SMA_M.2.2.004

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Genotyping protocols for commonly used SMA model mice

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1. OBJECTIVE

The described procedure serves as a protocol for PCR based genotyping of common mouse models of Spinal Muscular Atrophy (SMA). In this protocol we provide **1)** Procedures for isolating DNA from neonates and weaning age mice **2)** A 3 primer PCR assay for the *survival motor neuron* (*Smn*) knockout allele [1]. **3)** A 3 primer PCR genotyping assay specific to the genomic integration site of the *(SMN2)89Ahmb* transgene commonly used in lines 5024 and 5025 (aka: the delta 7 mouse) [1, 2]. **4)** A PCR assay for detection of the (SMN2)2Hung transgene used in line 5058 (aka: The Li or Taiwanese model) [3].

2. SCOPE AND APPLICABILITY

The provided protocols will provide their users with the ability to manage a mouse colony, track alleles in complex breeding experiments and determine mutants, even in the absence of overt phenotypes.

3. CAUTIONS

DNA concentration and purity can affect the efficiency of amplification. It is recommended that each lab validate the assay conditions with known controls before using this assay for colony maintenance. All PCR reactions detailed in this protocol, using the conditions provided, are optimized to work on a wide range of DNA concentrations (25-500ng).

4. MATERIALS

-50mM NaOH
-1mM Tris
- Invitrogen Recombinant Taq (Catalog #10342-020)
- Thermocycler
- Sterile PCR Tubes
- Sterile Microfuge Tubes
- Phenol
- Chloroform
- TE Buffer
- Roche Proteinase K
- Tail lysis buffer (200 ml) :
    8 ml NaCl 5M
    2 ml SDS 20%
    2 ml EDTA 0.5M, pH 8.0

20 ml 1M Tris-HCl pH 8.5
168 ml H_2O sterile

5. METHODS

5.1. DNA Extraction

Neonates:

- Prior to P4, remove less than 1-3 mm of tail using a razor blade or fine pair of scissors, and place it in a PCR tube
- Add 25ul of 50mM NaOH to each tube
- Denature tails in a thermocycler at 99°C for 50 minutes
- Once denatured, add 20ul of a 17:3, TE: 1mM Tris solution to each tube. Mix tubes thoroughly before proceeding to PCR.

Pre-weaning:

- 0.25-0.5 cm tail snip into 1.5 ml eppendorf tube. **Store at –20°C (manual defrost) or -80C if not directly processing.**
- For direct processing, add 400µL of Tail Lysis Buffer (100mM Tris-HCl pH8, 5mM EDTA, 200 mM NaCl, 0.2%SDS).
- Add 40-50 µl of 20 mg/mL solution of Proteinase K
- Mince tail using a small, sharp, stainless steel scissor that fits into the bottom of the tube. (This step is not absolutely necessary and should be done when there is large amounts of tissue)
- Incubate at 55-60 °C overnight or until tails are completely lysed. Invert tube occasionally if possible, this will help samples digest better.
- Remove tubes from 55 °C and perform organic extractions.

Organic extractions.

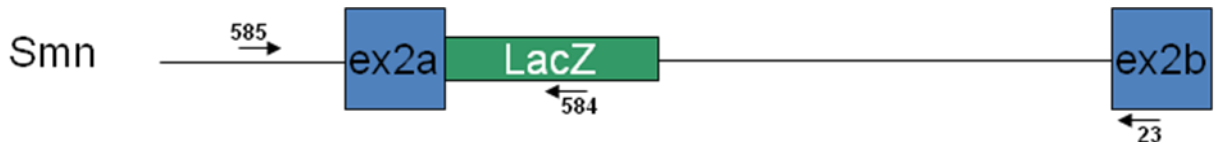
- Add to each tube:
  300 µl of phenol (Tris saturated)
  300 µl of chloroform/isoamyl alcohol (24:1)
- Cap tube and invert and shake by hand for ~15 sec.
- **DO NOT vortex** this will sheer the high molecular weight DNA.
- **Spin in microcentrifuge(16,100xg) to separate phases at least 5 minutes.**
- Transfer aqueous phase to clean tube using a wide bore pipette tip.
- IF the interphase still has excessive of protein, re-extract and move aqueous phase to a new tube. Fill tube with isopropanol (room temperature) and invert tube several times, until DNA precipitate forms.—**usually 450 µl is sufficient.**

- Remove DNA by touching it to the end of a pipette tip on your pipetman. Tap DNA to side of tube to remove excess alcohol and transfer to a new tube that contains TE (10mM Tris-HCl pH 7.5, 1 mM EDTA).
- Place tubes in a rack on a horizontal rotator set at 30-60 rpm. Leave O/N at RT

5.2 Specific geneotpying assays

***Smn* KO allele (Schrank et al. 1997[4])**

Details: DNA derived from mice or cell lines carrying the knockout allele can be genotyped using this protocol to determine whether mice are *Smn +/+, Smn +/-* or *Smn -/-*. The basis of the genotyping assay is the use of one forward primer in *Smn* intron 1 and two reverse primers, one in the *LacZ* selection cassette used to generate the knockout and one in *Smn* exon 2b.



-Primer 1- (585) mSmn multi Forward: CTCCGGGATATTGGGATTG
-Primer 2- (584) mSmn multi mut. Reverse: GGTAACGCCAGGGTTTTCC
-Primer 3- (23) mSmn exon 2b Reverse: CAAGGGAGTTGTGGCATTCTTC

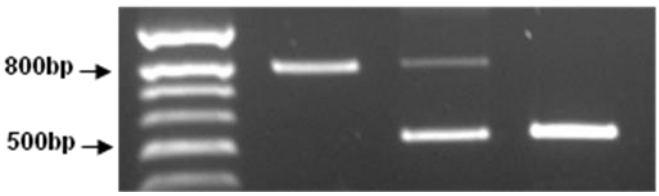
1. Set up PCR reaction in sterile PCR tubes using the following conditions:

13.3 µl	H2O
2.5 µl	PCR buffer
2.5 µl	MgCl (50mM)
2.5 µl	dNTPs (2.5mM)
1.0 µl	Primer 1 (50ng/µl)
1.0 µl	Primer 2 (50ng/µl)
1.0 µl	Primer 3 (50ng/µl)
0.2 µl	Taq (5U/µl)
1.0uL	DNA (~500ng)

2. Load reactions into the thermocycler and run with the following conditions: (conditions for an Eppendorpf mastergradient cycler)

Step 1: 94 ° - 3:00min
Step 2: 94 ° - 0:45sec

WT = 1 Band ~800bp
+/- = 1 Band ~800bp
1 Band ~500bp
-/- = 1 Band ~500bp

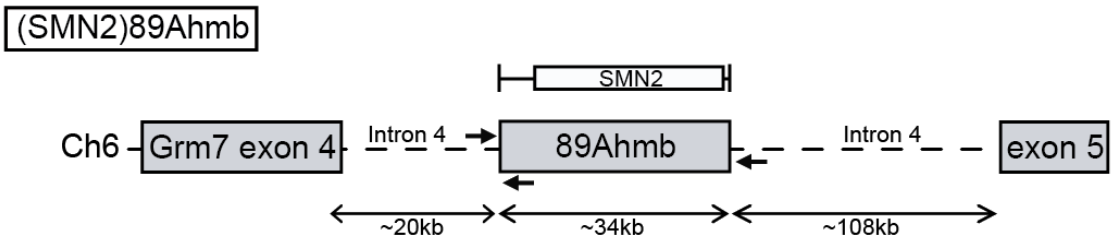


Step 3: 59 ° - 0:45sec
Step 4: 72 ° - 1:00sec
Go to Step 2, 39cycles
Step 5: 72 ° - 7:00min

3. Run PCR products out on a 1.2% -1.5% agarose gel.

(SMN2)89Ahmb (Monani, et al. 2000[1])

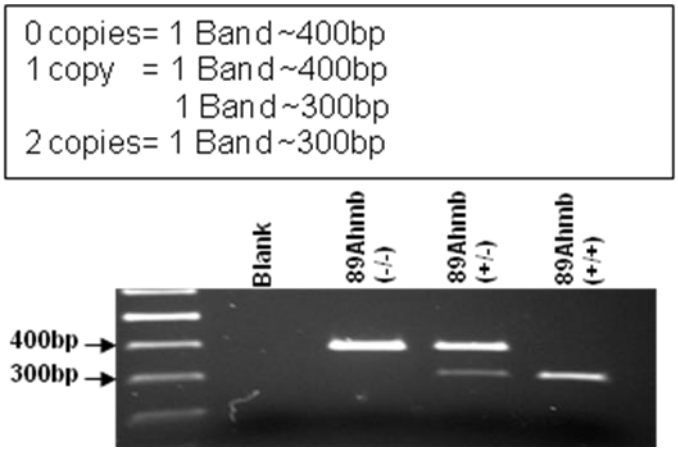
Details: DNA derived from mice or cell lines carrying the *(SMN2)89Ahmb* transgene can be genotyped using this protocol. *(SMN2)89Ahmb* is commonly used in The Jackson Laboratory strains 5024 and 5025 (aka: the delta-7 mouse). The *(SMN2)89Ahmb* transgene integrated in the murine metabatropic glutamate receptor 7 (*mGluR7* or *Grm7*), rendering the allele hypomorphic [5]. The 3 primer genotyping assay provided consists of one forward primer in *Grm7* intron 4 and two reverse primers, one in the 5' end of the *(SMN2)89Ahmb* transgene and one in *Grm7* intron 4.



- Primer 1- (591) 5024 TG Border (Grm7) Fwd: ctgacctaccagggatgagg
-Primer 2- (592) 5024 TG Border (SMN) Rev: ggtctgttctacagccacagc
-Primer 3- (597) 5024 TG negative Rev: cccaggtggtttatagactcaga

1. Set up PCR reaction in sterile PCR tubes using the following conditions:

11.6 µl	H2O
5.0 µl	PCR buffer
2.5 µl	MgCl (50mM)
2.5 µl	dNTPs (2.5mM)
1.0 µl	Primer 1 (50ng/µl)
1.0 µl	Primer 2 (50ng/µl)
1.0 µl	Primer 3 (50ng/µl)
0.2 µl	Taq (5U/µl)
1.0µl	DNA (~500ng)



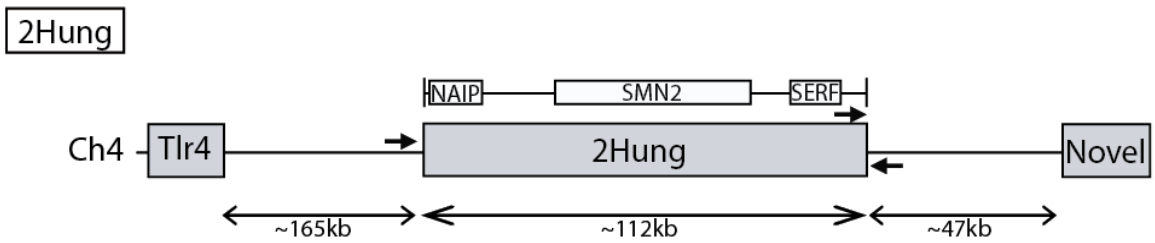
2. Load reactions into the thermocycler and run with the following conditions:

Step 1: 94 ° - 3:00min
Step 2: 94 ° - 1:00min
Step 3: 63 ° - 1:00min
Step 4: 72 ° - 1:00min
Go to Step 2, 39cycles
Step 5: 72 ° - 7:00min

3. Run PCR products out on a 1.2%- 1.5% agarose gel.

(SMN2)2Hung (Hsieh-Li et al. 2000[3])

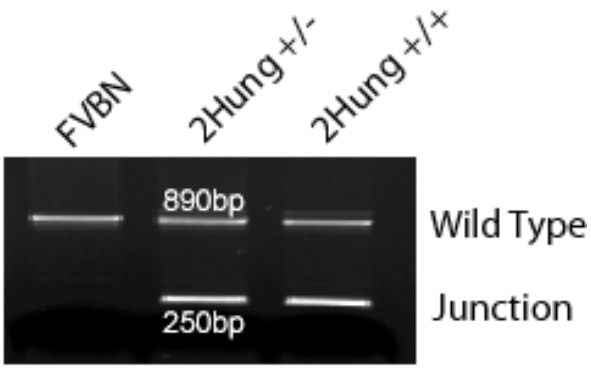
Details: DNA derived from mice or cell lines carrying the *(SMN2)2Hung* transgene can be genotyped using this protocol. *(SMN2)2Hung* is present in the Jackson Laboratory strain 5058 (aka: the Li or Taiwanese model) [3]. The *(SMN2)2Hung* transgene integrated into an intergenic region of murine Chromosome 4 [6]. The 3 primer genotyping assay provided consists of one reverse primer in *Chromosome 4* and two forward primers, one in *Chromosome 4* and the other at the 3' end of the *(SMN2)2Hung* transgene. ***NOTE: A complex rearrangement at the 3' insertion site, that is believed to contain a duplication of the insertion site, makes copy number analysis impossible with the provided assay.



-Primer 1- (690)-Li Transgene Fwd: tgtcttgagccaagttagcc
-Primer 2- (698)-Li Chrm 4 Rev: cctgctcctgcctatgaagt
-Primer 3- (737)-Li Chrm 4 Fwd: ttgctttatgactcttgatacctg

1. Set up PCR reaction in sterile PCR tubes using the following conditions:

13.3 µl	H2O
2.5 µl	PCR buffer
2.5 µl	MgCl (50mM)
2.5 µl	dNTPs (2.5mM)
1.0 µl	Primer 690 (50ng/µl)
1.0 µl	Primer 698 (50ng/µl)
1.0 µl	Primer 737 (50ng/µl)



0.2 µl Taq (5U/µl)
1.0 µl DNA (~500ng)

2. Load reactions into the thermocycler and run with the following conditions:

Step 1: 94 ° - 3:00min
Step 2: 94 ° - 0:45min
Step 3: 62 ° - 0:45min
Step 4: 72 ° - 1:00min
Go to Step 2, 39cycles
Step 5: 72 ° - 7:00min

3. Run PCR products out on a 1.2-1.5% agarose gel.

5.3. Notes

The above protocols were validated using both the neonatal and pre-weaning DNA extraction protocols provided above. If your lysis protocol differs from these conditions the assay may need to be re-optimized.

6. EVALUATION AND INTERPRETATION OF RESULTS

Results can be interpreted using the criteria described for each assay.

7. REFERENCES

1. Monani, U.R., et al., The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. Hum Mol Genet, 2000. 9(3): p. 333-9.
2. Le, T.T., et al., SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. Hum Mol Genet, 2005. 14(6): p. 845-57.
3. Hsieh-Li, H.M., et al., A mouse model for spinal muscular atrophy. Nat Genet, 2000. 24(1): p. 66-70.
4. Schrank, B., et al., Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci U S A, 1997. 94(18): p. 9920-5.
5. Gogliotti, R.G., et al., Characterization of a commonly used mouse model of SMA reveals increased seizure susceptibility and heightened fear response in FVB/N mice. Neurobiol Dis, 2011. 43(1): p. 142-51.

6. Gogliotti, R.G., et al., Molecular and phenotypic reassessment of an infrequently used mouse model for spinal muscular atrophy. Biochem Biophys Res Commun, 2010. 391(1): p. 517-22.

8. APPENDIX 1: BURGHES GENOTYPING

Low copy SMN transgene (005024)

Primers provided by Christine J. DiDonato

TG89 Border (Grm7) Fwd (591): ctgacctacagggatgagg

TG89_Grm7_negative Rev (597): cccaggtggtttatagactcaga 400 bp Grm7

TG89 Border (SMN) Rev (592): ggtctgttctacagccacagc 300 bp SMN

Multiplex reaction

94C 3 min

cycle 30 times

94C 1min

59C 1min

72C 1min

72C 5 min

multiplex, one common forward primer, two reverse primers

just top band, no SMN2

two bands, het

just bottom band, homozygous for SMN2

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mouse Smn/ KO

mSmn2AF TTTTCTCCCTCTTCAGAGTGAT

mSmn2BR2 GCTGTGCCTTTTGGCTTATCTGG 325 bp mSmn

Beta gal R GAGTAACAACCCGTCGGATTC 428 bp KO

95C 3 min

cycle 35 times

95C 1min

57C 1min

72C 1min

72C 5 min

multiplex, one common forward primer, two reverse primers

just top band, SMA mouse, homozygous for KO

two bands, het

just bottom band, no KO present

SMNdelta7 (005025)

SMNpromoterF TGGAGTTCGAGACGAGGCCTAAGC

1.2R (exon 1-2 junction) CAGAATCATCGCTCTGGCCTGTGCC 550bp

94C 5 min

cycle 30 times

94C 1 min

65C 1 min

72C 1 min

72C 2 min

9. APPENDIX 2: PROTOCOL FOR TATTOOING OF NEONATAL MICE

Material:

Syringe and Needle : 31 gauge insulin syringe

Tattoo Ink : Animal Tattoo Ink Paste (Ketchum : Stock No. KI-1475-039)

Procedure :

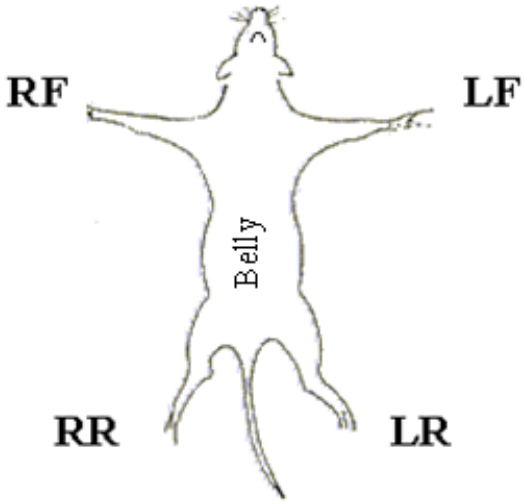
- Fill the syringe with the tattoo ink.
- Cut a piece of tail for genotyping.
- Hold the pup on its back in your palm. With the ankle of the concerned foot firmly between your thumb and index finger, insert the needle at an angle and inject a dollop of ink just beneath the skin of the footpad. Thus, number each pup of a litter.

- Wipe away the excess ink.
- Weigh the pup.

Note: It is better to cut the tail first and then tattoo to prevent the tail from getting stained.

The numbering of the pups is as follows:

1. LF
2. RF
3. LR
4. RR
5. 2F
6. 2R
7. LFLR
8. RFRR
9. LFRR
10. RFLR
11. No mark
12. 2FLR
13. 2FRR
14. LF2R
15. RF2R
16. All four!



LF = Left Front
RF = Right Front
LR = Left Rear
RR = Right Rear