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Experimental Analysis of Imprinted Mouse X-Chromosome Inactivation

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Abstract

X-chromosome inactivation is a dosage compensation mechanism that equalizes X-linked gene expression between male and female mammals through the transcriptional silencing of most genes on one of the two X-chromosomes in females. With a few key exceptions, once the X-chromosome is inactivated replicated copies of that X-chromosome are maintained as inactive in all descendant cells. X-inactivation is therefore a paradigm of epigenetic inheritance. Imprinted X-inactivation is a specialized form of X-inactivation that results in the silencing of the paternally derived X-chromosome. Due to its parent-of-origin-specific pattern of inactivation, imprinted X-inactivation is a model of mitotic as well as meiotic, i.e., transgenerational, epigenetic inheritance. All cells of the early mouse embryo undergo imprinted X-inactivation, a pattern that is subsequently maintained in extraembryonic cell types *in vivo* and *in vitro*. Here, we describe both high- and low-throughput approaches to interrogate imprinted X-inactivation in the mouse embryo as well in cultured extraembryonic stem cells.

Keywords

X-chromosome inactivation; Allele-specific gene expression analyses; Preimplantation mouse embryogenesis; Trophoblast stem cells (TSCs); Extraembryonic endoderm (XEN) stem cells

1 Introduction

X-inactivation begins during early embryonic development. The mouse embryo exhibits two distinct forms of X-inactivation, imprinted and random. Imprinted X-inactivation, which results in the exclusive silencing of genes on the paternal X-chromosome, initiates in the preimplantation embryo [1–3]. In the postimplantation embryo, the extraembryonic lineages of the trophoblast and the primitive endoderm, which generate the placenta and the yolk sac, respectively, stably maintain imprinted X-inactivation [4–6]. The embryonic epiblast lineage, on the other hand, reactivates the paternal-X [2, 3] and subsequently randomly inactivates either the maternal or the paternal X-chromosome [7]. Importantly, imprinted X-inactivation is stably maintained in cultured trophoblast stem cells (TSCs) and

extraembryonic endoderm (XEN) stem cells [8–10]. Random X-inactivation can be modeled in differentiating embryonic stem cells and is stably maintained in epiblast stem cells [11, 12]. Because the inactive-X is stably maintained in descendant cells, X-inactivation is a model of heritable transcriptional regulation during mitotic cell division. Notably, imprinted X-inactivation is a paradigm for not only mitotic heritability, but also meiotic, or transgenerational, epigenetic regulation, due to its parent-of-origin-specific inactivation pattern [1–3]. The dissection of how imprinted X-inactivation occurs therefore promises to elucidate the transmission of epigenetic information across reproductive as well as cell division cycles. Below, we delineate current approaches to experimentally interrogate imprinted X-inactivation in vivo and in vitro. We describe the isolation of preimplantation mouse embryos and extraembryonic tissues from postimplantation embryos, the derivation of TSCs and XEN cells, and allele-specific profiling of the X-linked transcriptome via Sanger sequencing, Pyrosequencing, and RNA-sequencing (RNA-Seq).

2 Materials

The analysis of imprinted X-chromosome inactivation requires determining which of the two X-chromosomes is the source of X-linked RNAs. The allele-specific discrimination of X-linked RNAs in turn requires that the profiled samples harbor divergent X-chromosomes. X-linked genes in hybrid embryos or cells will contain known single nucleotide polymorphisms (SNPs), which can be exploited to determine whether an RNA transcript arises from maternally vs. paternally inherited X-chromosome [8, 13, 14]. Hybrid embryos and stem cells can be generated from any two strains of mice whose genomes have been deep sequenced and are available for download. Common combinations of divergent strains include *Mus musculus* X *Mus castaneus* or *Mus musculus* X *Mus molossinus* [14, 15]. For every parental cross used, samples generated from the opposite parental cross (e.g., *Mus musculus* dam X *Mus molossinus* sire and *Mus molossinus* dam X *Mus musculus* sire) should also be analyzed to rule out any strain-specific effects [14].

2.1 Isolation and Analysis of Hybrid Embryonic Day (E) 3.5 Blastocyst Embryos

1. Dissecting microscope.
2. E3.5 pregnant female mouse (*see Note 1*).
3. Finely sharpened forceps. Use 9 µm and 3 µm lapping film (Ted Pella) to sharpen.
4. 23 G Needle and 3 ml syringe.
5. 1× PBS with 6 mg/ml bovine serum albumin (PBS/BSA): To make 50 ml, combine 4 ml of 7.5% (w/v) BSA, 5 ml of 10× PBS, and 41 ml of ultrapure water (to be used in the downstream analysis of embryos). Keep on ice or at 4 °C for up to a week.
6. M16 medium (to be used for the derivation of cell lines). Store single-use aliquots (4 ml) at –20 °C and thaw as needed.
7. 35 mm tissue culture dish for collecting embryos.

8. Fluorescent microscope to detect paternal X-linked GFP expression (*see Note 1*).
9. PicoPure RNA Isolation Kit (Life Technologies).
10. Dynabeads mRNA DIRECT Kit (Life Technologies).

2.2 Isolation and Analysis of Postimplantation Hybrid Embryonic Day (E) 5.5–6.5 Embryos

1. Dissecting microscope.
2. E5.5–6.5 pregnant female mice (*see Note 1*).
3. Sharpened forceps.
4. 6 mg/ml BSA in 1× PBS as in Subheading 2.1, **item 5** above.
5. Embryo dissecting dish (*see Note 2*).
6. PicoPure RNA Isolation Kit (Life Technologies).
7. Dynabeads mRNA DIRECT Kit (Life Technologies).
8. Fluorescent microscope to detect paternal X-linked GFP expression (*see Note 1*).

2.3 Derivation and Culture of Trophoblast Stem Cell (TSC) Lines

1. Mitotically inactivated male mouse embryonic fibroblast (MEF) feeder cells (*see Note 3*).
2. 96-well, 4-well and 6-well tissue culture dish.
3. Round-bottom 96-well dish (*see Note 4*).
4. Hybrid embryonic day (E) 3.5 blastocyst embryos.
5. Sterile 1× PBS.
6. 0.05% Trypsin-EDTA.
7. TSC growth medium: RPMI with 20% ES cell qualified fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 mM β -mercaptoethanol, 2 mM L-glutamine, 37.5 ng/ml FGF4, and 1.5 mg/ml heparin (*see Note 5*).
8. TSC derivation medium: TSC growth medium with 50 mg/ml penicillin/streptomycin (p/s) (*see Note 6*).
9. MEF medium: MEM α , 10% FBS (*see Note 7*).

2.4 Derivation and Culture of Extraembryonic Endoderm Stem (XEN) Cell Lines

1. Mitotically inactivated male mouse embryonic fibroblast (MEF) feeder cells (*see Note 3*).
2. 0.2% gelatin: 0.2 g gelatin powder per 100 ml of ultrapure DNase/RNase free water. Autoclave to dissolve and sterilize.
3. 96-, 4-, and 6-well tissue culture dish.

4. Round-bottom 96-well dish (*see Note 4*).
5. Hybrid embryonic day (E) 3.5 blastocyst embryos.
6. Sterile 1× PBS.
7. 0.05% Trypsin-EDTA.
8. XEN growth medium: MEM α , 20% ES cell qualified FBS, 1 mM sodium pyruvate, 100 mM β -mercaptoethanol, 2 mM L-glutamine, 100 mM nonessential amino acids (*see Note 5*).
9. XEN cell derivation medium: XEN cell growth medium, 50 mg/ml penicillin/streptomycin (p/s), 1000 units/ml Leukemia Inhibitory Factor (LIF, Millipore) (*see Note 6*).
10. MEF medium: MEM α , 10% FBS (*see Note 7*).

2.5 Cryopreservation of TSC and XEN Cell Lines

1. 2× freezing medium: 50% FBS, 20% DMSO, and 30% cell growth medium.
2. Cryogenic vials.

2.6 Sexing of TSC and XEN Cell Lines by PCR

1. DNA lysis buffer: 50 mM Tris pH 8.8, 1 mM EDTA, 0.5% Tween 20.
2. 20 mg/ml Proteinase K.
3. PCR master mix, for a 1× reaction: 1× Klentherm Buffer (0.2 M Tris pH 9.1, 0.1 M Ammonium sulfate, 0.1 M MgCl₂, 0.015% BSA), 0.4 mM dNTPs, 0.4 μ M forward primer (CCGCTGCCAAATCTTTGG), 0.4 μ M reverse primer (TGAAGCTTTGGCTTTGAG), 2.5 U Taq polymerase (*see Note 8*).
4. PCR Thermal cycler.
5. Materials for gel electrophoresis.

2.7 Molecular Characterization of TSC and XEN Cell Lines

1. TRIzol (Life Technologies) (*see Note 9*).
2. SuperScript III One-Step RT-PCR System (Life Technologies) (*see Note 10*).
3. Primers for lineage specific transcripts:
 - *Cdx2* forward: GCAGTCCCTAGGAAGCCAAGTGA.
 - *Cdx2* reverse: CTCTCGGAGAGCCCAAGTGTG.
 - *Fgf4*, forward: CGTGGTGAGCATCTTCGGAGTGG.
 - *Fgf4*, reverse: CCTTCTTGGTCCGCCGTTCTTA.
 - *Eomes*, forward: GTGACAGAGACGGTGTGGAGG.
 - *Eomes*, reverse: AGAGGAGGCCGTTGGTCTGTGG.

- *Gata4*, forward: GCCTGTATGTAATGCCTGCG.
 - *Gata4*, reverse: CCGAGCAGGAATTTGAAGAGG.
 - *Gata6*, forward: GCAATGCATGCGGTCTCTAC.
 - *Gata6*, reverse: CTCTTGGTAGCACCAGCTCA.
 - *Afp*, forward: TCGTATTCCAACAGGAGG.
 - *Afp*, reverse: AGGCTTTTGCTTCACCAG.
 - *Bmp-2*, forward: GTTTGTGTTTGGCTTGACGC.
 - *Bmp-2*, reverse: AGACGTCCTCAGCGAATTTG.
 - *Dab-2*, forward: GGCAACAGGCTGAACCATTAGT.
 - *Dab-2*, reverse: TTGGTGTGATTTTCAGAGTTTAGAT.
 - *Ttr*, forward: AGTCCTGGATGCTGTCCGAG.
 - *Ttr*, reverse: TTCCTGAGCTGCTAACACGG.
4. Materials for gel electrophoresis.
 5. Reagents for immunofluorescence (*see* Subheading 2.10).
 6. Primary antibodies:
 - α -NANOG (ReproCELL) (specific expression in pluripotent stem cells).
 - α -CDX2 (BioGenex) (specific expression in TSCs).
 - α -GATA6 (R&D Systems) (specific expression in XEN cells).

2.8 Sanger Sequencing to Detect Allelic RNA Expression of X-Linked Genes

1. TRIzol (*see* Note 9).
2. SuperScript III One-Step RT-PCR System (Life Technologies) (*see* Note 10).
3. Materials for gel electrophoresis.
4. Gel extraction kit.
5. Sanger sequencer.
6. Software for analysis of Sanger chromatograms (*see* Note 11).

2.9 RNA Fluorescence In Situ Hybridization (FISH)-Based Analysis of X-Linked Gene Expression

1. 100% Molecular biology-grade ethanol: Use filtered ddH₂O to make stocks of 70, 85, and 95% ethanol.
2. Fluorescently labeled gene-specific probes.

3. 6-well dish, or similar chamber to be used for dehydration and for washing coverslips (*see Note 12*).
4. Small glass plate (*see Note 13*).
5. Parafilm.
6. Forceps.
7. Hybridization chamber: A small humid chamber for incubating slides, humidity provided by 2× SSC/50% deionized formamide (*see Note 14*).
8. Incubator set to 37 °C, for overnight incubation.
9. 2× SSC/50% deionized formamide: 5 ml 20× SSC, 25 ml deionized formamide, 20 ml filtered ddH₂O.
10. 2× SSC: 5 ml 20× SSC, 45 ml filtered ddH₂O.
11. 1× SSC: 2.5 ml 20× SSC, 47.5 ml filtered ddH₂O.
12. 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI): dissolved in water at 5 mg/ml.
13. Incubator set to 39 °C, for washes.
14. Mounting medium (*see Note 15*).
15. Microscope slides.

2.10 IF-Based Detection of Proteins and Chromatin Marks Enriched on the Inactive X-Chromosome

1. 1× PBS.
2. 6-well dish, or similar chamber to be used for washing cover-slips (*see Note 12*).
3. Blocking buffer: 1× PBS with 0.5 mg/ml BSA, 50 µg/ml tRNA, and 0.2% Tween 20 (make and use a 10% Tween 20 stock). Prewarm to 37 °C.
4. Primary antibody of choice.
5. Small glass plate (*see Note 13*).
6. Parafilm.
7. Forceps.
8. IF chamber: A small humid chamber for incubating slides, humidity provided by 1× PBS (*see Note 14*).
9. Incubator set to 37 °C.
10. 1× PBS with 0.2% Tween 20.
11. Fluorescently conjugated secondary antibody: Alexa Fluor (Invitrogen) secondary antibodies work well with this protocol; AF488, AF555, and AF647 have very similar absorption and emission spectra to the Fluorescein-12, Cy3,

and Cy5 dyes used for FISH, respectively. These antibodies can be used in conjunction with FISH probes for multicolor imaging with the same set of fluorescence microscope filters.

12. 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (5 mg/ml).
13. Mounting medium (*see* Note 15).
14. Microscope slides.

2.11 Quantification of Allele-Specific RNA Expression by Pyrosequencing

1. Software: PyroMark Assay Design (Qiagen).
2. TRIzol (*see* Note 9).
3. SuperScript III One-Step RT-PCR System (Life Technologies) (*see* Note 10).
4. Materials for gel electrophoresis.
5. DNase-free 96-well Semi-Skirt PCR plates.
6. PyroMark Q96 ID sequencer (Qiagen).

2.12 Quantification of Allele-Specific Expression by RNA-Seq

RNA-Seq quantifies the normalized absolute as well as relative allelic expression of genes across the X-chromosome in female preimplantation embryos, extraembryonic tissues of postimplantation embryos, TSCs, and XEN cells. The analysis below is optimized for hybrid TSCs and XEN cells, but can also be applied to hybrid preimplantation mouse embryos and postimplantation embryo extra-embryonic tissues.

1. Sample preparation and sequencing.
 - a. Total RNA from hybrid samples that is either PolyA+ selected or ribosomal RNA depleted for sequencing library preparation.
 - b. Illumina TruSeq Library preparation kit (Illumina).
2. Reference genomes.

Our analysis used the mouse mm9 reference. Other versions may be used, but it is necessary for the version of the B6 reference to match that of the SNP annotation and GTF annotation.

- a. The murine C57BL/6J (B6) reference genome is used as a template for the *in silico* assembly of other strains' genomes (e.g., 129/S1 [*Mus musculus*], CAST [*Mus castaneus*], or JF1/Ms. [*Mus molossinus*]). The B6 reference genome sequence is available at: http://www.ensembl.org/Mus_musculus/Info/Index.
- b. Strain-specific SNP data: SNP data in the form of VCF files for the other parental strains can be downloaded from the Wellcome Trust (<http://www.sanger.ac.uk/science/data/mouse-genomes-project>) or the

RIKEN Institute (<http://www.riken.jp/en/research/labs/>). The SNP annotation should match the B6 reference genome version.

- c. Gene annotation file: RefSeq GTF annotation file can be obtained from NCBI. Available at <https://www.ncbi.nlm.nih.gov/refseq/>.

The GTF annotation should match the version of the B6 reference genome used.

1. Computational cluster.

All analyses were conducted in Flux, a shared, Linux-based high-performance computing cluster. Any similar computing cluster should suffice.

2. Software packages.

The following provides a list of bioinformatics tools that can be used to process RNA-Seq data for the detection of allele-specific transcript expression. Details regarding the purpose and application of each tool for this analysis can be found in Subheadings 3 and 4.

- VCFtools [16]: a program package designed to facilitate the manipulation of complex genetic variation data in the form of VCF files. VCFtools can be used to filter out or summarize variants, compare files, convert to different file types, validate and merge files, and create intersections and subsets of variants. Available at <https://vcftools.github.io/index.html>.
- STAR [17]: a C++ script that is used to align RNA-Seq reads to a reference genome. Available at <https://github.com/alexdobin/STAR>.
- SAMtools [18]: a package designed for manipulating alignments in the SAM format, including sorting, merging, indexing and generating alignments in a per-position format. Available at <http://samtools.sourceforge.net>.
- Bedtools [19]: can be used for a wide range of genomic analyses. Bedtools allows for the intersection, merging, counting, complementation and shuffling genomic intervals from multiple file types. Available at <http://bedtools.readthedocs.io/en/latest/>.
- HTSeq [20]: a Python package that provides infrastructure to process data from high-throughput sequencing assays. HTSeq allows for the writing of custom scripts to perform specific analysis tasks. Available at www-huber.embl.de/HTSeq/doc/overview.html.
- DESeq2 [21]: allows for the differential expression analysis of RNA-seq read count data. Available at <http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>.
- NGSUtils [22]: a suite of tools for working with high-throughput sequencing datasets. The module bamutils can be used for manipulating

and analyzing bam files. Available at <https://github.com/ngsutils/ngsutils/tags>.

3 Methods

3.1 Isolation of Hybrid Embryonic Day (E) 3.5 Blastocyst Embryos

1. Euthanize pregnant female mouse at E3.5 (*see* Note 16).
2. Dissect the abdominal cavity and place the uterus into a dish containing chilled PBS/BSA (for embryo analysis) or M16 Medium (for TSC or XEN cell derivation).
3. Using the needle and syringe, flush chilled PBS/BSA (for embryo analysis) or M16 Medium (for cell derivation) gently through the uterine horns into a 35 mm dish.
4. If available, microscopically check for GFP fluorescence to select for female embryos (*see* Note 1).
5. For total RNA isolation: Using a P20 pipette, pipet each E3.5 embryo into a microtube containing 10 μ l extraction buffer from the PicoPure RNA Isolation Kit. Purify RNA following the manufacturer's instructions. Purified total RNA can be resuspended in 30 μ l of elution buffer.
6. For mRNA isolation: Pipet each embryo into 100 μ l lysis/binding buffer of the Dynabeads mRNA DIRECT Kit. Purify mRNA following the manufacturer's instructions. Purified mRNA can be resuspended in 50 μ l of elution buffer.
7. Purified RNA can be used for Sanger sequencing, Pyrosequencing, and RNA sequencing (RNA-Seq) as described below.

3.2 Isolation and Analysis of Postimplantation Hybrid Embryonic Day (E) 5.5–6.5 Embryos

1. Euthanize pregnant female mouse at E5.5–6.5 (*see* **Notes 16** and **17**).
2. Dissect uterus as in Subheading 3.1, **step 2**.
3. Separate the individual decidua from the uterus by cutting the uterine wall between individual decidua.
4. Place the decidua into a 35 mm dish containing chilled PBS/BSA. Keep on ice.
5. Move one of the decidua to a glass, round-bottom dish containing chilled PBS/BSA. Open up the decidua in PBS/BSA under a dissecting microscope using a pair of sharpened forceps and carefully dissect out the embryos. Keep extra glass dishes on ice so they are chilled during the dissection. Use one dish per embryo (*see* **Note 2**).
6. Separate the extraembryonic and epiblast portions of each embryo through physical bisection at the junction of the epiblast and extraembryonic ectoderm using a pair of fine forceps.

7. If available, a GFP fluorescent microscope can mark the epiblast by expression of the paternal X-linked *GFP* transgene (*see* **Notes 1** and **18**).>
8. Lyse the extraembryonic compartment for total RNA or mRNA isolation as with the E3.5 embryos in Subheadings 3.1, **steps 5–7** above.
9. Purified RNA can be used for Sanger sequencing, Pyosequencing, and RNA-Seq as described below.

3.3 Derivation and Culture of Trophoblast Stem Cell (TSC) Lines

1. Prepare 4-well plates with MEFs at $\sim 2 \times 10^5$ cells/well in MEF medium 1 day before embryo collection (*see* **Notes 3, 19–21**).
2. Collect E3.5 embryos as described in Subheading 3.1, **steps 1–4**.
3. Remove MEF media from 4-well dishes with MEF cells and replace with ~ 750 μ l of TSC derivation medium.
4. Place one blastocyst per well and culture at 37 °C and 5% CO₂ (*see* **Note 22**).
5. Day 2: Examine under microscope that the blastocysts have attached to the feeder layer of the well. The attachment may take a couple days.
6. Day 3–4: Once the blastocyst has attached firmly to MEFs, aspirate media and add fresh TSC derivation media.
7. Day 4: Prepare a flat-bottom 96-well dish with MEFs plated with MEF media in as many wells as embryos (*see* **Notes 3, 19, and 20**).
8. Day 5: From the MEF-plated 96-well dish, remove MEF media and replace with 150 μ l of TSC derivation medium for as many wells as there are plated embryos.
9. Day 5: Dissociate the blastocyst outgrowth through the following steps, using sterile techniques.
 - a. Aliquot 20 μ l trypsin in each well of a round bottom 96-well plate.
 - b. Warm trypsin at 37 °C in tissue culture incubator.
 - c. Remove medium from each well of the 4-well plates containing the blastocyst outgrowth and rinse with sterile 1 $\times$ PBS.
 - d. Pipet 500 μ l sterile 1 $\times$ PBS into each 4-well well.
 - e. Using a P20 pipette set at 3 μ l scrape the blastocyst outgrowth under a tissue culture microscope and place in the 96-well with prewarmed trypsin.
10. Process all of the blastocyst outgrowths as above.
11. Incubate in the 37 °C incubator for 3–10 min (*see* **Note 23**).
12. Gently pipet each trypsinized outgrowth in the 96-well with a P20 pipette set at 10 μ l. Take care to avoid bubbles.

13. Check each of the 96-well wells under the microscope. The outgrowths should now be disaggregated in small clumps or single cells. Avoid generating single cells as well as large clumps.
14. Pipet individual dissociated blastocysts, in 20 μ l trypsin, into single wells of the MEF-plated 96-well dish.
15. Rinse the wells that contained the dissociated embryos with an additional 50 μ l of TSC media, add this to the 150 μ l
16. Day 6, early: Change media with fresh 150 μ l TSC derivation media to remove/neutralize residual trypsin.
17. Day 7: Plate MEFs in 4-well dishes (*see Notes 3, 19, and 20*).
18. Day 8–10: Appearance of TSC colonies.
19. Trypsinize in 30 μ l trypsin and dissociate the TSC colonies into very small clumps by pipetting, neutralize trypsin with 100 μ l TSC media and transfer into a MEF-plated 4-well well containing 750 μ l TSC media (*see Notes 20, 24, and 25*).
20. Once the 4-well wells reach confluency, passage the cells to a fresh 4-well well with MEFs (a particularly robustly growing TSC line can also be passaged onto individual wells of a 6-well dish).
21. Continue to culture the TSCs in TSC growth medium at 37 °C, 5% CO₂. Change the medium every day and passage the cells (1:6) every third day or when the culture has reached ~80–90% confluency (*see Notes 3, 6, 19, and 20*).

3.4 Derivation and Culture of Extraembryonic Endoderm Stem (XEN) Cell Lines

1. Prepare 4-well plates with male MEFs at $\sim 2 \times 10^5$ cells/well in MEF medium 1 day before embryo collection (*see Notes 3, 19–21*).
2. Day 1: Collect E3.5 embryos as described in Subheading 3.1, **steps 1–4**.
3. Remove MEF media from 4-well dishes with MEFs and replace with ~750 μ l of XEN derivation medium.
4. Place one blastocyst per well and culture at 37 °C and 5% CO₂ (*see Note 22*).
5. Day 2: Examine under microscope that the blastocysts have attached to the feeder layer of the well. The attachment may take a couple days.
6. Day 3–4: Once the blastocyst has attached firmly to MEFs, aspirate media and add fresh XEN cell derivation media.
7. Day 7: Prepare a flat-bottom 96-well dish with MEFs plated with MEF media in as many wells as embryos (*see Notes 3, 19, and 20*).
8. Day 8: From the MEF-plated 96-well dish, replace MEF media with 150 μ l of XEN derivation medium in as many well as the plated embryos.

9. Day 8: Dissociate the blastocyst outgrowths as described for TSC derivation in Subheadings 3.1, steps 9–15 above but using XEN cell derivation media.
10. Day 9, early: Change media with fresh 150 μ l XEN cell derivation media to remove/neutralize residual trypsin.
11. Day 11–12: Plate MEFs in 4-well dishes (*see Notes 3, 19, and 20*).
12. Day 12–13: Appearance of XEN cells.
13. When the XEN cells become confluent, trypsinize the cells into single cell suspension using 30 μ l trypsin, neutralize with 100 μ l XEN cell media, and transfer into a MEF-plated 4-well well containing 750 μ l XEN cell media (*see Notes 20, 24, and 25*).
14. Once the 4-well wells reach confluency, passage the XEN cells to a fresh well in a 4- or 6-well plate, depending on confluency and rate of growth (*see Notes 26–28*).
15. Continue to culture the XEN cells in XEN cell growth medium on gelatinized plates at 37 °C, 5% CO₂. Passage the cells (1:10) every third day or when the culture has reached ~80–90% confluency (*see Notes 6, 27, and 28*).

3.5 Cryopreservation of TSC and XEN Cell Lines

1. Resuspend cell pellet in cell growth medium (*see Note 29*).
2. Add in an equivalent amount of 2 $\times$ freezing medium.
3. Freeze 1 ml per cryo vial.

3.6 Sexing of TSC and XEN Cell Lines

The sex of the cell lines can be achieved in one of several ways. One preferred method is to generate embryos, including for the derivation of TSCs and XEN cells, by using sires harboring an *X-Gfp* transgene [23–25] (*see Notes 1 and 18*). Alternatively, for TSCs and XEN cells sexing can be done by PCR of genomic DNA for the X/Y gene pairs *Kdm5c/Kdm5d*.

1. Deplete MEFs in TSC culture via centrifugation for 30 s at 75 $\times g$ (XEN cell cultures do not harbor MEFs).
2. Harvest the supernatant, which contains the TSCs, and centrifuge for 5 min at 500 $\times g$.
3. Remove the supernatant and resuspend the cells in 50 μ l DNA lysis buffer containing 1 μ l Proteinase K (*see Note 30*).
4. Incubate the lysate at 65 °C in a water bath for 3 hr—overnight.
5. Heat the lysate at 95 °C in a heat block for 10 min (*see Note 31*).
6. 100–200 ng, or ~1 μ l genomic DNA is sufficient for PCR reaction.
7. Set up PCR reaction.

8. Program the thermal cycler the following way: one initial cycle of 94 °C for 5 min, 35 cycles of—94 °C for 45 s, 55 °C for 30 s, 72 °C for 30 s. Hold at 12 °C.
9. Analyze PCR reaction by agarose gel electrophoresis.
10. A female cell line will exhibit one 330 bp band and a male cell line will show two bands of sizes 330 and 300 bp.

3.7 Morphological and Molecular Characterization of TSC and XEN Cell Lines

New TSC and XEN cell lines should be morphologically examined (Fig. 1) and molecularly profiled to determine that they are indeed TSCs and XEN cells.

1. Collect cells equivalent to ~1 confluent 6-well well through trypsinization.
2. Deplete feeder MEFs by centrifuging in a microfuge for 30 s at 75 ~ *g*.
3. Collect the supernatant, which contains the TSCs, and centrifuge for 5 min at 500 ~ *g*.
4. Resuspend the cells in 1 ml TRIzol. These samples can be stored at –80 °C for later use.
5. Isolate the RNA following manufacturer's instructions.
6. Analyze the expression of cell specific markers [26] by RT-PCR:
 - TSC: *Cdx2*, *Fgf4* and *Eomes*.
 - XEN: *Gata4*, *Gata6*, *Afp*, *Bmp2*, *Dab2*, and *Ttr*.
7. In addition to via RT-PCR, the TSC and XEN cell lines can be characterized through immunofluorescence (IF) staining (*see* Subheading 3.10) of the marker genes as described in Subheading 2.7, **item 6** above (Fig. 2).

3.8 Sanger Sequencing to Detect Allelic Expression of X-Linked Genes

Sanger sequencing is a qualitative method to determine the preferential expression of X-linked genes from one of the two parental X-chromosomes.

1. Design amplicons spanning a SNP and an intron (*see* **Note 32**).
2. Perform one step RT-PCR using SuperScript III One-Step RT-PCR System from Life Technologies following the manufacturer instruction (*see* **Note 10**).
3. Run the PCR product on an agarose gel.
4. Purify specific product by gel extraction.
5. Prepare the DNA samples following Sanger sequencing requirements.
6. Submit for sequencing with desired sequencing primers (*see* **Note 33**).
7. Visualize chromatograms in region of SNP (*see* Fig. 3 for an example).

3.9 RNA Fluorescence In Situ Hybridization (FISH)-Based Analysis of X-Linked Gene Expression

Expression (or silencing) of X-linked genes can be detected in individual cells of embryos and cultured TSCs and XEN cells through RNA FISH protocols [28].

1. Start with permeabilized and fixed samples plated on cover-slips in 70% ethanol.
2. Dehydrate the coverslips by moving them through a room-temperature ethanol series (85, 95, and 100% ethanol) for 2 min each.
3. Remove the coverslips from the well and air-dry at room temperature for 15 min (*see Note 34*).
4. Set up the FISH hybridization. Use 8–10 μ l of probe per 22 $\times$ 22 mm coverslip. Pipet the probe onto a Parafilm-wrapped glass plate (*see Note 13*), invert the coverslip onto the droplet of probe, and tap lightly with forceps to help the probe spread across the coverslip.
5. Hybridize overnight at 37 °C in a humid chamber (humidity provided by 2 $\times$ SSC/50% formamide) (*see Note 14*).
6. Make all wash solutions (2 $\times$ SSC/50% formamide, 2 $\times$ SSC, and 1 $\times$ SSC), and warm the solutions to 39 °C.
7. Carefully peel the coverslip off of the Parafilm, reinvert, and place in a well containing prewarmed 2 $\times$ SSC/50% formamide. Be sure that the coverslip goes into the well with the sample-side up.
8. Wash with prewarmed 2 $\times$ SSC/50% formamide at 39 °C, three times for 7 min each.
9. Wash with prewarmed 2 $\times$ SSC at 39 °C, three times for 7 min each. Add 5 mg/ml DAPI into the last 2 $\times$ SSC wash at a 1:100,000–1:200,000 dilution (*see Note 35*).
10. Rinse once quickly with prewarmed 1 $\times$ SSC.
11. Wash with prewarmed 1 $\times$ SSC at 39 °C, two times for 7 min each.
12. Use mounting medium to mount the coverslip onto a labeled microscope slide. Invert the slide onto a paper towel and press gently but firmly to remove excess mounting medium from under the coverslip.
13. Seal the coverslip and let dry thoroughly before viewing under a microscope.
14. Slides can be stored in the dark at –20 °C to preserve the fluorescent signal.

3.10 IF-Based Detection of Proteins and Chromatin Marks Enriched on the Inactive X-Chromosome

The inactive X-chromosome is characterized by accumulation of chromatin modifying proteins as well as chromatin modifications themselves. These can be detected through the protocols included in ref. 28, as for the FISH protocols.

1. Begin with fixed, permeabilized cells or embryo samples, plated on gelatinized glass coverslips and stored in 70% ethanol.
2. Make blocking buffer and warm to 37 °C.
3. Place sample coverslip in a 6-well dish that contains 2 ml of 1× PBS in each well (*see Note 12*).
4. Wash briefly with three changes of 1× PBS to remove ethanol.
5. Wash with 1× PBS three times, 3 min each on a rocker.
6. Wrap a glass plate tightly with Parafilm for incubating cover-slips for subsequent steps.
7. Block slides for 30 min at 37 °C in 50 µl prewarmed blocking buffer in a humid chamber: Place a 50 µl drop of blocking buffer on the Parafilm-wrapped glass plate and invert the cover-slip, sample side down, into the blocking buffer. Place the Parafilm-wrapped plate in the humid chamber, and incubate for 30 min at 37 °C. All incubations in blocking buffer, primary antibody, or secondary antibody should be set up in this manner.
8. Carefully lift coverslip from blocking buffer with forceps and place into a 50 µl droplet of diluted primary antibody on a Parafilm-wrapped plate. Incubate with 50 µl primary antibody diluted in prewarmed blocking buffer (dilution based on primary antibody you are using) in a humid chamber at 37 °C for 1 h.
9. Remove coverslip from primary antibody solution and place, sample-side up, in a 6-well dish. Wash three times with 1× PBS/0.2% Tween 20 for 3 min on a rocker.
10. Incubate in 50 µl prewarmed blocking buffer on a Parafilm-wrapped plate in a humid chamber for 5 min at 37 °C.
11. Incubate with 50 µl secondary antibody diluted in prewarmed blocking buffer in humid chamber at 37 °C for 30 min. Antibody dilution depends on secondary antibody used; Alexa Fluor-conjugated secondary antibodies should be used at a 1:300 dilution.
12. Remove coverslip from secondary antibody and wash three times with 1× PBS/0.2% Tween 20 for 3 min each on a rocker. The first wash should contain a 1:100,000–1:200,000 dilution of DAPI (5 mg/ml) (*see Note 35*). Then, rinse once briefly with PBS/0.2% Tween 20 and wash two more times for 5–7 min each while rocking to remove excess DAPI.
13. Remove coverslip from dish, tap off excess liquid, and then mount on a slide, sample-side down, in mounting medium. Image samples or store at –20 °C for later imaging.

3.11 Quantification of Allele-Specific Expression by Pyrosequencing

Pyrosequencing quantifies the relative expression of individual X-linked genes from the two alleles in female cells.

1. Design amplicons which are intron-spanning and contain an SNP of interest using the PyroMark Assay Design software. Amplicon length should be less than 200 bp for optimal results (*see Note 32*).
 - a. Three primers should be designed for each gene.
 - A pair of gene specific reverse transcription/cDNA amplification primers (forward and reverse), one of which is tagged with a biotin tag (*see Note 36*).
 - The third primer is a sequencing primer that ends within 5 bp of the SNP.
 - b. Use the PyroMark Assay Design software to generate an .xml assay file. This file should include the primers and the sequence surrounding the SNP of interest and is uploaded into the Pyrosequencer.
2. Prepare total RNA or mRNA from the desired hybrid sample.
3. Synthesize cDNA using the Life Technologies SuperScript III One-Step RT-PCR System (*see Note 10*).
4. Following the PCR reaction, run 5 µl from the total 25 µl RT-PCR reaction on a 3% agarose gel to assess the efficacy of amplification (*see Note 37*).
5. Pipet 9 µl of each successful sample into a well of the DNase-free 96-well Semi-Skirt PCR plates for Pyrosequencing according to the standard recommendations for use with the PyroMark Q96 ID sequencer.
6. The remainder of the PCR reaction can be stored at 4 °C.
7. Analyze the ratio of allelic gene expression through the Pyrograms (Fig. 4).

3.12 Quantification of Allele-Specific Expression by RNA-Seq (Fig. 5)

1. Sample preparation and sequencing.
 - a. From F1 hybrid total RNA, purify Poly-A+ RNA and generate a stranded cDNA sequencing library using the Illumina TruSeq Library preparation kit.
 - b. Libraries have been sequenced on the Illumina HiSeq2000 platform to generate 100 bp paired-end reads. However, as new technologies become available, sequencing can be performed on more advanced platforms to yield more reads and longer read lengths, which will increase mapping of the reads.
2. Assembly of in silico reference genomes.
 - a. Use VCFtools “vcf-consensus” perl script to substitute identified SNPs into the B6 reference genome to generate in silico parental genomes.

- b. Create a list, in VCF format, of every SNP site from both in silico reference genomes. This list will be used to identify SNP-overlapping reads.
3. Mapping reads to reference genomes.
 - a. Sequencing reads in the FASTQ format should be separately mapped to each of the two in silico genomes using STAR, allowing for 0 mismatches in mapped reads and no multimapped reads.
 - Allowing for 0 mismatches (“--outFilterMismatchNmax 0”).
 - Allow no multimapped reads (“--outFilterMultimapNmax 1”).This ensures allele-specific mapping of most SNP-containing reads to only one strain-specific genome. STAR is a good option for read mapping due to its ability to handle structural variability and trim the ends of long reads (*see Note 38*).
 - b. Reads can be filtered based on map quality, and should also be checked for reads that map to both reference genomes, but in different locations, which may sometimes occur (*see Subheading 3.12, step 4* for details).
4. Read filtering after mapping.

Allele-specific analysis requires some special considerations for read filtering. While allowing 0 mismatches in mapped reads means that many SNP-overlapping reads will map to only a single reference genome, some additional filtering may be required depending on SNP density and the location of SNPs within the reads. This step is necessary because mapping algorithms will try to map as many reads as possible, even if a full-length perfect match cannot be found. If a contiguous, full-length perfect match is not found, STAR may either splice or soft-clip reads. A small number of reads that map to an SNP-containing region in one reference genome may then be erroneously mismapped in the second reference, where a contiguous perfect match does not exist (*see Note 39*).

A small number of reads may be mapped with numerous or abnormal splice sites, or soft clipped reads mapped to different locations due to the presence of homologous sequence. The small number of reads that map to both genomes, but with different map coordinates in each can be identified using the “diff” function in the program BamUtil (available at http://genome.sph.umich.edu/wiki/BamUtil:_diff) and either completely removed or filtered such that one of the two map locations is selected based on quality score or other filtering parameters (*see Note 40*).

For filtering reads, we found it useful to break BAM files into SNP-containing reads and non-SNP containing reads before filtering; *see* Subheading 3.12, **step 5** for how to identify SNP-containing reads using Bedtools. Non-SNP reads can be similarly identified using the “-v” option in the Bedtools intersect utility.

- ### 1. Allele-specific expression analysis.

- a. Files in the SAM format should be converted to the binary BAM format using Samtools to make them a more manageable size.
- b. Read files in BAM format should then be split into plus and minus strands and converted to BED format, which are smaller and easier to manipulate.
 - This conversion can be accomplished using Samtools to separate reads by strand, then using the Bedtools “bamtobed” function to convert the file to BED format.
- c. Only RNA-Seq reads overlapping known SNP sites that differ between the parental genomes should be used for allele-specific analysis. To identify reads overlapping known SNP sites, the reads in BED format should be sorted by chromosome and position using the Bedtools “sort” command, then compared to the VCF SNP list using the Bedtools “intersect” command to create files identifying the number of reads overlapping each SNP site.
 - Use the “-c” option to count the number of reads in the BED file overlapping each entry in the VCF file.
 - Use the “-split” option to ensure that intronic SNPs are not considered to be covered by reads spliced across the intron.
- d. These files should then be joined on SNP position using the Unix “join” command to make one plus and one minus strand file per sample.
 - Each joined file should contain, at a minimum, a column listing SNP positions, a column listing the counts of allele-specific reads overlapping each SNP site for reference genome #1, and a column listing the counts of allele-specific reads overlapping each SNP site for reference genome #2. Other columns will be present depending on the initial format of the VCF file, and can be removed if desired to minimize file sizes.
- e. Intersect the joined files from the previous step with the GTF annotation file. Split the GTF file by strand, then use the bedtools “intersect” command to identify the SNPs that reside within annotated genes. Finally, these GTF files with SNP information can be joined to create one GTF file per sample.
- f. For each exonic SNP site, the proportion of reads from each X-chromosome should be identified based on the counts of reference 1 and reference 2 allelic reads in the intersected files.
 - The number of allelic reads, and the proportion of allelic reads mapping to each reference, can be calculated using basic Unix commands.

- g. Allelic expression should be calculated individually for each SNP site. For genes containing multiple SNPs, the paternal-X allele percentage for all SNPs can be averaged to calculate gene-level allelic expression.
- Use Bedtools “groupby” function, grouping rows by gene name or gene ID. Depending on the source of the GTF, the file may need to be manipulated and columns split or trimmed to create a column containing the gene name or gene ID.

2. Differential Expression Analysis.

This step is necessary if quantifying allelic X-linked gene expression between two different genotypes.

- a. Use Samtools “merge” to create a single alignment file for differential expression analysis.
 - To minimize inclusion of duplicate reads, merge non-SNP containing reads from one mapping run + SNP containing reads from reference genome 1 + SNP containing reads from reference genome 2; this compilation ensures that the reads that do not contain SNPs will not be counted twice.
- b. Count the number of reads per RefSeq annotated gene using HTSeq-count.
- c. The total read count from HTSeq can be normalized by library size.
- d. Multiply the total number of normalized reads per X-linked gene by the proportion of SNP-containing reads mapping to the paternal vs. the maternal X-chromosome.
- e. Calculate differential expression using DESeq2.

4 Notes

1. If possible, the sire used for generating embryos should contain a *Gfp* transgene on the X-chromosome [23–25]. Only female embryos will inherit the paternal X-chromosome, including the paternal X-linked *Gfp* transgene. The sex of the embryos can therefore be determined by examining GFP fluorescence.
2. A glass dish works well for this purpose, as plastic dishes are easily scratched by the dissecting forceps. Alternatively, a 35 mm tissue culture dish could be used.
3. Male MEFs should be used in order to differentiate the feeders from female stem cells during downstream analyses. The strain of the MEFs should also be known to distinguish MEFs from stem cells in allele-specific analysis.
4. Round bottom 96-well dishes work better than flat bottom for dissociating embryos, the round bottom allows the small volume of trypsin to pool at the bottom of the well.
5. ES qualified FBS is required for TSC media and XEN media. Non-ES qualified FBS may not maintain TSCs and XEN cells in their undifferentiated state.

6. Antibiotics (penicillin/streptomycin) are used in the media during derivation of cell lines only. After cell lines have been established, pen/strep can be removed from the media.
7. MEF media does not require ES qualified FBS.
8. Other PCR master mixes and any basic Taq polymerases are likely to work.
9. RNA purification methods other than TRIzol can be used in place of TRIzol.
10. Any robust RT-PCR system should work.
11. A variety of sequencing analysis software are available for free. Any software that can visualize chromatograms will do for analyzing allelic expression.
12. Use 22×22 mm coverslips, which fit well within a single well of a 6-well dish. All the dehydration and washing steps can be performed in the wells of this dish.
13. Short glass plates designed for casting protein gels are a good size for hybridization. For example, Bio-Rad's Mini-PROTEAN Short Plates fit well in the humid chamber described in **Note 14**.
14. For humid chambers, a microscope slide box (i.e., one that holds 100 slides) works well. Place paper towels soaked in $1 \times$ PBS in the bottom of the box to create a humid chamber for immunofluorescence. For RNA and DNA FISH hybridization procedures, create a humid chamber by placing paper towels soaked in $2 \times$ SSC/50% formamide in the bottom of the box.
15. For mounting medium, use Vectashield (Vector Labs) or similar antifade mounting medium and seal coverslips with clear nail polish after mounting on slides.
16. Cervical dislocation should be used for euthanasia to avoid gene expression changes that can be caused by chemical or CO_2 -based methods.
17. The extraembryonic tissues of E5.5 and E6.5 embryos maintain imprinted X-inactivation. And at these stages embryonic cell types have not ingressed into the extraembryonic compartment of the developing embryo [29].
18. The *X-Gfp* transgene is mosaically expressed in the epiblast due to random X-inactivation but is silenced in the extraembryonic tissues because of imprinted X-inactivation of the paternal X-chromosome [24].
19. Concentration of MEFs should approximately be:
 - 96-well plates: 2×10^5 cells across 12 wells of a 96-well plate.
 - 4-well dish: 2×10^5 cells per well, 8×10^5 cells per dish.
 - 6-well dish: 8×10^5 cells per well, 5×10^6 cells per dish.
 - 10 cm dish: 5×10^6 cells per dish.

20. MEF cells should be thawed and plated in MEF medium the day before use. Prior to plating the stem cells onto MEFs, remove the MEF media and replace with appropriate growth media for TSCs or XEN cells.
21. The number of 4-well wells with MEFs should be estimated based on how many embryos are expected.
22. When pipetting embryos into the 4-well dishes, be careful not to disturb the MEF cells with the pipette. Try to pipet the embryo into the center of the well, as it will be easier for later manipulation.
23. The amount of time in the incubator will change depending on how many blastocysts you have to dissociate. If you have multiple embryos, the first dissociated embryo will have been in trypsin longer than the last embryo. Adjust incubation time accordingly.
24. The number of cells in 96-well wells is small. Trying to collect the cells by centrifugation risks losing a significant number of the cells. When splitting from a single well in a 96-well plate, transfer the cells in trypsin into the next larger well (a 4-well well) for plating in sufficient growth media to neutralize the trypsin. Once the cells reach confluency in 4-well wells, they can be collected through centrifugation.
25. If there are not enough cells in the 96-well well, do not split, as they will not survive splitting to a 4-well well. The dissociated embryos in the 96-well dishes may grow in clumps, depending on how well they were dissociated. Allow these clumps of cells to become as large as possible before splitting, but not too large since the cells will then begin to die.
26. If the XEN cells are growing well in the 4-well dish, they should be ready to be split to a well of a 6-well dish. If the 4-well dish is still clumpy and not confluent, a 1:1 split into a new 4-well well may be necessary to help dissociate the clumps and get the cells to start growing as a monolayer.
27. Once the XEN cells are growing well in culture (approximately when they are split to a 6-well well), they no longer require MEF feeder layer and also do not require LIF in the culture media. Start plating cells in dishes precoated with 0.2% gelatin in XEN growth medium without LIF or antibiotics.
28. To prepare gelatinized plates: pipet enough 0.2% gelatin to coat the bottom of well. Wait ~10 min then aspirate the gelatin out of the well. Leave the dish open in the hood to let the gelatin dry.
29. Freeze cells at a density of ~2 6-well wells into one cryo vial.
30. Adjust the volume of lysis buffer used depending on the size of the cell pellet being lysed. Keep the ratio of Proteinase K to lysis buffer constant.
31. Some PCR reactions require Proteinase K denaturation, other reactions may work fine without it.

32. Intron-spanning RT-PCR amplicons distinguish between genomic DNA vs. cDNA amplification. Genomic DNA bands will be larger due to presence of the intron.
33. Sanger sequencing can produce results of 800–1000 nucleotides long. Depending on the length of the amplicon, the primers used for PCR can also be used for sequencing. If the amplicon is much longer, new sequencing primers should be designed closer to the SNP of interest.
34. Do not leave glass coverslips to dry in 6-well dishes after aspiration of 100% ethanol; the glass coverslip may stick to the bottom of the well. Find a safe place to set the coverslip to dry.
35. Dilute 5 mg/ml DAPI 1:500 in RNase/DNase-free ultrapure water, and store in the dark at –20 °C. Add 6–10 µL of this dilution into each well while washing samples.
36. Biotin-labeled primers should not be frozen/thawed multiple times as this can cause the biotin to detach from the primer or degrade. Aliquot the biotin primer into single use aliquots and store at –20 °C.
37. If there is a single band on the gel, the remainder of the PCR reaction can be prepared for Pyrosequencing. Pyrosequencing reactions should not be gel-extracted, as the gel extraction process is detrimental to the biotin tag.
38. STAR was chosen for its features including soft-clipping the ends of reads, how it deals with splice junctions, its speed, and its good documentation. Other software may become available in the future which would improve further on these features.
39. In normal RNA-Seq analysis, soft-clipping is beneficial, allowing for reads with poor quality near the ends to be mapped.
40. Some reads which overlap SNP sites in one reference genome can also map to the second reference, due to the presence of the SNPs near the ends of reads. The dual map is because of soft-clipping of the sequences at the end of reads. This issue does not present a problem for allele-specific analysis, as those soft-clipped reads adjacent to the SNP of interest will not be counted as SNP-overlapping reads when the BED files and VCF file are intersected.

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Fig. 1. Comparative morphology of TSCs, XEN, and embryonic stem cells (ESCs). Viewed at 20 $\times$, TSC and XEN cells can be seen as individual nuclei, compared to the more densely packed colonies of ESCs. TSCs also grow in colonies, while XEN can grow singly as well as in clusters

Fig. 2. Characterization of TSC and XEN and ESCs through immunofluorescence staining. TSCs are marked by CDX2; XEN cells are marked by GATA6; and ESCs are marked with NANOG [26]. Nuclei are stained blue with DAPI

Fig. 3. Sanger sequencing distinguishes genes expressed in a parent-of-origin-specific manner. Top, the two parental strains are characterized by a single nucleotide polymorphism (SNP) in the X-linked *Xist* gene marked by the blue arrow. *Xist* encodes a long noncoding (lnc) RNA that is only expressed from the inactive X-chromosome [27]. In female blastocysts, Sanger sequencing of the *Xist* cDNA shows that only the paternal allele of *Xist* is expressed

Fig. 4.

Representative Pyrosequencing pyrograms for analysis of allele specific gene expression. **(a)** Pyrogram of *Xist* cDNA from a hybrid TSC line derived from a cross of the *Mus musculus* 129/Sv strain (dam) to the *Mus molossinus* JF1 strain (sire). *Xist* is only expressed from the paternal (JF1) X-chromosome in TSCs and marks the inactive X-chromosome [8] (“C” allele = 0% expression; “T” allele = 100% expression). The height of the peaks is proportional to the number of the indicated nucleotide at that position. **(b)** In TSCs from the reverse cross (sire = JF1; dam = 129/Sv), *Xist* is only expressed from the paternal 129/Sv X-chromosome (“C” allele = 100% expression; “T” allele = 0% expression)

Fig. 5.

A summary of the steps in allele-specific RNA-Seq analysis of imprinted X-inactivation