Published in final edited form as:

Mol Genet Metab. 2012 May ; 106(1): 62–67. doi:10.1016/j.ymgme.2012.02.009.

Acadl-SNP Based Genotyping Assay for Long-Chain Acyl-CoA Dehydrogenase Deficient Mice

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Abstract

The long-chain acyl-CoA dehydrogenase (LCAD) (*Acadl* = gene; LCAD = protein) deficient mouse model has been important in evaluating the role of mitochondrial fatty acid oxidation of long-chain fatty acids in metabolic disorders. The insertion vector-based gene targeting strategy used to generate this model has made it difficult to distinguish homozygous and heterozygous genotypes containing targeted *Acadl* alleles in LCAD-deficient mice. Herein, we describe the design and validation of *Acadl* SNP genotyping methods capable of distinguishing between heterozygous and homozygous LCAD-deficient mice. The *Acadl* SNP genotyping assays are effective at allelic discrimination of both C57BL/6 and 129 mouse strain-based *Acadl* alleles under conditions including, both low purity and quantity genomic DNA templates. This makes the method practical and provides the necessary tools for genotyping the LCAD-deficient mouse model.000

Keywords

Mouse model; long-chain acyl-CoA dehydrogenase; *Acadl* alleles; SNP genotyping assay; molecular genotyping

1. Introduction

Long-chain acyl-CoA dehydrogenase (*Acadl* = gene; LCAD = protein) deficiency in mice closely mimics human very long-chain acyl-CoA dehydrogenase deficiency and is a key enzyme involved in mouse fatty acid oxidation of long chain fatty acids [1-5]. LCAD-deficient mice have been distributed around the world for various studies. Unfortunately, due to the insertion vector-based gene targeting strategy used [1], a simple PCR-based genotyping assay is not possible. As previously described, LCAD-deficient (LCAD^{-/-}) mice were generated using a targeting strategy that resulted in an insertion duplication of a region containing exons 3 through 4 within the *Acadl* locus [1], which confounds the development of routine genotyping strategies that distinguish targeted and wild-type *Acadl* alleles in LCAD^{+/-} and LCAD^{-/-} mice.

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Conflicts of Interest

The authors have no conflicts of interest to report.

The generation of the LCAD-deficient mouse model led to construction of a chimeric *Acadl* locus comprising sequence regions originating from mouse strains of three distinct genetic backgrounds. An insertion-based targeting vector, p*Acadl*^{tm1Uab}, which contains 129X1 (formerly 129/SvJ) genomic sequence from the *Acadl* locus spanning intron 2 through intron 4 and harbors an 821 base pair deletion in exon 3 was used during the targeted deletion step in TC-1 embryonic stem (ES) cells derived from 129S6 (formerly 129/SvEvTacFBR) mice. Recombinant mice generated from targeted ES cell clones were then backcrossed to C57BL/6 (B6) mice (Taconic Farms) for 10 generations to produce B6 congenic LCAD-deficient (B6.LCAD^{-/-}) mice. Ultimately, this targeting approach led to the generation of B6.LCAD^{-/-} mice with an *Acadl* locus consisting of proximal 129X1 and 129S6 sequence and distal B6 sequence (Figure 1) [1].

In an effort to develop a simple genotyping method for LCAD-deficient mice, we have utilized the chimeric nature of the targeted *Acadl* locus and employed targeted mutant 129-based allele and non-targeted wild-type B6 allele-distinguishing single nucleotide polymorphisms (SNPs). We describe here the validation of a SNP-based genotyping method that enables reliable genotyping and discrimination between B6 wild-type and 129-targeted *Acadl* alleles in B6.LCAD^{+/-} and B6.LCAD^{-/-} mice.

2. Materials and Methods

2.1 Mouse genomic DNA

Genomic DNA samples were prepared from B6.LCAD^{+/+} wild-type (Taconic), B6.LCAD^{+/-}, B6.LCAD^{-/-}, and 129S6 (wild-type) mice. All procedures were approved by the Sanford-Burnham Medical Research Institute (SBMRI) Institutional Animal Care and Use Committee (IACUC). Genomic DNA was isolated from mice by tissue harvest. DNA was isolated from tail or from liver extraction following humane euthanasia methods. Mouse genomic DNA (spleen) from 129X1 (wild-type) mice (formerly 129/SvJ) was acquired from Jackson Laboratory. For genomic DNA extraction, tail tissue was incubated in 200 µl of solution A (0.2 mM EDTA pH 12, 25 mM NaOH) at 95° C for 45 minutes to 1 hour, followed by the addition of 200 µl of solution B (40 mM Tris pH 5), then vortexed. Liver genomic DNA was extracted using the Genomic DNA Mini Kit (IBI Scientific), followed by purification with Wizard® Genomic DNA Purification Kit (Promega), then further purification using standard phenol-chloroform-isoamyl alcohol (Sigma) and ethanol precipitation methods to ensure purity of the genomic DNA with a 260/280 ratio of 1.8-2.0.

2.2 SNP assay development

A region spanning 1.5 kilobases (kb) between exons 3 and 4 within the *Acadl* gene locus was amplified with forward primer 5'-GACTTGCTCTCAACAGCAGTTACTTGGG-3' (exon 3) and reverse primer 5'-GGCTATGGCACCGATACACT-3' (exon 4), using either genomic DNA isolated from liver of B6.LCAD^{-/-}, B6.LCAD^{+/+}, or 129S6 mice, or from 129X1 genomic DNA (Jackson Laboratory). The respective 1.5 kb products were amplified using the Expand Long Template PCR system (Roche). PCR products were separated by gel electrophoresis on a 1% agarose gel, the 1.5 kb fragments then excised and gel purified (Qiagen). Following quantification, the purified products spanning the *Acadl* exon 3 to 4 region were sequenced with primers 5'-GACTTGCTCTCAACAGCAGTTACTTGGG-3' or 5'-GGCTATGGCACCGATACACT-3' with an ABI 3730 sequencer (Retrogen). Sequence data generated was analyzed and aligned using Vector NTI 11 (Invitrogen Life Technologies). Sequence runs for B6.LCAD^{-/-}, B6.LCAD^{+/+}, 129S6, and 129X1 *Acadl* exon 3 to 4 regions were aligned together or to known *Acadl* sequence for C57BL/6J (Gene ID: 11363; GenBank: NC_000067.5) (NCBI). Single nucleotide polymorphisms (SNPs) identified in B6.LCAD^{-/-}, 129S6, and 129X1 sequences aligned with B6.LCAD^{+/+} sequence

for the *Acadl* exon 3 to 4 region were selected for SNP genotyping assay development, based on the following criteria: (1) quality of sequence reads, and (2) no other sequence anomalies (i.e. insertions, deletions, or SNPs) within 50 bases of the target SNP.

Primers (5' GGAGGATTGCCAAGAGCTCAAG 3' forward; 5' TTTGTTTTGAAAGCAGCTT CTTGCT 3' reverse) and probes (5' TGGCCTAGAACTGACTAT 3' FAM; 5' TGGCCTAGAA CTAAGTAT 3' VIC) were selected (*Acadl* SNP 1 assay) to target a 93 base pair amplicon within intron 3 of the *Acadl* locus with the genotyping SNP located specifically at position 66,900,911 (*Acadl* SNP 1) on chromosome 1 (Applied Biosystems). Primers (5' GTGACT GCGGGTACATTAGAGT 3' forward; 5' TCTTACCCTGACACTGCAATTGT 3' reverse) and probes (5' AAGGTGCCCTCCATAAT 3' FAM; 5' CAAGGTGCCCTCTATAAT 3' VIC) were selected (*Acadl* SNP 2 assay) to target a 70 base pair amplicon also within intron 3 of the *Acadl* locus with the genotyping SNP located specifically at position 66,900,172 (*Acadl* SNP 2) on chromosome 1 (Applied Biosystems). *Acadl* SNP 1 and SNP 2 were verified as registered SNPs as rs48198501 (dbSNP) and rs30323299 (dbSNP), respectively. Both *Acadl* SNP 1 and 2 assays were then validated by real time PCR using an Applied Biosystems StepOne instrument (ABI). A single reaction consisted of 10 μ l of Taqman® Genotyping Master Mix (Applied Biosystems; 4371355), 0.5 μ l of either *Acadl* SNP 1 or 2 assay, 7.5 μ l dH₂O, and 2 μ l of genomic DNA template. Reactions were set up in MicroAmp Fast Optical 96-well plates (Applied Biosystems; 4346906). A SNP genotyping program of (60° C 30 seconds (1X); 95° C 10 minutes (1X); 95° C 15 seconds then 60° C 1 minute (40X); 60° C 30 seconds (1X)) was used with genomic DNA samples for known B6.LCAD^{-/-}, B6.LCAD^{+/-}, B6.LCAD^{+/+}, 129S6, and 129X1 mouse strains, as well as no template controls. Genotyping results were represented as allele intensities and calculated as the endpoint fluorescence of reporter dyes normalized to the Rox passive reference dye, minus the starting background reporter fluorescence normalized to the passive reference dye (ΔR_n). An allelic discrimination plot of normalized reporter fluorescence (ΔR_n) for each allele was then generated using the StepOne algorithm function. The StepOne software then uses an algorithm to plot the normalized reporter fluorescence (ΔR_n) as an allelic discrimination plot. Genotype clusters were defined by the ratio of allele 1 (C) ΔR_n /allele 2 (T) ΔR_n with ratios ~1 being defined as LCAD^{+/-} genotype clusters, ratios >1 defined as LCAD^{+/+} genotype clusters, and ratios <1 defined as LCAD^{-/-} genotype clusters.

2.3 Serial dilution analysis

Serial dilution assays were performed for both *Acadl* SNP 1 and 2 assays. Genomic templates from B6.LCAD^{-/-}, B6.LCAD^{+/-}, and B6.LCAD^{+/+} mice were diluted serially 2-fold with a maximum of 140 ng/ μ l of genomic template per reaction and a minimum of 2.18 ng/ μ l of genomic DNA per reaction. All genomic samples used were purified from liver with a 260/280 ratio of 1.8-2.0. SNP genotyping PCR assays were performed as described above.

Statistical Analysis—Statistical analysis was performed using ANOVA with Tukey post test. Results showing a *p*-value of *p* < 0.05 were considered statistically significant.

3. Results

3.1 Development of an *Acadl* SNP genotyping assay

In order to develop a SNP-based genotyping method to discriminate between the targeted 129 chimeric *Acadl* allele in B6.LCAD^{-/-} mice from the wild-type *Acadl* allele in B6.LCAD^{+/+} mice, we evaluated sequences for both the targeted 129 and wild-type B6 *Acadl* alleles to identify candidate SNPs. In our sequence analysis of several *Acadl* genomic

regions, PCR amplification and sequencing of a 1.5 kb region located between exons 3 and 4 of the targeted 129 chimeric *Acadl* allele and the wild-type *Acadl* allele in B6.LCAD^{-/-} and B6.LCAD^{+/+} mice, respectively, produced sequence runs that could be aligned together or with known sequence for the C57BL/6 *Acadl* locus to search for the presence of candidate SNPs. *Acadl* sequence alignments of the exon 3 to 4 region from B6.LCAD^{+/+} and B6.LCAD^{-/-} genomic templates indicated the presence of two SNPs [chr 1: 66,900,904 (*Acadl* SNP 1) and chr 1: 66,900,180 (*Acadl* SNP 2)]. Alignment of the wild-type *Acadl* locus from the B6.LCAD^{+/+} sequence run with known C57BL/6J sequence (Gene ID: 11363; GenBank: NC_000067.5) (NCBI) verified the sequence of the B6 wild-type version of this allele for these two SNPs, where as alignment of the targeted 129 chimeric *Acadl* locus sequence run from B6.LCAD^{-/-} mice to the known C57BL/6 sequence verified the presence of the two single nucleotide differences [*Acadl* SNP 1, rs48198501 (dbSNP) and *Acadl* SNP 2, rs30323299 (dbSNP)]. The presence of these two allele-discriminating SNPs suggested likely candidates for SNP genotyping assay development. Thus, we chose *Acadl* SNP 1 and 2 for assay development in a SNP-based genotyping assay to distinguish between the targeted 129 chimeric and B6 wild-type *Acadl* alleles from B6.LCAD^{-/-} and B6.LCAD^{+/+} mice, respectively (Figure 1). Preliminary screening for SNP-assay design (Applied Biosystems) produced a candidate primer and probe combination for each SNP [*Acadl* SNP 1 assay and *Acadl* SNP 2 assay, respectively], that could then be assessed for genotyping validation using genomic templates (Figure 2).

The ABI StepOne software platform calculates normalized probe intensities (ΔR_n) and plots the values representing each allele on an allelic discrimination plot. In our *Acadl* SNP assays we assessed the presence of two possible alleles, 129 chimeric and B6, for *Acadl* SNP 1 and 2. Each axis of an allelic discrimination plot corresponds to the intensity (ΔR_n) of one of the two assayed alleles where the location of dots on the plot reflects the contribution of both allele intensities (ΔR_n) in a particular sample. In order to validate the ability of our *Acadl* SNP assays to distinguish the genotypes of mice harboring only B6 alleles (wild-type, B6.LCAD^{+/+}), only 129 chimeric alleles (B6.LCAD^{-/-} mice), or both B6 and 129 chimeric alleles (B6/129 heterozygous B6.LCAD^{+/-} mice), we isolated genomic DNA from B6.LCAD^{+/+}, B6.LCAD^{+/-}, and B6.LCAD^{-/-} mice and performed *Acadl* SNP genotyping analysis. Our results for *Acadl* SNP assay 2 indicated clear and reproducible discrimination of cytosine-containing B6 (red circles) and thymine-containing 129 (blue circles) allelic intensities (ΔR_n) between B6.LCAD^{+/+} and B6.LCAD^{-/-} mice (Figure 3, right), respectively. In addition, a third cluster representing B6.LCAD^{+/-} allelic intensities (ΔR_n) (green dots) showed an intermediate position on the allelic discrimination plot reflecting equal contributions from both B6 and 129 chimeric alleles in the genomic sample (Figure 3, right). Similar results were also seen with *Acadl* SNP 1 assay. Taken together, both *Acadl* SNP 1 and 2 assays serve as effective SNP genotyping methods for discriminating between B6 wild-type and targeted 129 chimeric *Acadl* alleles in LCAD-deficient mice.

As stated earlier, the *Acadl* targeted locus is a chimera of 129-based sequence originating from 129S6 ES cell genomic DNA and the 129X1 targeting vector. Specifically, the sequence containing exons 3 to 4, the region used for our *Acadl* SNP assay design, is duplicated within the targeted locus comprising of one exon 3 to 4 region originating from 129S6 ES cell DNA and one from the 129X1 targeting vector [1]. Given the duplicated nature of the targeted locus our initial validation of the SNP-based genotyping method does not indicate whether the allele intensity signal (ΔR_n) for the targeted chimeric *Acadl* allele originates from the 129S6 or the 129X1 sequence. In order to determine which 129-derived genetic background sequence harbors *Acadl* SNPs 1 and 2 detected from our initial analysis, genomic DNA from either 129S6 or 129X1 mice were subject to validation by the *Acadl* SNP 1 and 2 assays. Performing the *Acadl* SNP 2 assay with genomic DNA from both 129S6 and 129X1 strains showed a significantly higher detection of the 129 allele,

compared to the B6 allele in both 129S6 and 129X1 genomic samples, resulting in clustering of these 129-derived samples with B6.LCAD^{-/-} samples (blue circles) (Figure 3). Similar results were also seen with the *Acadl* SNP 1 assay. Furthermore, sequence analysis of the region spanning exons 3 and 4 of the *Acadl* locus in either 129X1 or 129S6 genomic DNA confirmed the presence of both 129 SNP variants as seen in genomic samples from LCAD-deficient mice. These results indicated that both 129X1 and 129S6 strain sequences contribute to the detection of 129 SNP variant alleles in our *Acadl* SNP analysis of the *Acadl* targeted locus from LCAD-deficient mice.

3.2 The *Acadl* SNP genotyping assays are reliable under conditions of low DNA purity

In order to validate the utility of the *Acadl* SNP assays with respect to DNA purity, we compared allelic detection of B6.LCAD^{+/+}, B6.LCAD^{+/-}, and B6.LCAD^{-/-} genomic DNA purified from liver (260/280 ratio~1.8-2.0; 50 ng/μl), crude genomic DNA from tail tips (260/280 ratio~1.50-1.69; 181-339.1 ng/μl), or diluted crude genomic DNA (50 ng/μl) (Figure 3a-c, respectively). Our results indicate that the lower purity crude tail genomic DNA compared to high purity purified genomic DNA showed lower individual allele intensity signals that were either at or just below the acceptable threshold of detection ($\Delta Rn=1$) as compared to the separate cluster of assay positive controls (Figure 3b, right) while purified controls gave the highest allelic intensities, well above the threshold of detection (Figure 3a, right). However, despite this more diffuse pattern on the allelic discrimination plot, the ratio of allelic intensities for the 129 allele (allele T) and the B6 allele (allele C) from crude DNA preps did not change significantly as compared to purified DNA samples (Figure 3a-b, left). The ratio of allelic intensities for the 129 allele (allele T) and the B6 allele (allele C) for diluted crude DNA preps were also similar to that of purified and crude preps (Figure 3c, left). However, dilution of the lower purity crude tail genomic DNA resulted in more cohesive clustering of sample reads on the allelic discrimination plot and individual allele signal intensities that were above the threshold of detection (Figure 3c, right). This trend more closely resembled sample clustering from purified genomic DNA (Figure 3a, right). Collectively, these results show that irrespective of the clustering pattern of the allelic discrimination plots associated with varying degrees of DNA purity, the 129 to B6 allele ratios showed consistent and expected values. However, crude DNA preps had a tendency to display individual allele signal intensities at or slightly below the acceptable threshold of detection. Similar results were seen with the *Acadl* SNP 1 assay.

3.3 The *Acadl* SNP genotyping assays are reliable under conditions of low DNA quantity

In addition, the effect of DNA quantity on assay performance was assessed using two-fold serial dilutions of liver purified DNA template (140 ng/μl to 2.18 ng/μl quantities; 260/280~1.8-2.0) from B6.LCAD^{+/+}, B6.LCAD^{+/-}, and B6.LCAD^{-/-} mice. SNP genotyping with *Acadl* SNP 2 assay using a range of DNA quantities showed little effect on the ratio of 129 to B6 allele intensities and modest decreases in signal intensities for each allele (Figure 4a). Individual signal intensities even at the lowest concentration (2.18 ng/μl) of template DNA were still well above the detectable threshold for the B6 allele in B6.LCAD^{+/+} and B6.LCAD^{+/-} genomic samples and for the 129 allele in B6.LCAD^{+/-} and B6.LCAD^{-/-} genomic samples (Figure 4b, c). This indicates that the *Acadl* SNP 2 assay is a robust genotyping approach under a broad range of template DNA quantities. Similar results were shown for *Acadl* SNP 1 assay. We also observed that detection of both B6 and 129 alleles in B6.LCAD^{-/-} and B6.LCAD^{+/+} samples, respectively, showed a small degree of residual signal (Figure 4b and 4c, respectively). This in part can be explained by a minimal signal produced in NTC (No Template Control) samples; $\Delta Rn = 0.0956 \pm 0.006$ for the 129 allele and $\Delta Rn = 0.105 \pm 0.005$ for the B6 allele. An obvious explanation for this phenomenon is due to trace amounts of non-specific DNA contamination. Another possible explanation may be due to a baseline level of spontaneous quencher-reporter dye dissociation from each

probe and further detection of this signal. However, the degree of signal arising in NTC samples accounts for only a small portion of the residual signal. The additional residual signal observed may result from the generation of cross-amplifying signals where a primer/probe set for a specific SNP allele will cross-amplify the non-specific allele, an inherent caveat associated with SNP-based genotyping. This likelihood is a result of non-specific annealing of probes that differ by only one nucleotide within SNP genotyping assays. Despite the residual signals imparted by the *Acadl* SNP genotyping assays, the individual allelic signal intensities for the respective dominant alleles are well above the threshold of detection while residual signal intensities from non-specific cross-amplification remain below this threshold of detection.

4. Discussion

The gene targeting method used to generate the LCAD-deficient mouse model led to an insertion duplication of exons 3 through 4 within the *Acadl* gene locus making traditional genotyping methods impractical. We describe here the development and validation of an effective SNP genotyping method for LCAD-deficient mice on a B6 background that reproducibly discriminates between heterozygous and homozygous genotypes by specifically detecting the 129-based sequence of the mutant *Acadl* locus or the B6-based sequence of the wild-type *Acadl* locus.

One of the limitations of this SNP genotyping assay is that its utility is only useful for distinguishing genotypes of LCAD-deficient mice on a B6 background. Since the assay is based on detection of 129 versus B6 alleles, genotyping would not be effective for LCAD-deficient mice on a congenic background that is of 129 origin or shows high conservation to 129 sequence at the *Acadl* locus (i.e. BTBR). Validation of the *Acadl* SNP genotyping assays for mouse strains on a mixed genetic background of 129 and B6, as is the case for the originally described LCAD-deficient mice [1, 6], would depend on the percentage of B6 genomic contribution. Due to lack of availability, the *Acadl* SNP genotyping methods have not yet been evaluated on the previous LCAD-deficient and corresponding LCAD wild-type models which were maintained as non-congenic mice at generation 2-3 on a mixed 129/B6 genetic background. The primary complication is possibly that the corresponding wild-type control may not harbor enough B6 sequence at the *Acadl* locus to effectively discriminate between targeted and wild-type *Acadl* alleles using this SNP assay. However, in additional studies, we have found that a minimum of two backcrosses of a NIH Swiss Black mouse strain, which shows detection of the 129 *Acadl* allele, to the C57BL/6 genetic background was sufficient to impart predominantly B6 *Acadl* allele detection using the *Acadl* SNP genotyping assays. This suggests that the *Acadl* SNP genotyping assays may be valid even for the original LCAD-deficient mouse model on generation 2-3 of the mixed 129/B6 genetic background.

In cases where standard endpoint PCR have been impractical, other alternatives have been used to distinguish genotypes which include copy number analysis of inserted neomycin resistance genes using quantitative PCR approaches. These methods have been reported to distinguish between heterozygous and homozygous mutant mice [7]. This approach would be ideal especially for LCAD-deficient mice on a 129 (or related) genetic background where the *Acadl* SNP assays would fail to discriminate mutant and wild-type *Acadl* alleles. However, genotyping mouse models containing disruptions at multiple gene loci all of which contain a neomycin-resistance cassette limit the utility of the QPCR method. In addition, this analysis relies on a single cycle threshold difference, which may not have the sensitivity and reliability necessary for routine genotyping and consistent maintenance of mutant mouse strains. In cases where the targeted *Acadl* allele contains discriminating SNPs compared to the corresponding wild-type genetic background, our studies suggest that the

Acadl SNP genotyping approach has greater sensitivity due to the specificity for targeted and wild-type alleles. In addition, the *Acadl* SNP assay is highly reproducible and reliable under a wide range of DNA purities and quantities adding to its superiority over the QPCR approach.

This study is a demonstration of alternate methods in genotyping that may have broad application for many other mouse strains where traditional methods are also not practical. As an example, medium chain acyl-CoA dehydrogenase (MCAD) deficient mice were also generated with a targeting method that resulted in an insertion duplication of exons 8 through 10 [8]. As a result, an earlier genotyping method (Shawn E. McCandless, data not published) used for this strain involved a multi-step process of PCR reactions followed by a restriction digest for differentiation of PCR products that are 147 and 235 base pairs in size to distinguish the B6 allele from the 129 allele. Efficiency and sensitivity of genotyping in MCAD-deficient mice on a B6 genetic background would be greatly improved with a SNP genotyping method as described for the B6.LCAD mutant line. In addition to mouse strains that are targeted at a single locus, studies evaluating phenotypes as a result of gene-gene interactions employ mouse strains that have been cross-bred to contain multiple gene loci that have undergone targeted disruption or replacement. Generation and maintenance of these novel multi-gene mutant models requires genotyping methods that are highly gene-locus specific, an essential feature where in many cases traditional PCR methods and especially simple neomycin-resistance cassette detection are inadequate. Taken together, the *Acadl* SNP assay reflects a viable approach that can serve to streamline rapid and specific genotyping for mouse strains that previously involved impractical techniques.

Acknowledgments

We thank Caron Stonebrook for mouse colony assistance for this project and Shawn E. McCandless for helpful discussion. This work was supported by National Institutes of Health (NIH) grant R01-RR02599, and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Center for Research Resources, or the NIH.

Abbreviations

Acadl	acyl-coenzyme A dehydrogenase, long-chain
LCAD	long-chain acyl-CoA dehydrogenase

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Highlights

- To date, genotyping of *Acadl* targeted alleles in heterozygous and homozygous LCAD-deficient mice has been impractical.
- We have developed two *Acadl* SNP genotyping assays that distinguish between heterozygous and homozygous *Acadl* targeted alleles.
- The *Acadl* SNP genotyping assays are effective at both low DNA purity and quantity.

Targeted 129X1-129S6 *Acadl* Locus Wild-type *Acadl* Locus

- 129S6
- 129X1
- PGKneobpA/pGEM-11zf(+) plasmid sequence
- C57BL/6

Figure 1. Chimeric Strain-Specific Sequence of the Targeted *Acadl* locus in LCAD-Deficient Mice

(Top) Targeted 129X1-129S6 *Acadl* locus. *Acadl* locus sequence in LCAD-deficient mice consists of targeted vector comprising 129X1-129S6 chimeric components. Sequence distal to the *Acadl* locus is of C57BL/6 origin. (Bottom) Wild-type *Acadl* locus. Wild-type C57BL/6 *Acadl* locus sequence and distal sequence. (Legend for sequence origin; no color (129S6); blue (129X1); grey (C57BL/6); red (plasmid sequence PGKneobpA/pGEM-11zf(+)). S1 and S2 refer to *Acadl* SNP 1 and *Acadl* SNP 2 locations, respectively, in the targeted *Acadl* (top) locus and wild-type C57BL/6 locus (bottom).

<i>Acadl</i> SNP 1 Assay	GGAGGATTGCCAAGAGCTCAAGGCTAGCTTAGGTTTACATAGT <u>[T/C]AGTTCTAGG</u> <u>CCA</u> GCCAGAGTGATATAGCAAGAAGCTGCTTTCAAACAAA
<i>Acadl</i> SNP 2 Assay	GTGACTGCGGGTACATTAGAGTGTCA <u>CCAAGGTGCCCTC</u> <u>[T/C]ATAA</u> TAGGACAATT GCAGTGTCAAGGTAAGA

Figure 2. Amplicon sequences for *Acadl* SNP assays 1 and 2

Lower case letters signify forward and reverse primer targets for amplification. Uppercase letters denote the remaining intervening sequence of the amplicon and underlined sequences represent allele-specific probe target sequence. Nucleotides within brackets indicate allele-specific SNP nucleotide [allele 1/allele 2] where allele 1 corresponds to the B6 allele and allele 2 corresponds to 129-derived alleles.

Figure 3. Acadl SNP 2 Assay Validation

B6.LCAD^{+/+}, B6.LCAD^{+/-}, B6.LCAD^{-/-}, and 129S6 genomic DNA samples were (A) purified from liver [There were no statistical differences among groups homozygous for the 129 allele (B6.LCAD^{-/-}, 129S6, and 129X1), however comparison between (B6.LCAD^{-/-}, 129S6, and 129X1), (B6.LCAD^{+/-}), and (B6.LCAD^{+/+}) groups were significant, $p < 0.001$], (B) crude preparations from mouse tail clippings [Statistical differences were seen between (B6.LCAD^{-/-}, 129S6, and 129X1), (B6.LCAD^{+/-}), and (B6.LCAD^{+/+}) groups, $p < 0.001$], or (C) crude genomic DNA from mouse tail clippings diluted to 50ng/ μ l using a 1:1 solution A/B mix [Statistical differences were seen between (B6.LCAD^{-/-}, 129S6, and 129X1), (B6.LCAD^{+/-}), and (B6.LCAD^{+/+}) groups, $p < 0.001$. Differences were also seen between

B6.LCAD^{-/-} and 129X1, $p < 0.01$, and between B6.LCAD^{-/-} and 129S6, $p < 0.05$]. (Left) Ratio of the signal intensity (ΔR_n) of the 129 and B6 alleles are shown for the indicated samples subject to *Acad*/SNP 2 assay. (Right) Allelic discrimination plots showing signal intensities (ΔR_n) for the B6 versus the 129 allele using *Acad*/SNP 2 assay. Each dot represents the genotype of an individual sample (blue circles are homozygous for the 129 allele (LCAD^{-/-}), green circles are heterozygous and contain both B6 and 129 alleles (LCAD^{+/-}), and red circles are homozygous for the B6 allele (LCAD^{+/+}, wild-type). Data are representative of 5 mice per genotype (B6.LCAD^{+/+}, B6.LCAD^{+/-}, B6.LCAD^{-/-}, and 129S6 strains) where both liver and tail clippings (A-C) were used internally from the same respective mice. Genomic DNA from the 129X1 strain was purified from spleen (Jackson Laboratory) and corresponding circles represent technical replicates.

Figure 4. Affect of DNA Quantity on *Acadl* Assay Allelic Detection

Affect of DNA quantity on the (A) ratio of signal intensity of the 129 and B6 alleles, (B) the signal intensity (ΔRn) of the B6 allele alone, and (C) the signal intensity (ΔRn) of the 129 allele alone for purified genomic DNA isolated from B6.LCAD^{+/+}, B6.LCAD^{+/-}, and B6.LCAD^{-/-} mice subject to *Acadl* SNP 2 assay. Two-fold dilutions of genomic DNA quantity were used (140 ng/ μ l-2.18 ng/ μ l) in addition to no template controls (NTC). Statistical differences were seen between all three genotypes at each dilution, $p < 0.001$. Data are representative of 5 different mouse genomic samples for each genotype and each sample was assayed with 3 technical replicates.