Письма в ВАВИЛОВСКИЙ ЖУРНАЛ ГЕНЕТИКИ И СЕЛЕКЦИИ
Letters to Vavilov Journal of Genetics and Breeding
2022 • 8 • 2 • 214-222

pismavavilov.ru
DOI 10.18699/LettersVJ-2022-8-10

Original article

Inexpensive protocol for single-cell X-chromosome inactivation analysis: *Xist* expression and H3K27me3 staining

I.E. Pristyazhnyuk[1], N.I. Meshcheryakov[2], A.G. Menzorov[1, 2]

Abstract: The X-chromosome status is an important marker of mammalian cell embryonic differentiation. Both female X-chromosomes are transcriptionally active (Xa) at the blastocyst stage, then one of the X-chromosomes undergoes random inactivation (Xi). Naïve pluripotent stem cells have both Xa, while primed pluripotent stem cells and differentiated cells – XaXi. When pluripotent stem cells are generated from non-model species, it is important to analyze X-chromosome status, as well as pluripotency marker gene expression at the early stages, to minimize time and cost. Thus, the X-chromosome state may point to the pluripotency status of the newly derived pluripotent cells, and early analysis of pluripotency markers would facilitate the cell selection. We present two detailed protocols of X-chromosome inactivation analysis: a single-cell analysis based on cell lysis with subsequent reverse transcription PCR, and immunofluorescent H3K27me3 staining. The H3K27me3 staining marks transcriptionally inactive chromatin. The single-cell lysis for RNA isolation is performed in a small volume of PCR-compatible non-ionic detergents, Nonidet P-40 and Tween-20, with BSA. Subsequent cDNA synthesis is performed in the same tube. This method could be used for *Xist* amplification, as an early X-chromosome inactivation marker, as well as for other genes, such as *Rex1*, expressed in mammalian pluripotent cells. We have successfully applied those protocols for X-inactivation analysis in the American mink embryonic fibroblasts and three lines of induced pluripotent stem cells. Thus, the proposed protocols allow fast and inexpensive testing of pluripotency and X-inactivation markers on a single-cell level.
Key words: single cells; *Neovison vison*; American mink; *Xist*; *Rex1*; *HK1*; H3K27me3.

Acknowledgments: The reported study was funded by RFBR, project number 20-04-00369, and the Ministry of Education and Science of the Russian Federation, state project FWNR-2022-0019. Cell lines are available at the Collective Center of ICG SB RAS "Collection of Pluripotent Human and Mammalian Cell Cultures for Biological and Biomedical Research" (https://ckp.icgen.ru/cells/; http://www.biores.cytogen.ru/icg_sb_ras_cell/).

For citation: Pristyazhnyuk I.E., Meshcheryakov N.I., Menzorov A.G. Inexpensive protocol for single-cell X-chromosome inactivation analysis: *Xist* expression and H3K27me3 staining. *Pisma v Vavilovskii Zhurnal Genetiki i Selektsii = Letters to Vavilov Journal of Genetics and Breeding.* 2022;8(2):214-222. DOI 10.18699/LettersVJ-2022-8-10

Оригинальное исследование

Экономичный протокол для анализа инактивации Х-хромосом: экспрессии *Xist* и иммунофлуоресцентной окраски H3K27me3

И.Е. Пристяжнюк[1], Н.И. Мещеряков[2], А.Г. Мензоров[1, 2]

Аннотация: Статус Х-хромосомы – важный маркер дифференцировки клеток эмбриона млекопитающих. Обе Х-хромосомы самок транскрипционно активны (Xa) на стадии бластоцисты, затем одна из них проходит случайную инактивацию (Xi). В «наивных» плюрипотентных клетках обе Х-хромосомы активны, в «праймированных» – одна из Х-хромосом инактивирована. Также «наивные» плюрипотентные клетки в процессе дифференцировки становятся XaXi. При получении плюрипотентных стволовых клеток из немодельных организмов необходимо проводить анализ статуса Х-хромосомы и определять наличие экспрессии генов-маркеров плюрипотентности на ранних стадиях, чтобы минимизировать время и стоимость. Таким образом, статус Х-хромосомы может указать на тип плюрипотентности, а ранний анализ экспрессии генов-маркеров плюрипотентности упрощает селекцию клеток. Мы представляем два протокола для анализа инактивации Х-хромосомы на уровне единичных клеток.

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Novosibirsk State University, Novosibirsk, Russia
Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
menzorov@bionet.nsc.ru

Первый протокол основан на лизисе единичных клеток с последующей ПЦР с обратной транскрипцией, второй – на иммунофлуоресцентном окрашивании H3K27me3. H3K27me3 маркирует транскрипционно неактивный хроматин. Лизис единичных клеток для выделения РНК проводят в маленьком объеме совместимых с ПЦР неионных детергентов, Nonidet P-40 и Tween-20, и BSA с последующей наработкой кДНК в той же смеси. Этот метод выделения РНК может быть использован для последующей амплификации как раннего маркера инактивации Х-хромосомы, *Xist*, так и других генов, экспрессирующихся в плюрипотентных клетках млекопитающих, таких как *Rex1*. Мы успешно использовали протоколы для анализа инактивации Х-хромосом в эмбриональных фибробластах и трех линиях индуцированных плюрипотентных стволовых клеток американской норки. Таким образом, описанные протоколы позволяют выполнять быстрый и недорогой анализ маркеров плюрипотентности и инактивации Х-хромосомы на уровне единичных клеток.

Ключевые слова: single cells; *Neovison vison*; American mink; *Xist*; *Rex1*; *HK1*; H3K27me3.

Introduction

Random X-chromosome inactivation takes place in the early stages of mammalian embryonic development. The cells with naïve pluripotency, i.e. mouse embryonic stem (ES) cells isolated from the blastocysts of the 129 mouse strain have both X-chromosomes active (Xa) (Barakat et al., 2010). The primed pluripotent stem cells, such as human ES cells generated by the standard protocols, could have different states of X-chromosome inactivation, including the presence of inactivated X-chromosome (Xi) (Fan, Tran, 2011). Recently, mouse ES cell single-cell RNA-seq analysis revealed heterogeneity between X-chromosome status as well as some asynchronism between X-chromosome inactivation and cell differentiation (Chen et al., 2016). Thus, the status of X-chromosome inactivation may clarify the developmental stage of pluripotent cells, point to the beginning of the differentiation process, or in some cell types be heterogeneous.

We have previously produced American mink (*Neovison vison*) ES and induced pluripotent stem (iPS) cells (Menzorov et al., 2015). The majority of the female ES cell line cells were negative for the inactive chromatin marker H3K27me3, thus suggesting naïve pluripotency with both X-chromosomes in an active state. We produced XX American mink iPS cells to determine whether the iPS cells have similar X-chromosome status to the ES cells (Pristyazhnyuk, Menzorov, 2020).

Here we describe an inexpensive protocol for the express analysis of X-chromosome inactivation in single cells. We applied the protocol for the X-inactivation analysis in the American mink embryonic fibroblasts and iPS cells. The protocol is easily adaptable to assessing the expression of the other genes in single cells and the analysis of the various chromatin marks.

Applications of the method

The overview of the single-cell gene expression analysis protocol is presented in fig. 1, *a*. The overview of the immunofluorescent marker staining – in fig. 1, *b*.

The proposed protocols of single-cell RT-PCR and immunofluorescent analyzes are suitable for a variety of purposes. Qualitative expression of any gene at a single-cell level could be revealed with certain limitations. The protocol could be modified to allow quantitative analysis (see *Advantages and limitations*). The immunofluorescent analysis could be used for histone modification or other antigen studies.

In this article, we applied the protocols for X-chromosome inactivation study in American mink fibroblasts and iPS cells. In addition, the protocols are well suited for the analysis of the iPS cell colonies in the early stages of reprogramming. During the iPS cell derivation, it is difficult to select the pluripotent cell colonies. The expected colony morphology is well known for mouse, human, or American mink iPS cells. For the other species, such as the pinnipeds or fox there is no data. Gene expression analysis of such pluripotency markers as *Nanog* and *Rex1* (*Zfp42*) from colony pieces could allow selection of iPS-like cells without prolonged culture before the "standard" tests for pluripotency, such as the embryoid body formation and teratomas in immunodeficient mice.

Immunofluorescent analysis has numerous applications. We used it to reveal the histone modification of the Xi, H3K27me3 (Matveeva et al., 2017). The H3K27me3 histone modification is scarcely present throughout autosomes and Xa, but Xi possesses an excess of it. That marker is routinely used to assess X-chromosome inactivation in different mammalian species. We have previously shown that American mink XX ES cells (Menzorov et al., 2015) and XX iPS cells (Pristyazhnyuk, Menzorov, 2020) have up to 87 % XaXa cells, in compliance with their pluripotent status.

Comparison with other methods

There are numerous technologies for single-cell isolation, i.e. fluorescence-activated cell sorting, microfluidics, lab-on-a-chip devices, laser microdissection, random seeding and dilution, and manual cell picking (Gross et al., 2015). We selected manual cell picking for its affordability and relative simplicity. While most sophisticated methods allow the subsequent analysis of gene expression and other parameters in thousands of cells, manual cell picking is enough for studying the expression of several genes in tens of cells.

One of the principal problems is RNA loss during isolation from limited material. Single-cell RNA isolation could be performed by spin column-based kits, i.e. GenElute Single Cell RNA Purification Kit (Sigma-Aldrich, cat. no. RNB300) or PicoPure RNA Isolation Kit (Applied Biosystems, cat. no. KIT0204). Another option is a direct lysis method. We decided to choose the latter option and used modified PBND (PCR buffer with nonionic detergents) to lyse cells with the addition of BSA. The PBND is a buffer with non-ionic detergents compatible with PCR that was originally developed for tissue lysis (Higuchi, 1989). We used 0.1x concentration of PCR compatible non-ionic detergents Nonidet P-40 and Tween-20 from the original formulation and BSA (bovine serum albumin). Similar protocols of direct lysis with different concentrations of BSA (Svec et al., 2013) and BSA and Nonidet P-40 (Le et al., 2015) were previously reported. Compared to these protocols we also used random hexamers for the total cDNA synthesis, not the gene-specific primers.

The cDNA was synthesized by the standard protocol with a couple of modifications: low reaction volume and lack of DNase

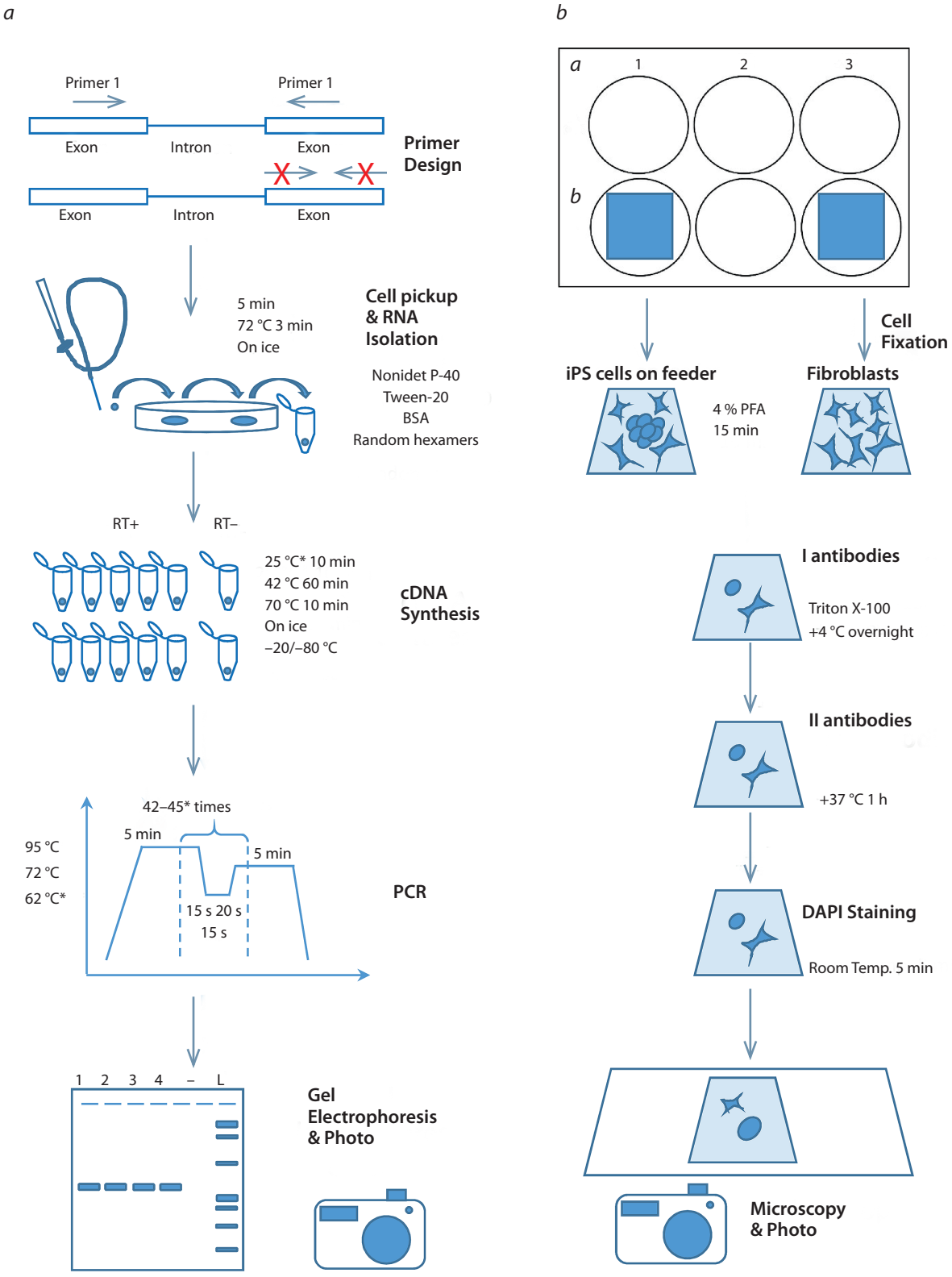

Fig. 1. Overview of the protocols.

a – single-cell gene expression analysis; *b* – immunofluorescent staining

* Those parameters should be determined experimentally

I treatment. The PCR was also performed under the standard conditions; we used Hot Start *Taq* DNA polymerase to prevent non-specific product formation.

There are many methods for Xi visualization in cell culture. Mainly, *Xist* transcript detection by the RNA fluorescent *in situ* hybridization (FISH). The *Xist* transcript is associated with the Xi and/or the nuclear matrix around it (Brown et al., 1992; Clemson et al., 1996). In addition, loss of such Xa-associated histone modifications as H3K9 acetylation and H3K4 methylation (Heard et al., 2001; Goto et al., 2002; Okamoto et al., 2004), and Xi-associated H3K9me2, H4K20me1, and H3K27me3 (Heard et al., 2001; Plath et al., 2003; Silva et al., 2003; Kohlmaier et al., 2004; Okamoto et al., 2004) are detected by immunofluorescence. The later ones are among the earliest events in the X-chromosome inactivation process after *Xist* transcript accumulation. The most widely studied repressive marks on the Xi chromosome are methylation of H3 lysine 9 (H3K9me2) and 27 (H3K27me3). H3K27me3 tends to be enriched on Xi, while H3K9me2 marks "inactive" more widely (Rougeulle et al., 2004; Marks et al., 2009). We identified Xi by the H3K27me3 immunofluorescent staining.

Advantages and limitations

The main advantages of the RT-PCR protocol are its affordability and simplicity. The direct lysis solution and other reagents are inexpensive compared to spin column-based kits. Manual cell picking is also affordable, though laborious.

The first limitation of the method is that the number of analyzed genes is limited to two or three. The cDNA volume is 5 µl, which is enough for one to three PCR. Further dilution may result in false-negative results due to the nonhomogeneous distribution of the low copy cDNA molecules. The single-cell whole-genome amplification would allow amplification of numerous transcripts, but that approach is beyond the scope of that article.

The second limitation is the qualitative "yes or no" results of the gene expression analysis. The quantitative PCR (qPCR) or its modification Droplet Digital PCR (ddPCR) would allow (semi) quantitative gene expression analysis. The "real" quantitative analysis of a gene expression is probably not achievable, as the RNA reverse transcription process efficiency varies.

The multiplex PCR of the whole cDNA volume should solve the problem of cDNA loss after the dilution. In our experience, multiplex PCR for two gene transcripts had not produced reliable results either due to a different initial number of cDNA copies or different primer annealing efficiency. To overcome that limitation fluorescent probe-based qPCR may be performed.

The third limitation is omitting the DNase I treatment, thus genomic DNA remains in the solution. The reason is that EDTA-based DNase I inactivation makes undiluted cDNA solution toxic for the *Taq* DNA polymerase and the dilution would lead to false-negative results. To overcome that drawback we propose to design primers for different exons of the gene of interest to prevent genomic DNA amplification or for distinguishing between cDNA and genomic DNA. This primer design approach does not always work. For instance, it appeared that the American mink *Hprt1* gene has an expressed pseudogene, and thus it is impossible to use it as an RT-PCR control. We used *HK1* housekeeping gene as an RT-PCR control for the XY fibroblasts. *HK1* does not have pseudogenes in human, mouse, and dog genomes (McDonell, Drouin, 2012); based on our data the same is true for American mink.

We had successfully extracted RNA and performed RT-PCR from the cell colonies of about 100 cells. Given the sensitivity of the method, it is possible to use DNase I with EDTA inactivation with a subsequent 10x dilution to remove EDTA toxicity. Similar protocols report successful usage of the direct lysis method for up to 512 (Svec et al., 2013) or about 1000 cells (Le et al., 2015).

The single-cell RNA-seq analysis outperforms RT-PCR in the number of genes as it allows to analyze expression of the majority of the highly expressed transcripts. On the other hand, when there are just a few genes of interest, RT-PCR is undoubtedly more cost-effective.

The immunofluorescent analysis of the cells on the coverslip allows the spatial analysis of X-chromosome status depending on the location inside or outside of the colony. Cells at the colony border may be differentiated and, thus, have XiXa status. The main limitation is a low permeability of multilayer colonies for the antibodies that result in a difference in the cell staining. Another protocol could be used to solve that problem. The cytocentrifugation spreads cells and facilitates access for antibodies. It also allows concentrating the cells approximately twenty-fold on the small spot of about 5 mm^2 (Jones, Cornbleet, 1997). That enables using very little cell amount, however, the centrifuge procedure also distorts the cell morphology making most of the cells similar to each other. As the iPS cells are cultures on feeder cells, after the centrifugation iPS and feeder cell nuclei would not be distinguishable. For immunofluorescent staining with antibodies against H3K27me3 Xi markers, both methods of cell preparation could be used with the fibroblasts cell culture if permeabilization is performed before the fixation procedure. Such procedure could not be used for the American mink iPS cells, as cells were massively detaching from the coverslip. Therefore, cytocentrifugation could be used for the American mink fibroblasts, but not for the iPS cells.

Experimental design

Primer design

The protocol overview is presented in fig. 1. The general guidelines for various application primer design are available (PCR Primer Design, 2015). We used Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al., 2012) to design primers for the American mink *Rex1*, *Xist*, and *HK1* genes. As the isolated RNA is not treated with DNase I, the primers and genes of interest should comply with the following criteria:

a) The primers are located in different exons of the gene of interest;

b) There are no pseudogenes with DNA amplification PCR product that has the same length as the cDNA product;

c) There are no expressed pseudogenes.

The primer pairs should be tested in advance on species-specific DNA, cDNA synthesized from DNase I treated RNA, and single-cell "cDNA" without reverse transcriptase treatment. There should be no PCR products from the DNA with the same length as for cDNA (pseudogene DNA amplification). The amplification from cDNA synthesized from DNase I treated RNA indicates pseudogene expression, thus contamination of desired

gene expression is expected. Finally, PCR of lysed cells that were subjected to all steps of the protocol except reverse transcriptase treatment is necessary to demonstrate the absence of contamination.

RNA isolation, cDNA synthesis, and PCR

Primers for cDNA synthesis are included in a 1x cell lysis buffer to minimize the number of steps. We used random hexamers for the total RNA conversion, as one of the genes of interest, *Xist*, does not have a poly(A) tail. If you need to analyze the expression of relatively short genes, oligo(dT) primers could be used. In the case of a single gene expression analysis, gene-specific primers are a good option.

Cells without reverse transcriptase treatment should be included in an experiment as a negative control.

The single-cell first-strand cDNA synthesis is performed according to the manufacturer's protocol with slight modifications, such as the decrease of the total reaction volume and the usage of the cell lysis buffer instead of RNA solution. The PCR is also performed according to the manufacturer's protocol with the decrease of the reaction volume.

Immunofluorescent analysis

The immunofluorescent analysis was performed according to the standard procedure (Matveeva et al., 2017). American mink fibroblasts were grown on the coverslips. Mink iPS cells were seeded on the feeder cells prepared from the mitomycin-treated embryonic fibroblasts (CD-1 mouse strain) (Menzorov et al., 2015). The feeder cells served as an inner control of the staining. Since mouse fibroblasts were a mixture of male and female cells, we observed the big nuclei with and without inactive histone marks surrounding tightly packed iPS cell colonies with smaller nuclei. Thus, both positive and negative control was present on the coverslips with fixed iPS cells.

Materials

Biological materials

- American mink embryonic fibroblasts NV4 (XY) and NV5 (XX) (embryos were provided by O.V. Trapezov, ICG SB RAS, Novosibirsk, Russia).
- American mink induced pluripotent stem cells iNV1XX1, iNV2XX5, and iNV5XX2 (Pristyazhnyuk, Menzorov, 2020).

The cell lines are available at the Collective Center of ICG SB RAS "Collection of Pluripotent Human and Mammalian Cell Cultures for Biological and Biomedical Research" (https://ckp.icgen.ru/cells/; http://www.biores.cytogen.ru/brc_cells/collections/ICG_SB_RAS_CELL).

Reagents

The reagents could be substituted by analogs from different producers.

- 0.2-ml PCR tubes (Axygen, cat. no. PCR-02-C)
- Phosphate Buffered Saline (PBS) Tablets (Amresco, Am-E404-100)
- Nonidet P-40 (Amresco, cat. no. Am-E109)
- Tween-20 (PanReac AppliChem, cat. no. A4974)
- Triton X-100 (VWR International, cat. no. Am-0694)
- Bacteriological Petri dishes (100×15 mm; not treated, aseptic; Falcon, cat. no. 351029)
- Filter pipette tips (10, 200, and 1000 µL; Axygen, cat. nos. TXLF-10-L-R-S, TF-200-L-R-S, and TF-1000-R-S)
- Sterile plastic tubes (Axygen, SCT-5ML-S)
- Autoclaved ddH_2O (Milli-Q filtered water)
- DEPC-treated ddH_2O (Thermo Fisher Scientific, cat. no. 10813012)
- Agarose (MP Biomedicals, cat. no. AGAF0500)
- Tris-acetate-EDTA buffer for gel electrophoresis
- Ethidium bromide (Sigma-Aldrich, cat. no. E1510)
- M-MuLV–RH First Strand cDNA Synthesis Kit (Biolabmix, Russia, cat. no. R01-50)
- BioMaster HS-Taq PCR-Color (2×) (Biolabmix, Russia, cat. no. MHC010-1020)
- 22 × 22 mm coverslips (BB022022A1, Menzel-Glaser, Thermo Fisher Scientific, Germany)
- Microscope slides (AGAA000001, MENZEL MICROSCOPE SLIDES, MENZEL, Thermo Fisher Scientific, Germany)
- Triton X-100 (VWR International, cat. no. Am-0694)
- Albumin Fraction V (pH 7.0) for Western blotting (BSA for immunofluorescence) (PanReac AppliChem, cat. no. A6588)
- Bovine serum albumin, molecular biology grade (BSA for RT-PCR) (New England Biolabs, cat. no. B9000S)
- Anti-trimethyl-Histone H3 (Lys27) (anti-H3K27me3) rabbit polyclonal antibody (Sigma-Aldrich, cat. no. 07-449)
- DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich, cat. no. D-9542)
- Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 (Thermo Fisher Scientific, cat. no. A11010)
- Goat anti-Mouse IgG/IgM (H+L) Secondary Antibody, Alexa Fluor 488 conjugate (Thermo Fisher Scientific, cat. no. A-10680)
- ProLong Gold Antifade Mountant (Thermo Fisher Scientific, cat. no. P10144)

Primers

- NvXistE1F: 5′-TCAAAAGATCCGCCCAGCTC-3′ (DNK-Sintez, Russia, custom order)
- NvXistE2R: 5′-TCTTGAGTGGGGCACAGAAC-3′ (DNK-Sintez, Russia, custom order)
- NvRex1E1F: 5′- TGGTTTCAGCTGCTGTAGGG -3′ (DNK-Sintez, Russia, custom order)
- NvRex1E3R: 5′-GCCCTTTTCTTCAGTTGCTGG-3′ (DNK-Sintez, Russia, custom order)
- NvHK1E11F2: 5′-AACTGGTGCGACTGATCCTG-3′ (DNK-Sintez, Russia, custom order)
- NvHK1E12R2: 5′-GTACACACGTGCTGGACTGA-3′ (DNK-Sintez, Russia, custom order)

Reagent setup

10x cell lysis buffer

Add and mix 437.25 µl DEPC-treated ddH_2O, 5 µl Nonidet P-40, 5 µl Tween-20, and 2.25 µl 20 mg/ml BSA in a 1.5 ml tube.

1x cell lysis buffer

Mix 2.45 µl DEPC-treated ddH_2O, 0.3 µl 10x cell lysis buffer, and 0.25 µl of 20x random hexamers (from the M-MuLV–RH First

Strand cDNA Synthesis Kit) per 0.2 ml tube. Prepare necessary amounts of the reagents for all cells in one tube.

NOTE: Oligo(dT) or gene-specific primers could be used instead of random hexamers depending on the experiment design.

PBS

Add 1 tablet of PBS to 100 ml of ddH_2O and autoclave.

4% PFA

Dissolve 4 g paraformaldehyde in 100 ml PBS buffer with heating on the magnetic stirrer. Add drops of 5N NaOH until the solution becomes clear, then cool down and adjust pH to 7.4 with 1N HCl and 1N NaOH. Freeze the solution in aliquots at –20 °C, use it after thawing immediately or within a few days.

Antibody Dilution Buffer

Add 0.2 % Triton X-100 and 2 % BSA to PBS.

PBST

Add 0.2 % Tween-20 to PBS.

DAPI stock solution

Dissolve 2 mg of DAPI (4′,6-Diamidino-2-Phenylindole) in 2 ml PBS, keep at +4 °C. To stain the chromatin, dissolve 1 µl of the DAPI stock solution in 1 mL of PBS.

Equipment

The producer of the equipment is not specified for some items, as there are various analogs.

- Glass capillary (Micropipettes, 50 µl) (Blaubrand intraMark, cat. no. 708733)
- Inverted microscope (Olympus, cat. no. CKX53)
- Carl Zeiss Axioplan 2 imaging microscope (Jena) with CoolCube1 CCD-camera with the ISIS (*In Situ* Imaging System; MetaSystems GmbH) software
- T100 Thermal Cycler (Bio-Rad, cat. no. 1861096)
- Mini-centrifuge/vortex Micro–Spin FV-2400 (Biosan, cat. no. BS-010201) with R-0.5/0.2M rotor (Biosan, cat. no. BS-010201-BK)
- Centrifuge/mixer ELMI Fugamix CM-70M-09 with ELMI spin/mix rotor 70.02 (ELMI SIA, Latvia)
- Molecular imager (Gel Doc XRS + with Image Lab software system; Bio-Rad, cat. no. 170-8265)
- Gel electrophoresis tank and power source (Sub-Cell GT Horizontal Electrophoresis System, Bio-Rad, cat. no. 1704484; Bio-Rad PowerPac basic power supply; Bio-Rad, cat. no. 1645050)
- Humidity Chamber Plus (Covance, cat. no. SIG-31031)
- Magnetic stirrer with heater (Biosan, cat. no. MSH 300)
- Thermostat (for incubation at +37 °Autoclave
- Pipettes (0.5–5, 0.5–10, 2–20, 20–200, and 100–1000 µL)
- Freezers and refrigerator for –80 °C, –20 °C, and +4 °C

Procedure

1. Analysis of *Xist* and *Rex1* expression in single cells

CRITICAL: All steps are performed using filtered tips and RNase- and DNase-free tubes. Use gloves to minimize RNase contamination. All reagents should be thawed, mixed, briefly centrifuged, and put on ice. Use metal rack on ice or PCR cooling rack for tubes during the protocol.

Cell preparation and reverse transcription

1.1. Prepare single-cell suspension following the routine protocol. Cell culture protocol for the mink embryonic fibroblasts and iPS cells was described previously (Pristyazhnyuk, Menzorov, 2017). Centrifuge and wash cells twice in PBS, then perform a serial dilution and put 50 µl drops on non-adhesive (i.e. bacteriological) Petri dishes. There should be 5–50 cells per field of view at 100x magnification under an inverted microscope.

1.2. Thaw an aliquot of 10x cell lysis buffer, prepare 1x cell lysis buffer, and add 3 µl per 0.2 ml tube on ice.
NOTE: RNase inhibitor could be added to the 1x cell lysis buffer to prevent RNA degradation. For example, RiboLock RNase Inhibitor (Thermo Fisher Scientific, cat. no. EO0381).

1.3. Put double rows of 50 µl PBS drops on a separate Petri dish.

1.4. Make capillary transfer mouth pipette by combining glass capillary, silicone tube, and aspirator mouthpiece (Nakagata, 2015). The aspirator mouthpiece could be substituted by a 200 µl filtered tip. Capillary sterilization is not necessary. The inner diameter of a pipette should be several times larger than the cell size.

1.5. Collect cells from the suspension by the capillary transfer mouth pipette into PBS drop, wash, transfer into the second drop, wash, and transfer into 0.2 ml tube with 1x cell lysis buffer in a minimal volume.

1.6. Wait at least 5 min after the last cell transfer.

1.7. Heat the solution with cells at 70 °C for 3 min in the Thermal Cycler with lid temperature 105 °C. Put on ice.

1.8. Mix 1 µl 5x reaction buffer, 0.5 µl 0.1 M DTT, 0.25 µl 10 mM dNTP mix, and 0.25 µl M-MuLV–RH reverse transcriptase (100 U/µl) per cell (all reagents from the M-MuLV–RH First Strand cDNA Synthesis Kit). Prepare necessary amounts of the reagents for all cells in one tube.
NOTE: Add 0.25 µl DEPC-treated ddH_2O instead of reverse transcriptase for the negative control – cells without reverse transcriptase treatment.

1.9. Add 2 µl to each tube with a lysed cell.

1.10. Mix and spin cell lysate using centrifuge/mixer 1F4 program (1 min of medium intensity mixing and spinning).

1.11. Incubate in the Thermal Cycler with lid temperature 105 °C: 10 min at 25 °C, 60 min at 42 °C, and 10 min at 70 °C.
NOTE: Incubation at 25 °C is not necessary when oligo(dT) or gene-specific primers are used. If the RNA is expected to be CG-reach, the reaction temperature can be increased up to 50 °C.

1.12. The cDNA could be directly used in PCR, stored at –20 °C for less than one week, and at –80 °C for longer storage.

PCR

1.13. Thaw, mix, and briefly centrifuge BioMaster HS-Taq PCR-Color (2×), ddH_2O, primers, and cDNA.

1.14. Mix 5 µl BioMaster HS-Taq PCR-Color (2×), 2.6 µl ddH_2O, and 0.4 µl template-specific primers (10 pM each) per tube, vortex, and briefly centrifuge. Prepare necessary amounts of the reagents for all cells in one tube.

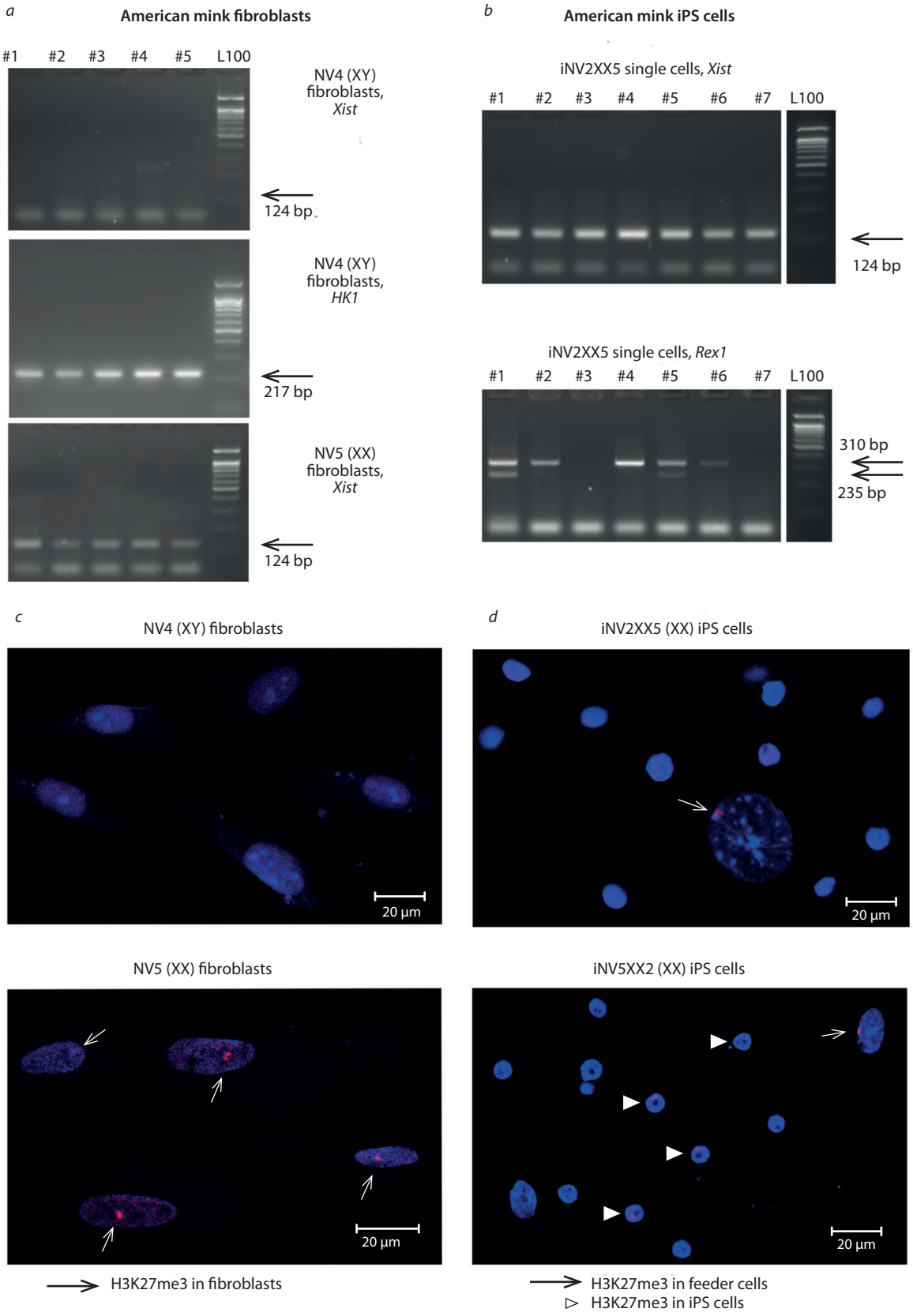

Fig. 2. Examples of RT-PCR and immunofluorescent analyses of the American mink fibroblasts and iPS cells.

a – *Xist* and *HK1* expression in American mink XX and XY fibroblast single cells; *b* – *Xist* and *Rex1* expression in American mink iNV2XX5 iPS single cells; XX and XY fibroblast single cells; *c* – immunofluorescent staining of H3K27me3 in American mink XX fibroblasts; *d* – immunofluorescent staining of H3K27me3 in American mink iPS cells

1.15. Mix 8 µl PCR mix and 2 µl isolated cDNA, briefly centrifuge.
NOTE: As 0.1x PBND is fully PCR compatible, 4.6 of 5 µl cDNA could be used for amplification if only one amplification per cell is planned.
1.16. Perform DNA amplification in a T100 thermal cycler using the following program: 95 °C for 5 min, 42 cycles consisting of 95 °C for 15 sec, 62 °C for 15 sec, 72 °C for 20 sec, and a final extension step of 72 °C for 5 min.
NOTE: The optimal number of cycles and annealing temperature should be defined in advance for each pair of primers.
1.17. Perform gel electrophoresis in TAE buffer in 3 % agarose gel and document results.

2. H3K27me3 immunofluorescent staining

2.1. Grow the fibroblasts or iPS cells on the coverslips.
2.2. Fix the cells with 4 % PFA at room temperature for 15 min.
2.3. Rinse with PBS 3 times for 5 min.
2.4. Dissolve the primary antibody (1:100) in the antibody dilution buffer, 200 µl per coverslip.
NOTE: The optimal antibody dilution ratio should be determined experimentally. Usually, it is between 1:100 and 1:1000.
2.5. Put antibody solution on the coverslip with the cells. The solution should be held on the glass by surface tension. Place the coverslip carefully into the humidified chamber. Incubate overnight at 4 °C.
2.6. On the next day, rinse the coverslip in PBST 3 times at room temperature for 5 min. It is preferred to use an orbital shaker.
2.7. Dissolve the secondary antibody in PBS (1:500). Incubate in the humidified chamber at 37 °C for 1 h.
NOTE: The optimal antibody dilution ratio should be determined experimentally. Usually, it is between 1:100 and 1:1000.
2.8. Rinse with PBST 3 times at room temperature for 5 min.
2.9. Stain with DAPI at room temperature for 5 min.
2.10. Rinse with PBS.
2.11. Mount the coverslip on the microscope slide in the drop of ProLong Gold Antifade Mountant. The coverslip surface with the cell should be beneath.
2.12. Photo the nuclei using a confocal laser scanning microscope.

Results and discussion

The single-cell gene expression protocol is expected to provide "yes or no" information on a gene of interest expression. In the case of qPCR, the results could be (semi)quantitative.

We applied the proposed protocol to X-inactivation analysis in female-derived (XX) American mink embryonic fibroblasts and iPS cells (Pristyazhnyuk, Menzorov, 2020). The expected *Xist* expression was detected in 19 out of 20 XX fibroblasts, but not in XY fibroblasts (Fig. 2, *a*). We used the American mink *HK1* as a positive control for cDNA quality in XY fibroblasts. We detected *HK1* expression in 16 single cells out of 20 and no *Xist* expression. For XX fibroblasts, *Xist* expression was expected and such control was not necessary. Then we estimated *Xist* expression in American mink iPS cells with pluripotency marker *Rex1* as a control (see Fig. 2, *b*). In this experiment design, the presence

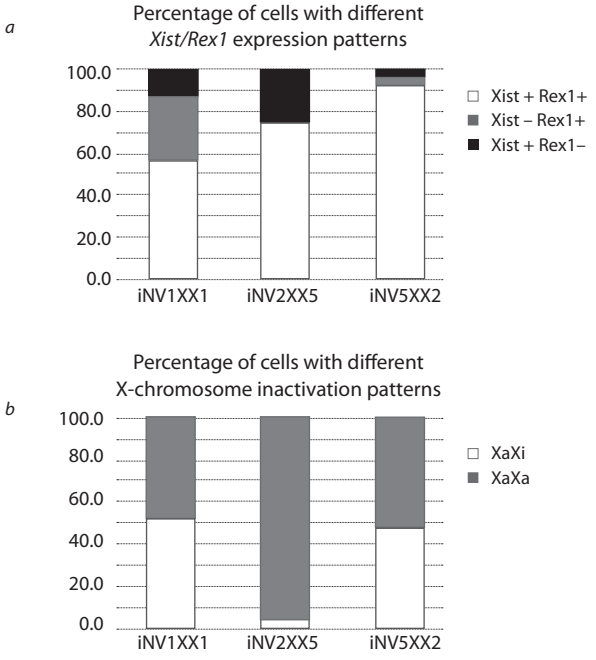

Fig. 3. X-chromosome inactivation analysis in American mink iPS cells. *a* – RT-PCR analysis; *b* – H3K27me3 immunofluorescent staining analysis

of the *Rex1* expression was considered a sign of pluripotency, and its absence in the presence of the *Xist* transcript – a sign of the differentiation. Interestingly, we were able to find two *Rex1* isoforms, homologous to the California sea lion (*Zalophus californianus*) (NCBI Reference Sequences XM_027599281.1 and XM_027599282.1). In different cells, we were able to see PCR products of either one of the isoforms or both. Thus, simultaneous amplification of different gene isoforms in single cells is possible with that protocol.

The immunofluorescent staining of the American mink fibroblasts is presented in fig. 2, *c*. The XY fibroblasts had no fluorescent signal as expected (data not shown). The diploid XX fibroblasts had one signal, and tetraploid – two, representing one or two Xi, respectively. The American mink iPS cells formed islands of cells with small nuclei surrounded by the bigger nuclei of the feeder cells (see Fig. 2, *d*). It should be noted, that the H3K27me3 signal was located mostly on the periphery of the colonies. We explain it by the partial differentiation of the peripheral cells.

Interestingly, the percentage of the American mink iPS cells with *Xist* expression exceeded that of the H3K27me3 mark (Fig. 3). There could be several possible reasons. First, the iPS cells could have changed XaXa status to XaXi without any changes in morphology and/or gene expression. It is known that cell density, duration of *in vitro* culture, and other factors could promote pluripotent stem cell differentiation. Second, though *Xist* is a well-known X-chromosome inactivation marker, its expression is one of the first steps of the X-chromosome inactivation process (Pinheiro, Heard, 2017). There may be an onset of *Xist* expression in XaXa cells that is revealed by RT-PCR but does not coincide with H3K27me3 chromatin modification, as it has not happened yet. Third, there might be a certain

threshold of expression of *Xist* and other genes to trigger X-chromosome inactivation (Barakat et al., 2010). Fourthly, X-chromosome inactivation and loss of pluripotency are not always synchronized on a single cell level in mouse ES cells with different pluripotency statuses. *Xist* expression appeared to be heterogeneous as well (Chen et al., 2016). That may be the case for American mink iPS cells. Due to our protocol constraints, we were not able to quantify the level of *Xist* expression, though we presume that it was lower than in fibroblasts. Overall, the presence of *Xist* transcript may not be enough to conclude the X-chromosome inactivation.

There is another well-known marker of Xi, ubiquitinated histone H2A (uH2A). We had tried to detect it as well. It appeared that in the case of the anti uH2A antibody (Ubiquityl-Histone H2A (Lys119) (D27C4) XP® Rabbit mAb; Cell Signaling Technology, cat. no. 8240) permeabilization should be done before fixation since the standard procedure had not provided the appropriate staining. Fixation with the subsequent permeabilization had worked successfully for the mink fibroblasts, but mink iPS cells detached, thus we had not included uH2A staining in the protocol.

Conclusion

We described detailed protocols for the analysis of gene expression and antigens in single cells. In particular, we have shown that those protocols could be successfully applied to X-chromosome inactivation in the American mink iPS cells.

References

Barakat T.S., Jonkers I., Monkhorst K., Gribnau J. X-changing information on X inactivation. *Exp. Cell Res*. 2010;316(5):679-687. DOI 10.1016/j.yexcr.2010.01.015.

Brown C.J., Hendrich B.D., Rupert J.L., Lafrenière R.G., Xing Y., Lawrence J., Willard H.F. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell*. 1992;71(3):527-542. DOI 10.1016/0092-8674(92)90520-m.

Chen G., Schell J.P., Benitez J.A., Petropoulos S., Yilmaz M., Reinius B., Alekseenko Z., Shi L., Hedlund E., Lanner F., Sandberg R., Deng Q. Single-cell analyses of X Chromosome inactivation dynamics and pluripotency during differentiation. *Genome Res.* 2016;26(10):1342-1354. DOI 10.1101/gr.201954.115.

Clemson C.M., McNeil J.A., Willard H.F., Lawrence J.B. XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *J. Cell Biol*. 1996;132(3):259-275. DOI 10.1083/jcb.132.3.259.

Fan G., Tran J. X chromosome inactivation in human and mouse pluripotent stem cells. *Hum. Genet*. 2011;130(2):217-222. DOI 10.1007/s00439-011-1038-1.

Goto Y., Gomez M., Brockdorff N., Feil R. Differential patterns of histone methylation and acetylation distinguish active and repressed alleles at X-linked genes. *Cytogenet. Genome Res.* 2002;99(1-4):66-74. DOI 10.1159/000071576.

Gross A., Schoendube J., Zimmermann S., Steeb M., Zengerle R., Koltay P. Technologies for single-cell isolation. *Int. J. Mol. Sci.* 2015;16(8):16897-919. DOI 10.3390/ijms160816897.

Heard E., Rougeulle C., Arnaud D., Avner P., Allis C.D., Spector D.L. Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X inactivation. *Cell*. 2001;107(6):727-738. DOI 10.1016/s0092-8674(01)00598-0.

Higuchi R. Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications (Perkin Elmer Cetus). 1989;2:1-3.

Jones C.D., Cornbleet P.J. Techniques for optimal cytocentrifuge slide preparation. *Laboratory Medicine*. 1997;11(28):713-716.

Kohlmaier A., Savarese F., Lachner M., Martens J., Jenuwein T., Wutz A. A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. *PLoS Biol*. 2004;2(7):E171. DOI 10.1371/journal.pbio.0020171.

Le A.V., Huang D., Blick T., Thompson E.W., Dobrovic A. An optimised direct lysis method for gene expression studies on low cell numbers. *Sci. Rep*. 2015;5:12859. DOI 10.1038/srep12859.

Marks H., Chow J.C., Denissov S., Françoijs K.-J., Brockdorff N., Heard E., Stunnenberg H.G. High-resolution analysis of epigenetic changes associated with X inactivation. *Genome Res*. 2009;19(8):1361-1373. DOI 10.1101/gr.092643.109.

Matveeva N.M., Fishman V.S., Zakharova I.S., Shevchenko A.I., Pristyazhnyuk I.E., Menzorov A.G., Serov O.L. Alternative dominance of the parental genomes in hybrid cells generated through the fusion of mouse embryonic stem cells with fibroblasts. *Sci. Rep*. 2017;7(1):18094. DOI 10.1038/s41598-017-18352-4.

McDonell L., Drouin G. The abundance of processed pseudogenes derived from glycolytic genes is correlated with their expression level. *Genome*. 2012;55(2):147-151. DOI 10.1139/g2012-002.

Menzorov A.G., Matveeva N.M., Markakis M.N., Fishman V.S., Christensen K., Khabarova A.A., Pristyazhnyuk I.E., Kizilova E.A., Cirera S., Anistoroaei R., Serov O.L. Comparison of American mink embryonic stem and induced pluripotent stem cell transcriptomes. *BMC Genomics*. 2015;16 Suppl. 13:S6. DOI 10.1186/1471-2164-16-S13-S6.

Nakagata N. Reproductive engineering techniques in mice. 3rd edn. Japan, Tokyo, Cosmo Bio Co.: 2016;4-5.

Okamoto I., Otte A.P., Allis C.D., Reinberg D., Heard E. Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science.* 2004;303(5658):644-649. DOI 10.1126/science.1092727.

PCR Primer Design (Methods in Molecular Biology). Basu S. (Ed.). 2nd edn. New York: Humana Press, 2015. DOI 10.1007/978-1-4939-2365-6.

Pinheiro I., Heard E. X chromosome inactivation: new players in the initiation of gene silencing. *F1000Res*. 2017;6:F1000 Faculty Rev-344. DOI 10.12688/f1000research.10707.1.

Plath K., Fang J., Mlynarczyk-Evans S.K., Cao R., Worringer K.A., Wang H., de la Cruz C.C., Otte A.P., Panning B., Zhang Y. Role of histone H3 lysine 27 methylation in X inactivation. *Science*. 2003;300(5616):131-135. DOI 10.1126/science.1084274.

Pristyazhnyuk I.E., Menzorov A.G. Generation of American mink induced pluripotent stem cells: a protocol. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding.* 2017;21(6):701-709. DOI 10.18699/VJ17.288. (in Russian)

Pristyazhnyuk I., Menzorov A. X-chromosome inactivation in American mink iPSCs. Proceedings of the Bioinformatics of Genome Regulation and Structure/Systems Biology (BGRS/SB-2020): The Twelfth International Multiconference, 06–10 July, Novosibirsk, Russia. 2020;508-509. DOI 10.18699/BGRS/SB-2020-310.

Rougeulle C., Chaumeil J., Sarma K., Allis C.D., Reinberg D., Avner P., Heard E. Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. *Mol. Cell Biol*. 2004;24(12):5475-5484. DOI 10.1128/MCB.24.12.5475-5484.2004.

Silva J., Mak W., Zvetkova I., Appanah R., Nesterova T.B., Webster Z., Peters A.H., Jenuwein T., Otte A.P., Brockdorff N. Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. *Dev. Cell*. 2003;4(4):481-495. DOI 10.1016/s1534-5807(03)00068-6.

Svec D., Andersson D., Pekny M., Sjöback R., Kubista M., Ståhlberg A. Direct cell lysis for single-cell gene expression profiling. *Front. Oncol*. 2013;3:274. DOI 10.3389/fonc.2013.00274.

Ye J., Coulouris G., Zaretskaya I., Cutcutache I., Rozen S., Madden T.L. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics.* 2012;13:134. DOI 10.1186/1471-2105-13-134.

Conflict of interest. The authors declare no conflict of interest.

Received March 11, 2022. Revised April 9, 2022. Accepted April 13, 2022.