Generation of SPRR3 TARGATT Knockin Mouse Model

Customer information

Project Title	CAG-L4SL-SPRR3
Job Ticket	MT060
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1. Summary

The generation of CAG-L4SL-SPRR3 (SPRR3) transgenic mouse model involved four steps. The first step was to generate a genetically modified founder mouse line, designated H11-3XattP (H11P3), by knocking in three tandem attP sequences, namely landing pad, into H11 locus of mouse C57BL/6 (B6) strain. The second step was to integrate SPRR3 transgene sequence into H11P3 sites by microinjecting integration cocktail into the pronuclei of heterozygous H11P3 embryos harvested from B6 mice. The integration cocktail consisted of a plasmid pBT378-CAG-L4SL-SPRR3 donor vector DNA (refer to report 1) and *in vitro* transcribed ϕC31 integrase mRNA. Because the SPRR3 donor construct contains two attB sites, and the mouse genome has three attP sites, ϕC31 integrase catalyzes site-specific DNA integration between attB and attP sequences, resulting in SPRR3 cassette integration into the H11P3 locus in the mouse genome. The third step was to implant the zygotes injected with the integration cocktail into CD1 foster mice to produce new mice. The fourth step was to identify SPRR3 transgenic founder by PCR-based genotyping.

On 10/26/2015, 120 H11/B6 embryos were injected with the integration cocktail, containing pBT378-SPRR3 DNA and integrase mRNA, 76 of which were implanted into three surrogates. From this round of microinjection, six mice were produced on 11/14/2015. Among them, one male mouse (1708#2) was identified as a SPRR3 founder with 1, 2-attP site integration.

The founder was mated with wild type B6 females. On 02/02/2016, ten mice were born. Genotyping results showed that seven of them are positive SPRR3 transgenic models.

2. Method - Identification of SPRR3 models

Tail tissues from F0 and F1 mice were collected and DNA extraction was performed individually. A panel of PCR primer pairs was designed to identify and confirm 1) attP site-specific insertion by the amplification of novel junction sequences at the SPRR3-targeted H11P3 allele; and 2) the insertion of SPRR3 (Table 1 and Figure 1). Following PCRs were performed and their expected sizes of PCR products are listed in Table 2.

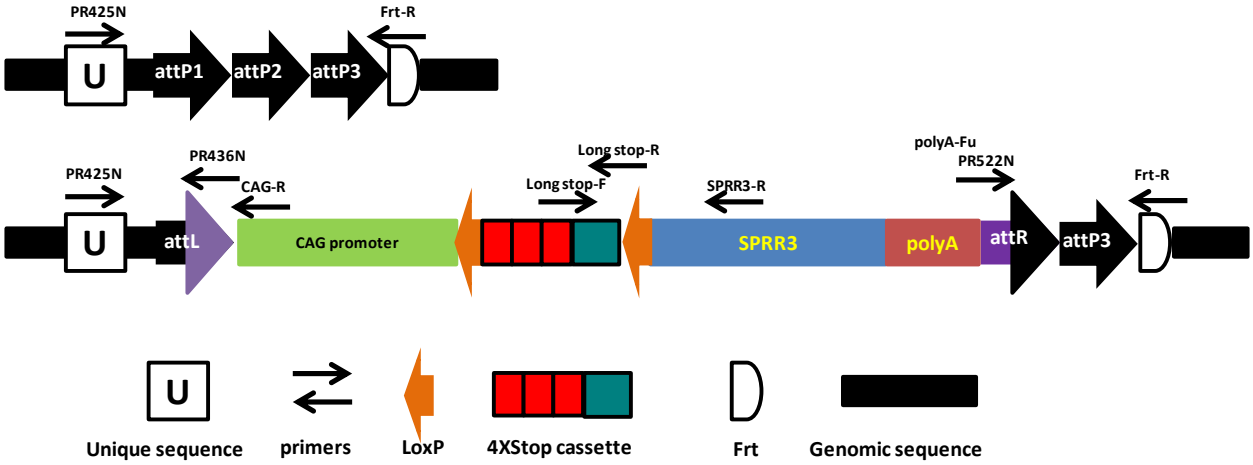


Figure 1. Transgene integration and genotyping scheme of SPRR3 transgenic model. Upper panel: genomic sequence with H11P3 landing pad; lower panel: founder genotype with SPRR3 transgene inserted at 1, 2-attP sites

PCR1: PR425N/PR436N (or 436N-1). PR425N (forward primer) recognizes a unique sequence on H11 allele, while PR436N (reverse primer) recognizes the attL sequence. The attL sequence is generated only when did ϕC31 integrase-mediated insertion occur at attP site of the 5'-end of H11P3 landing site (Figure 1). A PCR amplification using PR425N/PR436N detects site-specific insertion at 5' insertion site. The expected size of the amplicon is 136bp for 1, 2- or 1, 3-insertion, or 206bp for 2, 3-insertion when PR436N was used, and 147bp for 1, 2- or 1, 3-insertion, or 287bp for 2, 3-insertion when PR436N-1 was used (Figure 1). Two reverse primers were used to ensure positive amplification.

PCR2: PR425N (or R10N)/CAG-R. R10N (forward) primer recognizes Rosa26 locus sequence upstream of the engineered unique sequence. CAG-R (reverse primer) binds to CAG promoter sequence (Figure 1). The PCR amplification using PR425N/CAG-R can detect the insertion of SPRR3 transgene at the 5'-junction. The expected fragment size is 377bp for 1, 2- or 1, 3-

insertion, or 447bp for 2, 3-insertion when 425N/CAG-R was used, and 517bp for 1, 2- or 1, 3-insertion, or 587bp for 2, 3-insertion when R10N/CAG-R was used (Figure 1).

PCR3: long stop-F/SPRR3-R. Primer "long stop-F" recognizes the fourth SV40 intron-polyA sequence and SPRR3-R recognize sequences specific to the transgene (Figure 1). Positive amplifications are indications of the presence of the transgene in the mouse genome. DNA amplification using this primer set produces a 772bp fragment.

PCR 4: long stop-F/long stop-R. Alternatively, primer "long stop-F" and "long stop-R" recognize the fourth SV40 intron-polyA (Figure 1). DNA amplification using these primers produces a 570bp fragment, indicating the presence of the transgene in the mouse genome.

PCR 5: polyA-Fu/Frt-R (R1 or R2). Forward primer, polyA-Fu, recognizes the SV40 polyA sequence downstream of SPRR3 gene. Frt-R (R1 and R2) recognizes the FRT sequence that is part of TARGATT landing site in the mouse genome (Figure 1). A PCR amplification using polyA-Fu/Frt-R1 or R2 primer pair detects SPRR3 transgene insertion at 3' site of the H11 locus. The expected sizes using the polyA-Fu/Frt-R1 or R2 primer pair are 399bp or 437bp for the 2, 3- or the 1, 3- insertion or 469bp or 507bp for 1, 2- insertion, respectively.

PCR 6: PR522N/Frt-R1 or R2. PR522N (forward primer) binds to attR sequence. The attR sequence is present only when did ϕC31 integrase-mediated insertion occur at any of the attP sites of H11P3. A PCR amplification using the PR522N/Frt-R1 or R2 primer pair detects site-specific insertion at the 3'-end of H11 landing site (Figure 1). The expected sizes using the PR522N/Frt-R1 or R2 pair are 117bp or 155bp for 2, 3- or 1, 3- insertion, or 187bp or 225bp for 1, 2- insertion (Figure 1).

Table 1. PCR primers used for genotyping

Primers	Sequence
PR425N	5′-GGTGATAGGTGGCAAGTGGTATTCCGTAAG-3′
R10N	5′-AGTTCTCTGCTGCCTCCTGGCTTCT-3′
PR436N	5′-CCACCTCGACCCGTTCATCATGATG-3′
PR436N-1	5′-ATCAACTACCGCCACCTCGACC-3′
CAG-R	5′-CATATATGGGCTATGAACTAATGACCCCGT-3′
Long stop-F	5′-CTGACTCTCAACATTCTACTCCTCC-3′
Long stop-R	5′-GGACAAACCACAACTAGAATGCAG-3′
SPRR3-R	5′-GCTGACAAGGTTGCTTCACTTGATG-3′
polyA-Fu	5′-CTAGATAACTGATCATAATCAGCCATACCACAT-3′
PR522N	5′-GACGATGTAGGTCACGGTCTCGAAG-3′
Frt-R1	5′-GGAATAGGAACTTCGTCGACA-3′
Frt-R2	5′-CCGCGAAGTTCCTATACCTTTTG-3′

Table 2. Descriptions for PCR genotyping

Description	Forwad primer	Reverse primer	Fragment size(s), bp
5'-junction	PR425N	PR436N	136bp/206bp
	PR425N	PR436N-1	147bp/217bp
5'-Gene-of-Interest	R10N	CAG	517bp/587bp
	PR425N	CAG	377bp/447bp
Gene-of-Interest	Long stop-F	SPRR3-R	722bp
	Long stop-F	Long stop-R	570bp
3'-Gene-of-Interest	polyA-Fu	Frt-R1	399bp/469bp
	polyA-Fu	Frt-R2	437bp/507bp
3'-junction	PR522N	Frt-R1	155bp/225bp
	PR522N	Frt-R2	117bp/187bp

All PCR amplifications were prepared in 25 µL using MyTaq™ Red Mix (Bioline, Cat#, BIO-25044) (Table 3), and the amplifications were carried out using the following program: 95°C, 2 min; 35 cycles of [95°C, 15 s; 60°C, 15 s; 72°C, elongation duration varies by amplicon size], 72°C, 5 min; 4°C, indefinite.

Table 3. Preparation of PCRs

	25 µl reactions
MyTaq™ Red Mix 2X	12.5 µl
Nuclease-free water	8.5 µl
Downstream Primer (20µM)	1 µl
Upstream Primer (20µM)	1 µl
Template DNA	2 µl
Total Volume	**25 µl**

3. Results and interpretation- Identification of SPRR3 model

3.1. Identification of SPRR3 founder

On 10/26/2015, a mixture of plasmid pBT378-SPRR3 and ϕC31 integrase mRNA was microinjected into 120 H11P3/B6 embryos. Seventy-six of them developed well *in vitro* and were implanted into three CD1 foster mice. Six mice were produced from this round of microinjection on 11/14/2015. One male (1708#2) was identified as a founder (Figure 2). Four PCRs, #1, 2, 5 and 6, described in the method, showed positive amplifications for this mouse (Figure2a, 2e, 2f and 2b), suggesting that the founder had SPRR3 transgene inserted at 1, 2-attP sites of the H11 locus (Figure 1). Amplification of PCR3 indicated that mouse 1708#1 has the transgene inserted at a random site and #2 is a site specific founder (Figure 2c). PCRs testing whether the bacterial back (BB) sequence is integrated in the mouse genome showed negative for 1708#2 (data not shown).

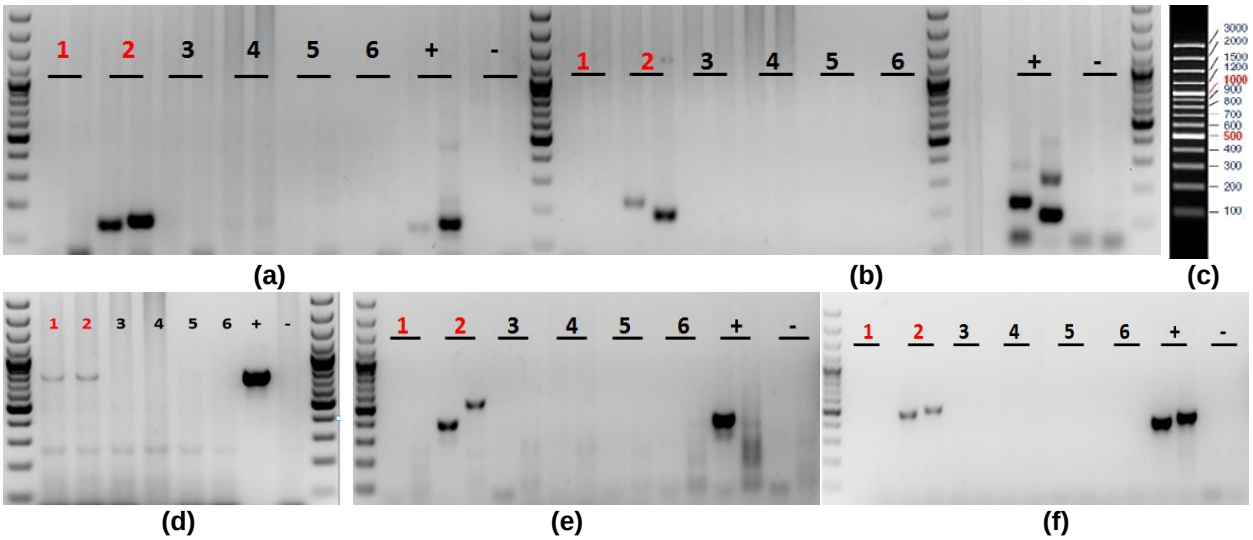
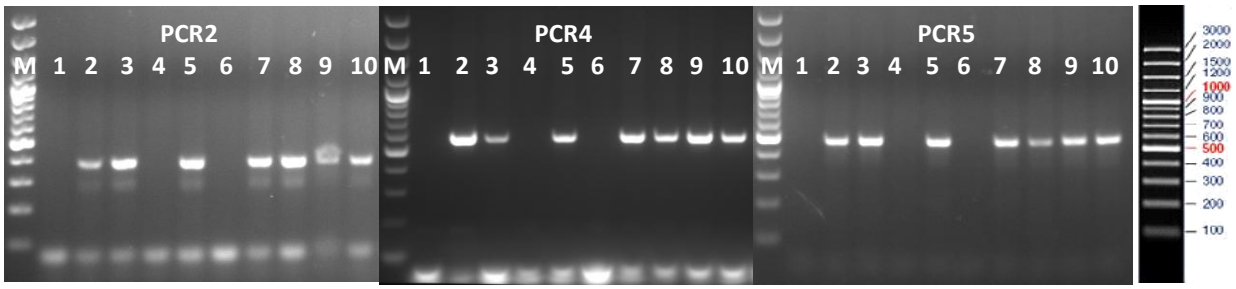


Figure 2. Identification of SPRR3 founder, 1708#2 among the six littermates. Genotyping PCRs as described in method (a) PCR1; (b) PCR6; (c) GeneRuler 100bp Plus DNA marker used in a to f; (d) PCR3; (e) PCR2; (f) PCR5

3.2. Identification of positive SPRR3 F1 mice

Founder 1708#2 was bred with wild type B6 female mice, which produced ten F1 mice on 02/02/2016. They were tested for PCRs 2, 4 and 5 to confirm the presence of SPRR3 transgene at the H11P3 locus. Genotyping results showed that seven of them are SPRR3 positive (Figure 3).



(a) (b) (c) (d)

Figure 3. Genotype identification of SPRR3 F1 mice by PCRs (a) PCR 2: PR425N/CAG-R; (b) PCR 4: Long stop-F/Long stop-R; (c) PCR 5: polyA-Fu/Frt-R1; (d)100bp ladder DNA marker used in lanes "M"

4. Shipping information for SPRR3 mice

The identifications of the SPRR3 founder and positive F1 mice are entailed in Table 4.

Table 4. SPRR3 founder and positive F1 mice identification

Mouse ID	DOB	Gender
1708#2*	11/14/2015	Male
1708#2A#2	02/02/2016	Male
1708#2A#3	02/02/2016	Male
1708#2A#5	02/02/2016	Female
1708#2A#7	02/02/2016	Female
1708#2A#8	02/02/2016	Female
1708#2A#9	02/02/2016	Female
1708#2A#10	02/02/2016	Female

*suggested to be retained at ASC as a backup