This supplemental text file contains a detailed summary of the approaches used to successfully create the pCARGO-*Airn* plasmid.

**Attempts to capture *Airn* lncRNA from BAC clone RP23-309H20 with pCARGO capture vector:** BAC clone RP23-309H20 harboring the *Airn* lncRNA gene was obtained from the BACPAC Resources Center and was modified by recombineering to generate the tetracycline-inducible pCARGO-RMCE-*Airn* donor vector. The pCARGO-RMCE-*Xist*-RFP capture vector containing a medium copy origin-of-replication (ori) and visual RFP marker mScarlet-I as described in 1 was modified by Gibson assembly to retrofit with *Airn* 200-400-bp homology arms and to retrieve the *Airn* lncRNA from the BAC. Details for capture vector assembly were slightly modified from 1 to improve scarless assembly of multiple fragments simultaneously and included 4 ng/µl extremely thermostable single-stranded DNA binding protein (ET SSB) isolated from a hyperthermophilic microorganism near Pozzuoli, Italy 2. RFP served as a tool for visually examining background transformants, namely red colonies, following recombineering. Plasmid DNA, which was purified using the conventional miniprep technique involving alkaline lysis with NaOH and SDS as initially described by Birnboim and Doly, contains roughly 3% denatured plasmid DNA. This DNA is resistant to restriction digestion and contributes to the emergence of background transformants, manifested as red colonies. To reduce background transformants 10 micrograms of pCARGO-RMCE-*Airn*-RFP capture vector was (1) initially treated with 25 U/ul T5FEN 3 for 30 minutes in NEB CutSmart buffer in a 37⁰C water bath (2) linearized by digestion and electrophoresed at 20 V/cm overnight to improve resolution of plasmid DNA topologies (linearized, open circular, covalently closed circular), and (3) excised from the gel and purified by gel extraction.

*Airn* BAC clone RP23-309H20 was infected with a replication-defective λ phage containing *exo*, *bet* and *gam* under the control of its native phage operon containing the pL promoter and temperature-sensitive repressor, cI857. Briefly, BAC clone RP23-309H20 was made competent for recombineering by infecting with 10 ul replication-defective λ phage [λ cI857 ind1 CroTYR26amber PGLN59amber rex< >tetRA] 4, and then incubated with shaking at 32°C for 1 h. A detailed description of making BAC clones proficient for recombineering is provided in 1. Despite noticing very few red background transformants, many white recombinants were examined using junction PCR to screen for successful recombineering. Subsequently, they were analyzed through restriction fingerprinting and pulsed-field gel electrophoresis (PFGE; data not shown). Several attempts to retrieve full-length ~89-kb *Airn* lncRNA from the BAC using the linearized capture vector pCARGO-RMCE-*Airn*-RFP failed with both ~200 bp homology and then extended ~400 bp homology arms. We conjectured that insertion of large recombinant genetic payloads into the capture vector with a medium copy ori contributed to its instability 5.

**Attempts to capture *Airn* lncRNA from BAC clone RP23-309H20 by shaving:** After failed attempts to transfer *Airn* from its BAC to the pCARGO-RMCE-Airn-RFP capture vector, we changed our strategy and opted to use dual antibiotic cassettes to remove unnecessary upstream and downstream sequences, e.g. ‘shaving’ by recombineering, directly within the BAC. The pCARGO-RMCE-*Airn*-RFP capture vector was altered using Gibson assembly, dividing it into two separate recombineering cassettes, each with distinct antibiotic resistance genes: 1) aminoglycoside phosphotransferase (APH) *aph(4)-Ia* to confer resistance to hygromycin B and 2) *bla* to confer resistance to ampicillin/carbenicillin. Each vector was also modified to include the R6K gamma origin-of-replication, which allowed for separation of R6K vector replication from hosts that do not contain replication initiator protein pi (in our case, the BAC-containing DH10B *E. coli* host; 6,7). Each cassette was processed as previously described, using T5FEN treatment and digestion to linearize the cassette. The KpnI-LHA *Airn*-AmpR-5'shave-KpnI fragment was obtained from the gel extraction of clone AMC868 – AmpR B11, while the Bsu36I-RHA *Airn*-HygroR-3'shave-Bsu36I fragment was acquired from the gel extraction of clone AMC868 – HygroR B7.

The induction of lambda red recombineering protein expression (exo, bet, gam) was carried out as follows: lambda-infected RP23-309H20 BAC containing cells were grown overnight at room temperature in no-salt LB with 12.5 µg/ml chloramphenicol and 10 µg/ml tetracycline. The next day, they were diluted 100-fold to an OD600 of approximately 0.1. The cells were then grown in 100 ml no-salt LB with 12.5 µg/ml chloramphenicol at 30°C until the OD600 reached approximately 0.3. The temperature was then raised to 44°C for 20 minutes (not 42°C for 15 minutes), following the protocol by Lyozin et al., 2017 8.

After heat induction at 44°C in a shaking water bath, the cells were immediately cooled on ice with swirling for 90 seconds. The media was removed through centrifugation at 4°C, and the cells were collected at around 6,000xg for 10 minutes. The 250-ml centrifuge bottle was rinsed with 4°C Milli-Q type I water (resistivity 18.2 MΩ-cm and conductivity less than 0.056 µS/cm) to eliminate residual media. The cells were then resuspended in 2 ml 20% glycerol at 4°C. The microbial cells underwent three washes with 20% glycerol, and were collected in a 2 ml microcentrifuge tube at approximately 7,000xg for each 2-minute spin. The 100 ml starter culture was reduced to a final volume of around 250 µl in 20% glycerol at 4°C, resulting in an approximately 400% increase in cells per microliter.

We observed that using 20% glycerol improved the number of transformants during electroporation (data not shown). Wu et al., 2019 observed that extra glycerol protected human hematopoietic stem cells' viability during therapeutic gene editing via electroporation 9. However, we were unable to increase electroporation efficiency through room temperature incubation, as described by Tu et al., 2016 10. The use of osmolytes, such as glycerol and trehalose, has been reported to enhance salt tolerance and potentially increase halotolerance viability in the presence of high NaCl levels after membrane disruption caused by electroporation.

A recombineering step was performed to remove the lox511 site in the pBACe3.6 vector backbone along with the ~56 kb of mouse genomic sequence located upstream of *Airn*/NR\_027772 exon 1 (mm10 chr17:12741311). The removed sequences were replaced by an ampicillin resistance cassette, a lox71 site, and a TreTight/CMV minimal promoter immediately upstream of *Airn* exon 1. A second recombineering step was performed to remove ~119 kb of mouse sequence downstream of the transcription end site for *Airn*/NR\_027772 (mm10 chr17:12830123) as well as the loxP site in the BAC vector backbone. The removed sequences were replaced by an SV40 polyadenylation sequence, an FRT-flanked PGK-EM7-Hygromycin resistance cassette, and a lox2272 site. Recombineering with multiplexing dual cassettes is challenging due to its low efficiency. Roughly 120 ng/µl of each cassette or 60 ng/µl of both AmpR and HygroR cassettes and 40 µl of induced lambda-infected RP23-309H20 BAC containing cells were electroporated at 1.65 kV in pre-chilled 1-mm gap cuvettes, with electroporator settings at 1.65 kV and a time constant of around 4.3 microseconds (E. coli Pulser™ Transformation Apparatus, Bio-Rad) . Traditional recombineering protocols necessitate a 1-hour recovery post electroporation, but we observed that lengthier recovery times (from 6 h to occasionally overnight) improved viability and the appearance of novel recombinants. Intrigued by this finding, we speculated that the added stress from dual selection, and heat-induction for expression of recombineering proteins necessitated an extended recovery period. While this manuscript was in preparation, a technical report was published by Wang et al 2022, describing an improved recombineering protocol with multiplexing double-stranded DNA cassettes 11. This technical advancement became an essential component for multiplexing in a process later coined as double-stranded DNA Recombineering-assisted Multiplex Genome Engineering (dReaMGE) 11. In that work it was shown that enhanced multiplex dsDNA recombineering could be achieved by increasing recovery time and by both increasing recovery time and dNTP concentration (approximately 10 nanomolar) by extending the number of replication cycles (time) or accelerating the speed of bacterial replication (increased dNTP concentration). We successfully shaved the BAC with individual cassettes by a two-step procedure and contemporaneously shaved both extraneous upstream and downstream sequences by single-step dReaMGE. The final BAC clone was fully sequence-validated. Shaving resulted in a modified pBACe3.6 vector that eliminated one of two 30-bp repeats 5’-tcgatgataagctgtcaaacatgagaattg-3’ to generate a BAC capture vector similar to pBAC2015 12 for future recombineering work and partially truncated sopC although still rendered functional 13.

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