A rapid, microplate SNP genotype assay for the leptin\textsuperscript{ob} allele

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Abstract A nonsense mutation in the mouse leptin gene causes genetic obesity. As a result of extensive research in the field of obesity, the use of leptin\textsuperscript{ob} mice is widespread. This mutation renders mice sterile, creating the need to breed heterozygous mice. For this reason, leptin\textsuperscript{ob} genotyping is necessary. To date, gel-based assays have been used for genotyping.\textsuperscript{a} Using the Invader Plus\textsuperscript{®} assay for single nucleotide polymorphism (SNP) detection, we have developed a gel-free microplate SNP assay for genotyping leptin\textsuperscript{wt} and leptin\textsuperscript{ob} alleles.—Oler, A. T. and A. D. Attie. A rapid, microplate SNP genotype assay for the leptin\textsuperscript{ob} allele. J. Lipid Res. 2008, 49: 1126–1129.

Supplementary key words obesity • obese • single nucleotide polymorphism

Leptin is a 167 amino acid peptide hormone produced primarily in adipose tissue. It binds to a cell surface receptor, where it elicits a variety of physiological responses. It is a potent suppressor of appetite when it signals in the hypothalamus.

Leptin was discovered by positional cloning of a locus underlying the morbid obesity of the obese mutant mouse (1), a spontaneously occurring mutant that was discovered at the Jackson Laboratory in 1950 (2). This mouse has a nonsense mutation in the leptin gene, leading to a null allele. The obese mouse is widely used in studies of obesity and diabetes. In fact, it is the most widely used animal model of obesity in the entire Jackson Laboratory collection.

The pioneering studies of Coleman and others (3–5) showed that certain mouse strains become diabetic when made obese through the introgression of the leptin\textsuperscript{ob} allele. These studies have inspired numerous investigators to combine the leptin\textsuperscript{ob} allele with other mutations to gain new insights into the molecular, genetic, and physiological processes underlying resistance and susceptibility to obesity-induced type 2 diabetes (6, 7). This strain is also used extensively in nutritional and pharmacological studies aimed at the treatment of obesity and diabetes.

The leptin\textsuperscript{ob} allele has a C→T mutation that changes an arginine codon to a stop codon at position 105 (1). At present, the wild-type and mutant alleles are most commonly genotyped by restriction endonuclease digestion of a PCR-amplified sample of genomic DNA, followed by agarose gel electrophoresis (8, 9). This procedure takes \( \sim 8 \) h to perform, of which \( \sim 1 \) h involves hands-on time. Because of the need for gel electrophoresis, the length of this procedure increases considerably with the number of samples. An alternative method, single-stranded conformational polymorphism analysis, also involves gel electrophoresis and is quite labor-intensive (10).

We have developed a genotyping assay for leptin\textsuperscript{wt} and leptin\textsuperscript{ob} alleles that eliminates the need for gel electrophoresis, using the Invader Plus\textsuperscript{®} assay for single nucleotide polymorphism (SNP) detection (11). The presence of the targeted SNP yields a fluorescent signal that can be quantitated in a standard plate reader. The entire process takes 0.5 h hands-on and a total of 2.5 h and can be scaled to hundreds of samples with minimal additional time.

MATERIALS AND METHODS

Genomic DNA extraction

One centimeter tail clips from mice were digested overnight at 55°C in 300 \( \mu \)l of tail buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 \( \mu \)g/\( \mu \)l proteinase K). The digests were brought to room temperature, and 100 \( \mu \)l of Gentra Puregene Protein Precipitation Solution (Qiagen) was added. Samples were vortexed for 5 s and incubated on ice for 20 min. Samples were spun in a microcentrifuge at 4°C for 5 min at maximum speed. Supernatants were poured into a new tube containing 300 \( \mu \)l of isopropanol. Samples were inverted 20 times, or until a string of precipitated DNA was visible. Samples were spun again at room temperature for 2 min. Supernatants were poured off, and 300 \( \mu \)l of 70% ethanol was added. Tubes were gently inverted once and spun again at room tem-
perature for 2 min at maximum speed. Again, the supernatants were poured off, with care to not lose the DNA pellets. Samples were spun at room temperature for 2 min at maximum speed. The supernatants were removed with a pipette tip, and the samples were air-dried for 30 min at room temperature. After 30 min, 200 μl of 1X Tris-EDTA was added to each pellet, and samples were stored overnight at 4°C before use. In preparation for the Invader Plus® assay, genomic DNA samples were first diluted on a 96-well plate: 4 μl (~200 ng to 1 μg of DNA) of genomic DNA into 120 μl of water (final volume, 30X). Samples were carefully mixed by pipetting to avoiding cross-contamination of wells.

DNA sequences

All sequences are shown 5’ to 3’. The target DNA sequences for leptin (NM_008493) surrounding the SNP are GATGGAGGAGGTCTCAAGAGATTCTCCAGGTCATTGGT for leptinwt and GATGGAGGAGGTCTCAAGAGATTCTCCAGGTCATTGGT for leptinob, with the SNP nucleotide in lowercase. Primer sequences used to amplify this target region are Forward (CAGGTCCTCCACCAGCCTGCCTTCC) and Reverse (CAGGGAGCAGCTCTTGGAGAAGGCC). The Invader® oligonucleotide sequence is AATGACCTGGAGAATCTCA. The Invader® probe sequences are as follows: for leptinwt, gacgcggagCGAGACCTCCT; for leptinob, cgccgaggTGAGACCTCCT, with the 5’ flap region in lowercase.

Invader Plus® Assay

A Leptin probe/primer mix was made with 2.0 μM each of Forward and Reverse primers, 2.5 μM each of leptinwt and leptinob probes, and 0.25 μM Invader® oligonucleotide. An Invader Plus® core kit containing fluorescence resonant energy transfer (FRET) mix, Invader Plus® MgCl2, Cleavase® VIII, and no-target control was purchased from Third Wave Technologies, Inc. For the Invader Plus® assay, hard-shell skirted 96-well plates with conical bottoms were used (Bio-Rad). To each well, 7.7 μl of cocktail was added. This cocktail contains 3 μl of Leptin probe/primer mix, 3 μl of FRET mix, 0.375 μl of Invader Plus® MgCl2, 0.3 μl of 1 mM deoxynucleoside triphosphates, 1 μl of Cleavase® VIII, and 0.06 μl of Hot Start Taq DNA polymerase (Fermentas) per sample. Then, 7.3 μl of each genomic DNA sample that was diluted and mixed on the 96-well plate was added to each well. The 15 μl cocktail/genomic DNA mixture was overlaid with 15 μl of mineral oil. To each plate, one sample each of leptin1/1, leptin1/2, leptin2/2.

![Invader Plus® assay for leptin single nucleotide polymorphism (SNP) genotyping. After amplification of the target region for the leptinwt allele (A) and for the leptinob allele (B), the Invader® oligonucleotide anneals to the target DNA. The respective probes anneal to the target DNA. This creates a mismatch in a DNA triplex (CAG and TAA for leptinwt and leptinob, respectively), which allows cleavage by Cleavase®. The 5’ flap of the probe is released and anneals to its specific fluorescence resonant energy transfer (FRET) cassette. This creates a new DNA triplex (TCA and TTA for leptinwt and leptinob, respectively), which is also a substrate for cleavage. This cleavage reaction removes the quench from the fluorophore, resulting in a fluorescent signal. Bases in lowercase represent DNA sequence not complementary to the leptin target DNA, and bases in uppercase not involved in the DNA triplexes are for the orientation of DNA binding. Nn and nx represent the number of bases in between. FAM, fluorescein.](image-url)
leptin\textsuperscript{wt}, and leptin\textsuperscript{ob/+} known genomic DNA and three no-target controls were added. The plate was run in a thermal cycler with the following protocol: 2 cycles of 95°C for 2 min, 65°C for 1 min, and 72°C for 1 min; 33 cycles of 95°C for 15 s, 65°C for 1 min, and 72°C for 30 s; and one cycle of 99°C for 10 min, 50°C for 15 min, and 10°C hold.

The plate was read on a Tecan Ultra 384 fluorescent plate reader with multilabeling capability. To detect the leptin\textsuperscript{wt} allele, a fluorescein (FAM) excitation/emission wavelength of 485/535 nm was used, and for the leptin\textsuperscript{ob} allele, a Red excitation/emission wavelength of 570/620 nm was used. Data are represented as relative fluorescence units (RFU). A z optimization was initially done to determine the best z position and mirror setup for each excitation/emission wavelength.

Calculations

Data were analyzed according to the following calculation:

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\text{FAM/Red ratio} = \frac{\text{RFU}_{\text{FAM sample}} - 1}{\text{RFU}_{\text{FAM average target control}}} - \frac{\text{RFU}_{\text{Red sample}} - 1}{\text{RFU}_{\text{Red average target control}}}
\]

If the FAM/Red ratio is <0.5, the genotype is leptin\textsuperscript{+/+}. If the ratio is >0.5 but <15, the genotype is leptin\textsuperscript{ob/+}. If the ratio is >15, the genotype is leptin\textsuperscript{ob/ob}.

RESULTS

We developed two genotyping assays, for the leptin\textsuperscript{wt} and the leptin\textsuperscript{ob} alleles. Two primers were designed to amplify the target region containing the SNP. One Invader\textsuperscript{®} oligonucleotide was made that is complementary to the target region 5' of the SNP while containing a mismatch at the terminal 3' end. Two Invader\textsuperscript{®} probe oligonucleotides were made, one containing the leptin\textsuperscript{wt} nucleotide and one containing the leptin\textsuperscript{ob} nucleotide. These probe oligonucleotides are complementary to the target region at the SNP nucleotide and 3' of the SNP, but they are not complementary 5' of the SNP. Instead, this 5' flap is complementary to a specific FRET cassette. During the 50°C incubation at the end of thermal cycling, the Invader\textsuperscript{®} oligonucleotide and the probe oligonucleotide anneal to their complementary target DNA, creating a mismatch triplex of DNA at the SNP nucleotide (Fig. 1). This DNA triplex is recognized and cleaved by Cleavase\textsuperscript{®} 3' of the SNP nucleotide, releasing the 5' flap of the probe oligonucleotide containing the SNP nucleotide at the terminal 3' end. Allele-specific 5' flaps will be released for each leptin\textsuperscript{wt} or leptin\textsuperscript{ob} allele. These 5' flaps will anneal to their specific FRET cassettes, creating another mismatch triplex of DNA, creating a substrate for Cleavase\textsuperscript{®}. Cleavage releases the quencher from the fluorophore. Applying thermal cycling, the reaction and signal are amplified. We used a FRET cassette with Red fluorophore for the C nucleotide (leptin\textsuperscript{wt} allele; Fig. 1A) and a FRET cassette with FAM fluorophore for the T nucleotide (leptin\textsuperscript{ob} allele; Fig. 1B). The assay measures all three possible genotypes: Red signal only (leptin\textsuperscript{+/+}), FAM signal only (leptin\textsuperscript{ob/+}), and both Red and FAM signals (leptin\textsuperscript{ob/ob}).

We obtained leptin\textsuperscript{+/+}, leptin\textsuperscript{ob/ob}, and leptin\textsuperscript{ob/+} mice (n = 7 in each group) from our breeding colony. They were genotyped using single-stranded conformation polymorphism analysis. The samples were then subjected to the Invader Plus\textsuperscript{®} assay, and the fluorescence was detected on a plate reader (Fig. 2). The FAM RFU corresponding to the leptin\textsuperscript{wt} allele are shown in Fig. 2A. The Red RFU corresponding to the leptin\textsuperscript{ob} allele are shown in Fig. 2B. The FAM/Red ratios corresponding to the genotype are shown in Fig. 2C. The mean FAM/Red ratios for the leptin\textsuperscript{+/+}, leptin\textsuperscript{ob/+}, and leptin\textsuperscript{ob/ob} mice were 0.07 ± 0.02, 1.4 ± 0.2, and 41 ± 10 (P < 0.0001 by one-way ANOVA). Ratio thresholds were determined empirically by evaluat-

![Fig. 2. Genotyping of leptin alleles. FAM relative fluorescence units (RFU) are shown in A and Red RFU are shown in B. The FAM/Red ratio is shown in C. In C, the thresholds used to distinguish the ob/ob from the ob/+ and the ob/+ from the +/+ are shown as dashed lines. All values are for seven different mice tested on seven individual assays, with a line indicating the mean. For the FAM/Red ratio in C, P < 0.0001 by one-way ANOVA.](image-url)
ing the lowest ratio for leptin$^{ob/ob}$ and the lowest ratio for leptin$^{ob/+}$ (Fig. 2C). There were no ambiguous results within the samples tested. These ratio thresholds can change if there is a differential decrease in fluorescence signal strength. This can occur with time and light exposure. An Excel template was used for ease in the analysis of genotypes. This template and a detailed protocol are available in the supplementary material.

**DISCUSSION**

We have developed and validated a gel-free, microplate fluorescence-based genotyping assay for the wild-type and obese alleles of leptin. This method is extremely reliable, is able to identify heterozygotes unambiguously, and is quite rapid. The method is amenable to automation and can easily be scaled to perform large numbers of assays in parallel. When this method was used to test our unknown samples, the failure rate was <1%. A failure will not result in a genotyping error because it results in the absence of both the FAM and Red signals. A single plate can handle 90 samples plus controls. Because of the extensive use of leptin$^{ob}$ mice, this method is an invaluable tool.

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