SUPPLEMENTAL APPENDIX

Methods

Cell culture
Primary porcine fibroblasts were established from ear biopsies of newborn Yucatan minipigs. Following isolation, the porcine fibroblasts were cultured at 37°C in DMEM (Lonza Biowhittaker, Walkersville, MD, USA, cat. no. BE12-604F) supplemented with 60 µg/mL penicillin, 100 µg/mL streptomycin, 292 µg/mL glutamine, and 15% heat-inactivated fetal calf serum. For gene targeting experiments, the cell culture medium was additionally supplemented with basic fibroblast growth factor (bFGF, 5 ng/mL).

rAAV/*APOE* KO virus packaging
Packaging of the rAAV/*APOE* KO virus (serotype 1) was performed by the Vector Core Facility at the Gene Therapy Center, Chapel Hill, NC, USA. Briefly, the pAAV/*APOE* KO plasmid was co-transfected into 293 cells with pXX680 and pHelper plasmids. Twenty-four hours post-transfection, medium from the transfected cells was removed and replaced with fresh medium. Virus was harvested 48-72 hours post-transfection and purified using gradient purification. For a detailed protocol please refer to the protocol published by Grieger et al (1).

Transduction and selection
Primary male fibroblasts isolated from newborn Yucatan minipigs (1.5×10^6) were seeded onto a gelatin coated 10 cm cell culture dish one day before transduction. Prior to transduction, the culture medium was changed and supplemented with bFGF (5 ng/µL). The rAAV/*APOE* KO virus particles (5×10^{11} particles) were incubated with the cells for 24 hours. The cells were subsequently trypsinized and 6/10 of the total volume of the cell suspension was seeded into thirty gelatin-coated 96-well plates resulting in approximately 300 cells per well. Three days post transduction (2 days after splitting into 96-well plates), cells were selected with G418 (1 mg/mL) for two weeks with the medium being changed every 3 to 4 days. Following selection, individual G418-resistant cell clones were trypsinized, and 1/3 of the resulting cell suspension from each cell clone was transferred to one well of a 96-well PCR plate for PCR screening, 1/3 was cultured in one well of a gelatin-coated 96-well cell culture plate for Southern blot analysis, and 1/3 was cultured in one well of a gelatin-coated 96-well plate for freezing at early passages and subsequent usage as nuclear donor cells for SCNT.

PCR screening of gene targeted donor cells
G418-resistant cell clones in 96-well PCR plates were harvested, centrifuged, and re-suspended in 25 µL lysis buffer (50 mmol KCl, 1.5 mmol $MgCl_2$, 10 mmol Tris-Cl, pH 8.5, 0.5% Nonidet P40, 0.5% Tween, 400 µg/mL Proteinase K) (2). The cells were lysed (65°C for 30 min, 95°C for 10 min) and 1 µL lysate was used for PCR screening. PCR conditions were as follows: (1) *APOE* 5' targeting screening: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 20 s, 58°C for 30 s, and 68°C for 2 min followed by 1 cycle of 68°C for 7 min; (2) *APOE* 3' targeting screening: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 20 s, 62°C for 30 s, and 68°C for 1.5 min followed by 1 cycle of 68°C for 7 min. Primers for screening of the *APOE* targeting events are listed in Table S1. Based upon PCR, a gene targeting frequency (targeted cell clones/G418-resistant cell clones) of 5.4% was achieved.

Southern blot analysis
Genomic DNA (15 µg), isolated from cultured gene targeted fibroblasts or ear biopsies from newborn piglets, was digested with *XmnI* restriction enzyme overnight, followed by gel electrophoresis on a 0.7% agarose gel and vacuum blotting onto a nitrocellulose membrane. A neo^r-specific probe (detecting the neo^r-cassette in the targeting vector) and an *APOE*-specific probe (detecting the porcine *APOE* gene upstream of the targeting region) were both generated by PCR and subjected to random labeling using a Prime-It II Random Primer Labeling Kit according to the manufacturer's instructions. Pre-hybridization and

hybridization were carried out at 42°C, and all washing procedures were performed at 53°C. Primers for generating the *APOE* and *neo*^{r} probes, respectively, are listed in Table S1.

Genotyping

Genotyping of the *APOE*$^{+/+}$, *APOE*$^{+/-}$, and *APOE*$^{-/-}$ piglets was performed by standard PCR. The primers used for genotyping are listed in Table S1.

Karyotyping

One of the three cloned *APOE*$^{+/-}$ founders was karyotyped using Q-banding and the software Quips Karyotyper version 3.1 (Vysis, Downers Grove, IL). Briefly, fibroblasts were isolated from the cloned piglet and cultured in slideflasks. The cells were arrested in metaphase by addition of colcemid and subjected to fixation and staining with DAPI prior to analysis.

Reverse transcriptase PCR

Total RNA was isolated from liver tissues using an RNeasy kit (Qiagen, Hilden, Germany, cat no. 74104). First strand cDNA was synthesized with 1 µg total RNA using a RevertAid First Strand cDNA Synthesis kit (Fisher Scientific, Hampton, NH, USA, cat. no. K1622) following the manufacturer´s instructions. One µL of cDNA was used as template for the *APOE* RT-PCR using the following PCR program: 94°C for 3 min for 1 cycle, 35 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 20 s followed by 1 cycle of 72°C for 7 min. The porcine β-actin gene *ACTB* was used as control for successful cDNA synthesis. For the *ACTB* RT-PCR, 1 µL cDNA was used as template using the following PCR program: 94°C for 3 min for 1 cycle, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s followed by 1 cycle of 72°C for 7 min. Primers used for the RT-PCR are listed in Table S1. Sequencing of the resulting RT-PCR products confirmed normal *APOE* expression in *APOE*$^{+/+}$ and *APOE*$^{+/-}$ pigs in contrast to *APOE*$^{-/-}$ pigs in which *APOE* expression was absent.

Western blot analysis

Plasma samples were diluted 1:25 in reducing sample buffer, boiled for 6-7 minutes, separated by SDS-PAGE in a 3-8% NuPAGE Novex Tris-acetat gel (Thermo Fisher Scientific, Waltham, MA, USA), and subsequently blotted onto an Immobilon transfer membrane (Millipore, Billerica, MA, USA). Blots were stained with rabbit monoclonal anti-human apolipoprotein B antibody (1:5000, ab139401, Abcam, Cambridge, United Kingdom) followed by HRP-conjugated goat anti-rabbit antibodies (1:4000, p0448, DAKO Denmark A/S, Glostrup, Denmark). Chemiluminescence detection was achieved with ECL Western Blotting Detection Reagents (GE Healthcare Europe GmbH, Brøndby, Denmark, cat. no. RPN2106) and analysis was performed using a ChemiDoc imager.

Cholesterol analysis

Blood plasma was analyzed for total cholesterol and triglycerides by standard procedures (Siemens® Clinical Methods) using an autoanalyzer ADVIA 1800 ® Chemistry System (Siemens Corporation, Tarrytown, NY, USA). *Intra-* and *inter* assay precision were in all instances below 3 and 4 CV%, respectively.
Size-exclusion chromatography of plasma pools (250 µL) was performed at room temperature using PBS-EDTA and Superose 6 fast protein liquid chromatography (FPLC) columns (GE Healthcare Europe GmbH, Brøndby, Denmark). The flow rate was 0.4 mL/min. Fractions of approximately 250 µL were collected in 96-well plates and used for cholesterol measurements. Cholesterol was measured in each fraction (100 µL) with an enzymatic cholesterol reagent (CHOD-PAP, Roche Diagnostics, Indianapolis, IN, USA).
Cholesterol carried in IDL/VLDL, LDL and HDL was determined after density gradient ultracentrifugation. Briefly, 1.2 mL plasma was adjusted to 1.019 g/mL with 5 mL of a NaCl solution (1.0221 g/mL) and 1.2 mL plasma to 1.063 g/mL with 5 mL of a NaBr solution (1.0767 g/mL). The density solutions were centrifuged in an ultracentrifuge (Optima™ XPN-90, Beckman Coulter, Ramcon A/S, Birkerød, Denmark) at 50,000 rpm overnight at 4°C. Afterwards, the tubes were sliced on the middle and the top and bottom fractions were collected for cholesterol measurements. The top of the 1.019 g/mL density tube contained the VLDL fraction while the bottom contained the LDL and HDL fractions. With respect to the 1.063 g/mL density tube, the top contained the VLDL and LDL fractions. The bottom contained the HDL fraction. Cholesterol was measured

on density fractions in duplicate with an enzymatic cholesterol reagent (CHOD-PAP, Roche Diagnostics Indianapolis, IN, USA) and the cholesterol values of lipoproteins calculated.

C-reactive protein and haptoglobin

Haptoglobin concentrations were determined by a sandwich ELISA using an in-house mouse anti-porcine haptogobin monoclonal antibody in the coating layer and biotinylated commercial rabbit anti-human haptoglobin (DAKO Glostrup, Denmark, cat. no. A0030) as the detection antibody as described previously (3) with a detection limit of 130 mg/L.

C-reactive protein (CRP) concentrations were analysed by a sandwich ELISA using dendrimer-coupled cytidine diphosphocholine in the coating layer as described previously (4), and polyclonal rabbit anti-human antibodies with cross-reactivity towards porcine CRP (5) followed by peroxidase-conjugated goat anti-rabbit antibody for detection (both antibodies from DAKO, Glostrup, Denmark). Pooled pig serum calibrated against a human CRP calibrator (DAKO, Glostrup, Denmark, cat. no. A0073) was used as standard. The detection limit was 0.35 mg/L (human equivalents).

Development of plates was done with a tetramethylbenzidine (TMB) peroxide color substrate from Kem-En-Tec (Taastrup, Denmark), following the manufacturer’s instructions, reading optical densities of wells at 490 nm subtracting unspecific coloration at 650 nm using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples including standards were determined in duplicate. Sample values were calculated from the curve fitted to the readings of the standard (using Ascent software v. 2.6, Thermo Scientific).

References

1. Grieger JC, Choi VW, Samulski RJ. Production and characterization of adeno-associated viral vectors. Nat Protoc 2006;1:1412-1428.

2. McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 2000;405:1066-1069.

3. Sorensen NS, Tegtmeier C, Andresen LO, et al. The porcine acute phase protein response to acute clinical and subclinical experimental infection with Streptococcus suis. Vet Immunol Immunopathol 2006;113:157-168.

4. Heegaard PM, Klausen J, Nielsen JP, et al. The porcine acute phase response to infection with Actinobacillus pleuropneumoniae. Haptoglobin, C-reactive protein, major acute phase protein and serum amyloid A protein are sensitive indicators of infection. Comp Biochem Physiol B Biochem Mol Biol 1998;119:365-373.

5. Heegaard PMH, Pedersen HG, Jensen AL, Boas U. A robust quantitative solid phase immunoassay for the acute phase protein C-reactive protein (CRP) based on cytidine 5'-diphosphocholine coupled dendrimers. J Immunol Methods 2009;343:112-118.

Supplemental Figures

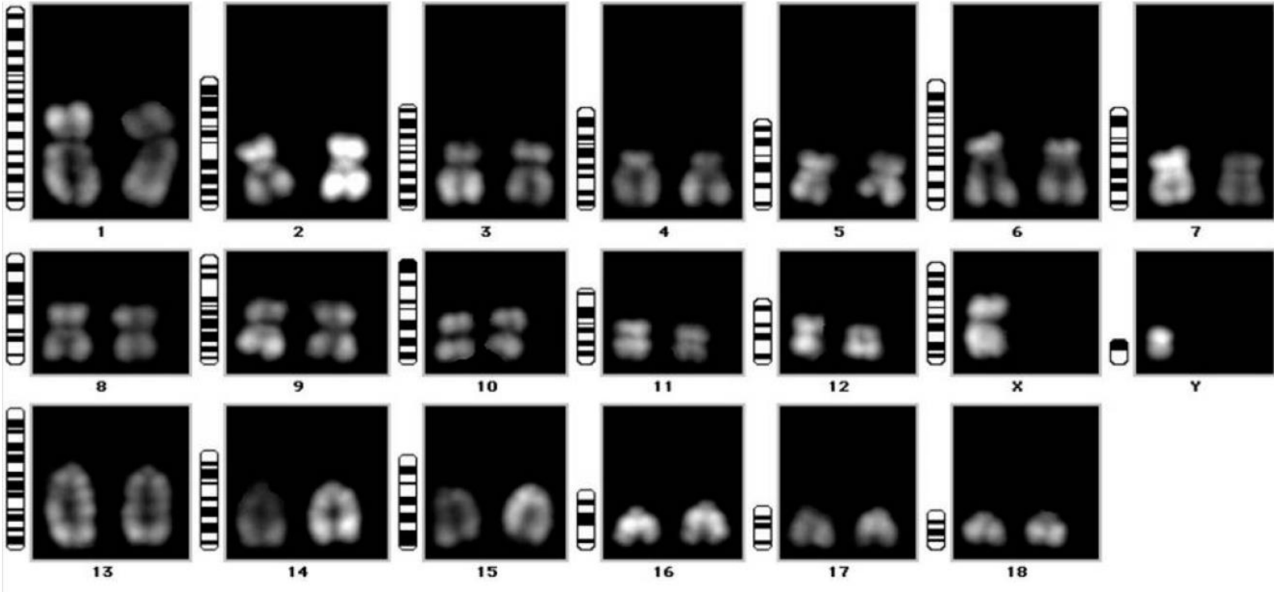

Figure S1. Karyotyping of cloned male founder pig. Fibroblasts isolated from the cloned *APOE*$^{+/-}$ pig were cultured and the cells were arrested in metaphase, fixed, and stained with DAPI prior to karyotyping using Q-banding and the software Quips Karyotyper version 3.1 (Vysis).

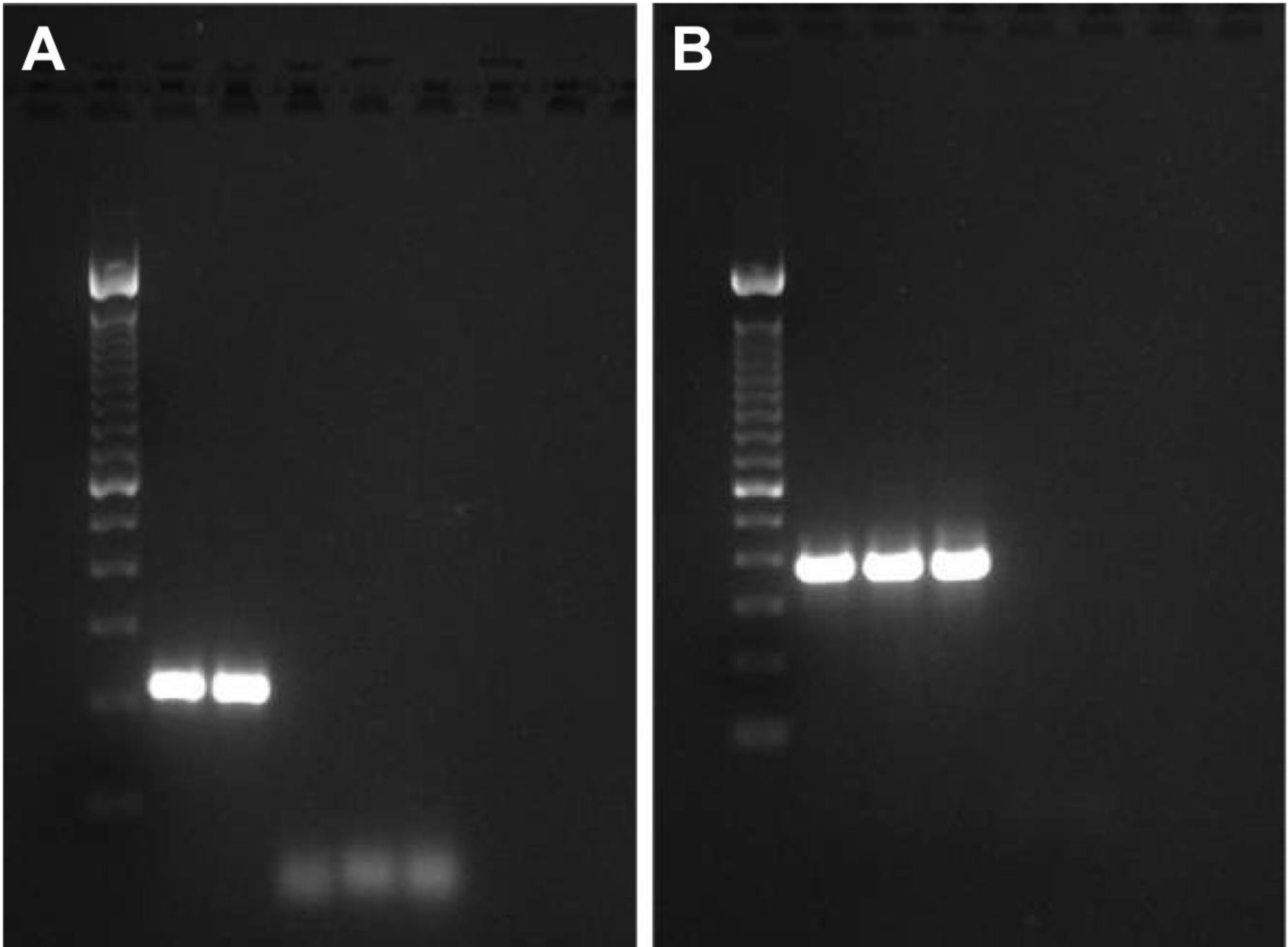

Figure S2. Reverse transcriptase PCR. Raw data used for presentation of Figure 1C. Total RNA was isolated from liver tissues and used for first strand cDNA synthesis. **A.** RT-PCR was performed as described in the methods section using *APOE*-specific primers amplifying the *APOE* exon 2-3 region. **B.** RT-PCR using porcine β-actin gene (*ACTB)*-specific primers amplifying *ACTB* as reference for successful cDNA synthesis from all samples. A 100 bp DNA ladder was used as DNA marker in both **A** and **B**.

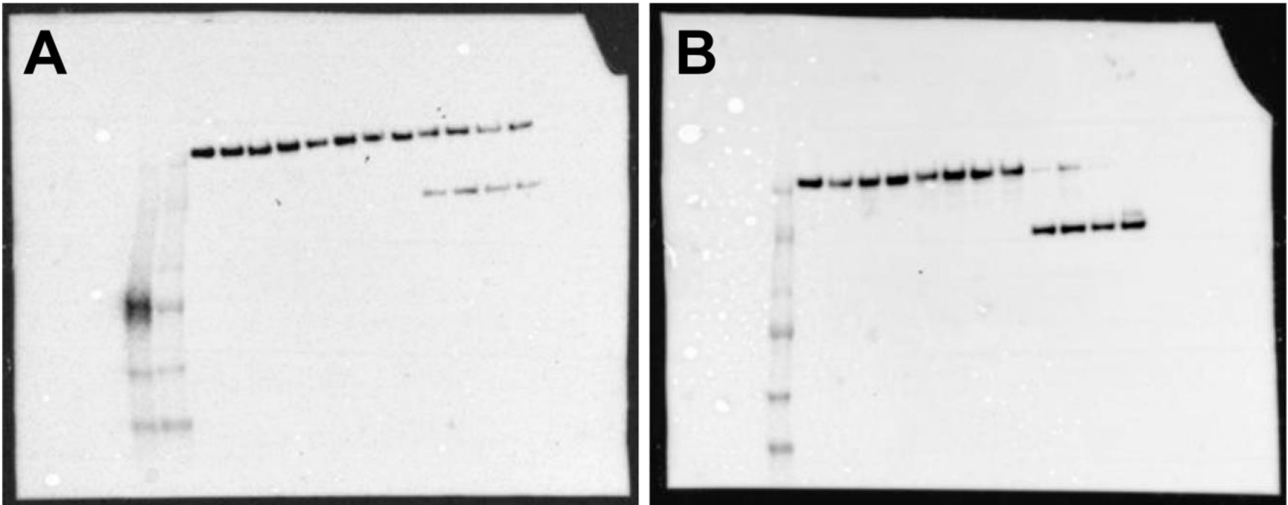

Figure S3. Western blot on plasma from *APOE*$^{+/+}$, *APOE*$^{+/-}$, and *APOE*$^{-/-}$ pigs showing ApoB-48 accumulation in *APOE*$^{-/-}$ pigs. Raw data used for presentation of Figure 2C. **A.** Plasma from pigs on low-fat diet, 8 weeks of age. **B.** Plasma from pigs after 8 weeks on HFHC diet, 16 weeks of age. Analyses were performed on individual plasma samples from four representative animals in each group using an anti-human anti-apolipoprotein B antibody (abcam #ab139401).

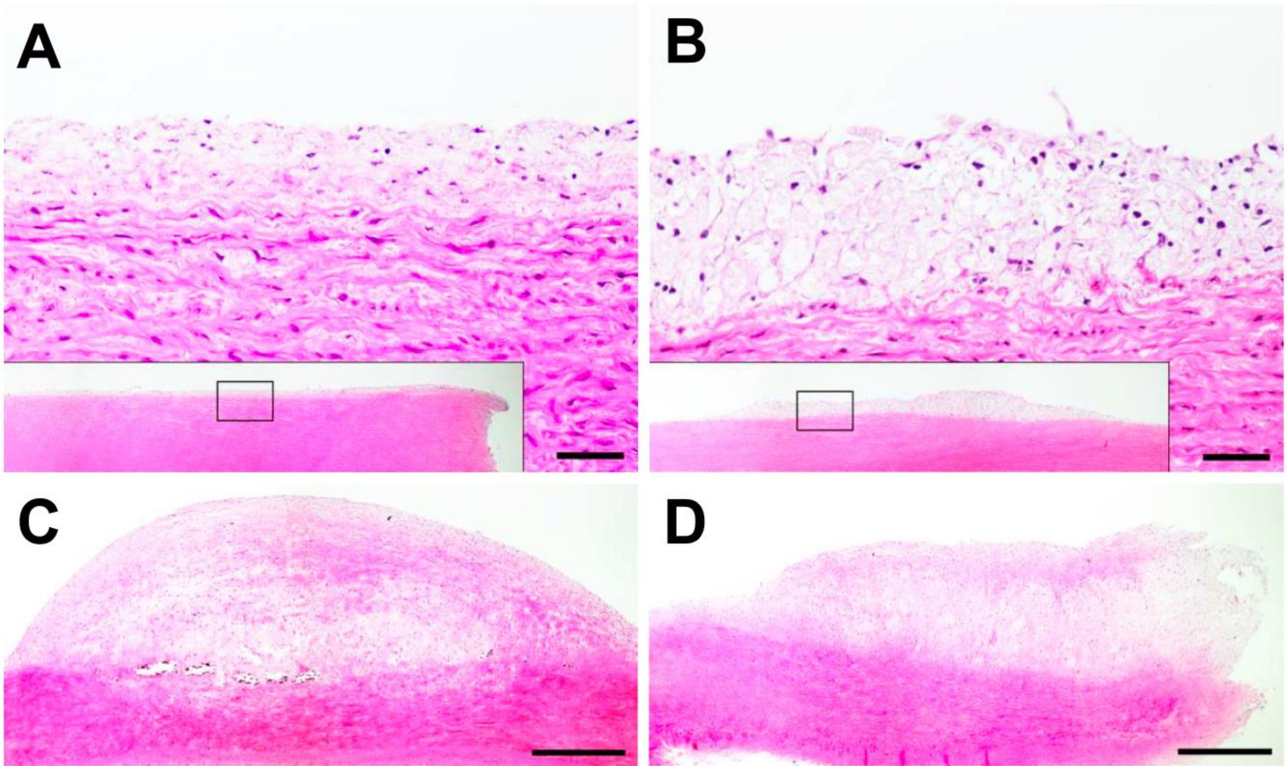

Figure S4. Lesions in thoracic aortas and iliofemoral arteries. A-B. Representative examples of lesions in thoracic aortas of *APOE*$^{-/-}$ Yucatan minipigs demonstrating xanthomas (non-raised lesions). Scale bar = 50 µm. **C-D.** Representative examples of lesions in iliofemoral arteries of *APOE*$^{-/-}$ Yucatan minipigs demonstrating early fibroatheroma (**C**) and pathological intimal thickening (**D**) (raised lesions). Scale bar = 500 µm.

Table S1. Primer Sequences

Amplicon	Forward primer (5´-3´)	Reverse primer (5´-3´)	Amplicon size (bp)
rAAV/*APOE* KO vector			
LHA	ATACATAC**GCGGCCGC**GGATCTGCTCGGGAAATATC	GCTCCAGCTTTTGTTCCCTTTAGCAGCACACGGTACCTGGTAC	960
RHA	CGCCCTATAGTGAGTCGTATTACCGGACCGTGTGTTCTGGAC	ATACATAC**GCGGCCGC**CCTCGGCCTGCAGGCGCAAC	910
PCR screening (gene targeting)			
APOE 5´KO PCR (F1+R1)	GGGATGGGGAGATAGGAGAAGAC	AGGTAGCCGGATCAAGCGTATGCAG	1973
APOE 3´KO PCR (F2+R2)	CCAATTCGCCCTATAGTGAGTCG	GGCGCCGCATGTCTTCCACCAGAG	988
Southern blot			
neo^r probe	GAAGCCCGGCATTCTGCACGC	CAGAAGCCATAGAGCCCACCGCA	1162
APOE probe	GGTCCTGCTCCTTCTCCCTGCCCAG	GCCAGTACAATTCGTCGGCTCTGGCTCGTCTG	603
Genotyping			
APOE KO allele	CCAATTCGCCCTATAGTGAGTCG	GGCGCCGCATGTCTTCCACCAGAG	988
APOE WT allele	GGACAGAGGACGAGCCGGGGCCG	GGTGCTGAGCAGCTCCTCCTGCAC	164
RT-PCR			
APOE exon 3 region	TCGCAAGCCAGAAGATGAGG	CTGAGCAGCTCC TCCTGCAC	221

Underlined sequence = linker regions for fusion PCR

Sequence in bold = *NotI* restriction site