat	450
Loading control	β -actin (D89627)	Am1	ttcaacagccctgccatgta	650
		Am2	cctccaatccagacagagtatt	
Geno- typing	nanos3 5'- 1st PCR	Fw9	caccagatgccgctgttcgc	4118
		neoR3	gtctgtgtgccagtcatagcc	
	nanos3 5'- nested PCR	GN1	cccacccctagcagaaggtaag	3195
		GFPR	catggtggcgaccggtggatcccgtag	
	nanos3 3'- 1st PCR	GFPFw1	atggtgagcaagggcgaggagc	7359
		Rv17	gagctcgtctggatggagctgaac	
nanos3 3'- nested PCR	NeoFw4	caggacatagcgttggtaccgg	5585	
	Rv24	gcatgatgcctgaaaatg		
Allelic analysis	nanos3 5' upstream	Rv50	ggttcctgattcgactcagctgtgtcc	1739
		Fw51	gcgatcggatcaaactctggccgc	
	Nanos3 3' downstream	Fw23	ggcgatcttcgagcggcaagg	1115
		Rv26	ccatgaatctgtccagagcaccg	
	nanos3 exon1	Fw1	ctttgacatgtggcacgactacatg	7969
outside nanos3 3'- arm	Rv27	gaagggagctgggatgaaggtctg		
Southern probes	Int	Fw11	cggtcatatagcctgctgtg	709
		Rv11	cttagcgcacctctgaggtccgc	
	Ext	GFPFw1	cctttgccgctgcaagatcgcc	503
		Rv17	gagctcgtctggatggagctgaac	
	gfp:neo	GFPFw1	atggtgagcaagggcgaggagc	1800
NeoRv2	gctagcgttgacggatccgaac			

Table S2. Analysis of PNS-resistant colonies.

Experiment	Colonies, n (%)		
	PNS resistant	PCR positive	Southern positive
1	3	0	0
2	7	1	0
3	17	3	1
4	14	2	0
5	15	5	3
total	56	9	4