

Video Article

Non-invasive Chromosome Screening (NICS) of Human Preimplantation Embryos: Sample Collection and Chromosomal Ploidy Analysis by MALBAC-NGS

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Abstract

Chromosomal abnormalities are common in human embryos and cause implantation failure, early pregnancy losses, and birth defects in practice of assisted reproductive technology (ART). Non-invasive chromosome screening (NICS) is an emerging technology that enables the selection of chromosomal-balanced embryos, without performing invasive embryo biopsy. Here we report the full protocol of NICS, which includes culture medium pretreatment, whole genome amplification (WGA) by multiple annealing and looping-based amplification cycles (MALBAC), library preparation for next generation sequencing (NGS) and NGS data analysis. To validate the reliability and efficiency of NICS, we have already performed NICS on 27 transfer cycles in 23 couples with balanced translocation, azoospermia, recurrent pregnancy loss (RPL), or recurrent implantation failure (RIF), 17 of them have achieved successful clinical pregnancies, and 9 among them have already obtained healthy live births. No pregnancy loss has been reported thus far. The NICS method avoids the need for embryo biopsy and therefore substantially increases the safety of its use.

Introduction

Assisted reproductive technologies (ART) have been increasingly used for treatment of infertility. However, the success rate of ART, such as in-vitro fertilization (IVF) has been limited and the pregnancy loss rate has been significantly higher than the normal population¹. The main cause of these problems are chromosomal abnormalities, which commonly exist in preimplantation human embryos². PGS came as an effective way to screen the embryos for chromosomal balance before implantation^{3,4}. And some studies have proved PGS can reduce the rate of abortion and improve the rate of pregnancy^{5,6,7,8}. However, PGS requires complex technical expertise, which requires specific training and experience. The invasive embryo biopsy procedure could also potentially cause damage to the embryos^{9,10}. Although long-term biosafety issue of embryo biopsy has not been evaluated thoroughly in human yet, animal studies have shown its negative influences on embryo development^{11,12}.

It was previously reported that trace amount of DNA materials are secreted into the culture medium during embryo development, and efforts have been made to perform comprehensive chromosome screening (CCS) using spent embryo culture medium^{13,14,15,16,17}. However, the detection rate and the accuracy of the tests have not met the requirement of extensive clinical use. We reported an improvement of using MALBAC for increasing the detection rate as well as the accuracy of the NICS test¹⁸. Here we report a detailed protocol including spent media sample preparation, NGS preparation and data analysis. By carefully removing cumulus cells from the oocytes, we perform intracytoplasmic single sperm injection (ICSI) and blastocyst culture. We then collect the Day 3-Day 5/Day 6 spent medium for MALBAC WGA and NGS library preparation. By using the NICS technology, we streamline the WGA and NGS library preparation steps in about 3 h, which enables obtaining CCS results noninvasively in about 9 h.

Protocol

Ethics statement: Institutional review board (IRB) approvals (Nanjing Jinlin: 2014NZKY-005; Wuxi Maternity: 2014-04-0515-02) were obtained. All of the embryos were voluntarily donated by patients, with informed consent obtained before performing the experiments on each embryo.

1. Preparation

1. Prepare the following reagents

1. Prepare fertilization medium plus 10% serum protein substitute (SPS) (IM) by adding 1 mL SPS to 9 mL Quinn's fertilization medium.
2. Make cleavage medium plus 10% SPS (GM) by mixing 9 mL Quinn's cleavage medium and 1 mL SPS.
3. Make blastocyst medium with 10% SPS (BM) by mixing 9 mL Quinn's blastocyst medium and 1 mL SPS.

4. Equilibrate all the above medium and 10 mL m-HTF medium with HEPES within 37 °C, 5% CO₂, 5% O₂ for more than 6 h.
2. (Optional) Prepare denudation and transfer pipettes by pulling glass Pasteur pipettes to generate fire polished open fine tips. The internal diameter of is 135-150 µm for denudation pipettes and 180-200 µm for transfer pipettes.
 1. Prepare manipulation pipettes by pulling glass Pasteur pipettes to generate fine fire polished and sealed end tips as show in **Figure 1**.

2. Sample Collection: Protocol 1

1. Pretreatment of oocyte-corona-cumulus complex (OCCC) before digestion with hyaluronidase.
NOTE: Here, ovarian stimulation was achieved with both Follicle Stimulating Hormone (FSH) and human menopause gonadotropin (hMG) preparations. When the lead follicle was >18 mm, 10,000 IU of chorionic gonadotropin (hCG) was used for final oocyte maturation. Oocytes retrieval was performed 36 h after trigger shot.
 1. Pick up oocytes and transfer to tissue culture dishes with 2.5 mL pre-warmed m-HTF covered with mineral oil. Carefully remove as many cumulus cells as possible without damaging these oocytes using 1 mL injection needle to cut out cumulus granulosa cells. Gently aspirate and release OCCCs in IM. Gently aspirate 2-3 times with transfer Pipette.
 2. Rapidly transfer these OCCCs to the central well of organ culture dish containing 1 mL IM using transfer Pipette. Incubate the oocytes in a 37 °C, 5% CO₂ and 5% O₂ incubator.
2. Digestion of OCCCs with hyaluronidase
 1. Add 1 mL 37 °C pre-warmed hyaluronidase (80 IU/mL) to the central well of organ culture dish containing OCCCs and mix thoroughly. Keep the final concentration of hyaluronidase at 40 IU/mL.
 2. Incubate the OCCCs on 37 °C thermal platform for 2 min. Observe the change under microscope every 30 s until only 1 or 2 layers of granulosa cells are left.
3. Denudation of granulosa cells
 1. Rapidly transfer the digested OCCCs to a 4-well IVF dish pre-filled with 0.5 mL GM covered by mineral oil. Observe the separated granulosa cells under the microscope.
 2. Gently aspirate and release the oocytes 5 times to remove the residual granulosa cells around the oocytes. Repeat this step with the remaining 3 wells to completely remove the granulosa cells.
4. Evaluation of the oocyte
NOTE: Granulosa cells need to be completely removed to avoid maternal contamination from the NICS results. If granulosa cells are still attached to the oocyte, perform either of the following procedure for complete removal of these cells.
 1. Evaluate the removal completeness of granulosa cells using a 20×10 microscope. If granulosa cells are still attached to the oocyte, perform one of the following procedures for complete removal of these cells:
 1. Transfer the oocyte with residual granulosa cells to the central well of the organ culture dish. Digest for 30 s (step 2.2.1) and gently aspirate and release the oocyte to remove residual granulosa cells using denudation pipette.
 2. Transfer the oocyte with residual granular cells to the central well of tissue culture dish with 2 mL pre-warmed m-HTF without serum. Place SPS on the round end of the manipulation pipette and roll the oocytes back and forth using the pipette as shown in **Figure 2** to completely remove granulosa cells around the oocyte.
 2. After performing ICSI¹⁹, transfer the oocytes into 30 µL GM micro-droplets (one oocyte to one micro-droplet) using transfer pipettes and incubate in a 37 °C, 5% CO₂ and 5% O₂ incubator. Record the day of ICSI as Day 0.
 1. Check the embryos at 8-9 am the next morning using 20×20 microscope. Score the embryos according to the Istanbul consensus workshop on embryo assessment¹⁸ on Day 1 for fertilization (about 18 h), Day 2 (about 45 h) and Day 3 (about 68 h) for embryo cleavage.
5. Wash the embryo.
 1. Prepare the 30 µL BM micro-droplets for each embryo covered with mineral oil in tissue culture dishes at 8-9 am of Day 3. Prepare another three 50 µL BM micro-droplets covered with mineral oil in new tissue culture dishes for washing. Incubate all micro-droplets at 37 °C before use.
 2. Transfer the Day 3 embryos into the BM micro-droplets. Gently aspirate and release the embryos 3 times in each droplet using denudation pipettes.
NOTE: This procedure can also help remove the residual granular cells attached to the embryo.
6. Transfer each embryo into a single BM micro-droplet. Perform blastocyst embryo culture to Day 5/Day 6 at 37 °C, 5% CO₂, 5% O₂.
7. Select the Day 5 blastocysts for vitrification based on laboratory criteria.
NOTE: This is when the embryos are fully expanded through to the hatched blastocyst with the grading of inner cell mass (ICM) and trophoblast reaching level B or above according to the Istanbul consensus workshop on blastocyst assessment²⁰. The whole blastocysts were given a score from 1 to 6 to assess their degree of development. The blastocysts were graded in three ranks based on morphological appearance. For example, the ICM was graded as A (many tightly packed cells), B (several loosely grouped cells) or C (few cells), and the trophoblast was graded as A (many cells forming a cohesive epithelium), B (fewer cells forming a loose epithelium) or C (very few large cells). The grade of selected embryo was 4-6 AA or AB or BA or BB or BC or CB for vitrification²⁰.
8. Perform vitrification at room temperature (RT).
 1. Gently adjust the ICM at a considerable distance from the targeted point of the 200 ms laser beam. Focus at the cell junction of the trophoblast to generate a small hole to release the fluid from the blastocoel cavity.
 2. After 5-7 min, transfer the blastocyst (with a minimum volume of spent BM) using a transfer pipette to equilibrated solution (ES) (vitrification kit) for equilibration. To prevent cross contamination, transfer each embryo using a new disposable transfer pipette.

3. After 15 min, place the embryo in vitrification solution (VS) for 1 min. Place the embryo in the vitrification device and immediately store it in liquid nitrogen.
9. For sample collection, transfer 15-20 μ L of spent BM (step 2.8.2) from each cultured embryo into a RNase/DNase-free PCR tube containing 5 μ L of cell lysis buffer.
NOTE: The same amount of BM that was not used for embryo culture is collected as a negative control. All collected samples are frozen immediately in liquid nitrogen then stored at -80°C until ready for NICS assay. Incubate early-stage blastocysts to Day 6 for sample collection.

3. Library Construction: Protocol 2

1. Culture Medium Pre-treatment
 1. Dilute 1 μ L positive control (10 ng human gDNA) in 199 μ L fresh culture medium. Mix thoroughly and centrifuge the tube briefly (2000 x g for 5 s).
 2. Transfer 10 μ L Day 5-Day 6 blastocyst culture medium, diluted positive control, and fresh culture medium to new 0.2 ml PCR tubes. Add 1 μ L MT enzyme mix to each PCR tube. Mix thoroughly and centrifuge briefly.
 3. Place the PCR tubes in a pre-heated NICSInst sample prep station and run lysis program as below using the following cycle: 75°C , 10 min; 95°C , 4 min; 22°C , forever.
 1. Click the "Lysis" icon to enter the setup screen. Select "Tube" for "Control mode", input 10 μ L for "Sample volume", select "On" for "Hotlid control", enter 105°C for the temperature, and select "No" for "Pause at the first seg". Click "OK" to proceed.
 2. Wait until "Remain time" shows "--:--:--", indicating the end of the program. Click "Stop" to terminate the program.
NOTE: Stop the program when the process is done. Proceed to the next step immediately.
2. Pre-Library Preparation
 1. Thaw the pre-library (pre-lib) buffer to RT. Mix thoroughly and centrifuge briefly.
 2. Prepare a master mix for pre-library reaction as follows: add 2 μ L pre-lib enzyme mix to 60 μ L pre-lib buffer. Mix thoroughly and centrifuge briefly.
 3. Add 60 μ L pre-library reaction mix into each pre-treated medium sample from step 3.1.4. Mix thoroughly and centrifuge briefly.
 4. Put the PCR tube(s) from step 3.2.3 in the sample prep station and run the pre-library program as below using the following cycles: 95°C , 2 min; (15°C , 40 s; 22°C , 40 s; 33°C , 30 s; 65°C , 30 s; 72°C , 40 s; 95°C , 10 s; 63°C , 10 s) \times 12 cycles; 4°C , forever.
 1. Click the "Pre_Lib" icon to enter the set-up screen. Select "Tube" for "Control mode", input 70 μ L for "Sample volume", select "On" for "Hot lid control", enter 105°C for the temperature, and select "No" for "Pause at the first seg". Click "OK" to proceed. Wait until "Remain time" shows "--:--:--". Click "Stop" to terminate the program.
NOTE: Stop the program when the process is done. Proceed to the next step immediately.
3. Library Preparation
 1. Thaw the library buffer to RT. Mix thoroughly and centrifuge briefly.
 2. Prepare a master mix for library reaction as follows: add 1.6 μ L library enzyme mix to 60 μ L library buffer. Mix the reaction thoroughly and centrifuge briefly.
 3. Add 60 μ L library reaction mix and 2 μ L barcode primer to each pre-library product (step 3.2.3). Mix the reaction thoroughly and centrifuge briefly.
 4. Place the PCR tube(s) in the thermal cycler and run the library preparation program as below program using the following cycles: 94°C , 30 s; (94°C , 25 s; 62°C , 30 s; 72°C , 45 s) \times 17 cycles; 4°C , forever.
 1. Click the "Lib_Prep" icon to enter the set-up screen. Select "Tube" for "Control mode", input 130 μ L for "Sample volume", select "On" for "Hotlid control", enter 105°C for corresponding temperature, and select "No" for "Pause at the first seg". Click "OK" to proceed. Wait until "Remain time" shows "--:--:--". Click "Stop" to terminate the program.
4. Library Purification
 1. Take out magnetic beads (see Table of Materials) from $2-8^{\circ}\text{C}$ at least 20 min before the purification step. Vortex and mix the beads for 20 s. Dispense enough beads for the purification step into a new 1.5 mL micro-centrifuge tube and warm beads to RT.
 2. Add 1 \times magnetic beads to each library. Mix by pipetting up and down at least 10 times and incubate at RT for 5 min. Centrifuge briefly and place it on the magnetic stand.
NOTE: For example, add 100 μ L magnetic beads to 100 μ L library sample.
 3. Wait for about 5 min until the solution becomes clear. While on the magnetic stand, carefully aspirate the solution and discard. Add 200 μ L freshly prepared 80% ethanol to the tube. Incubate at RT for 30 s and carefully remove the supernatant. Repeat the wash step one more time.
 4. Remove as much ethanol as possible. Air dry the beads on the magnetic stand for about 5-10 min at RT. Remove the tube from the magnetic stand and add 17.5 μ L elution buffer. Vortex to resuspend and centrifuge briefly. Incubate at RT for 5 min.
 5. Place the tube onto the magnetic stand and wait till the solution becomes clear. Carefully transfer 15 μ L supernatant to a new tube.
5. Quantify purified libraries using a fluorometer according to the user guide of dsDNA HS assay kit²¹. The yield of libraries ranges from ~ 15 to 300 ng. Take 10 ng of each library sample for pooling.
6. Perform sequencing as per the system user guide²².
NOTE: Purified libraries sequence at single end 50 bp on the platform, yielding about 2 million reads for each sample. A 0.03 x sequencing depth is recommended.
7. Data Analysis

1. Enter the users' Name and Password in the login page (**Figure 3A**). In the *YKPGS_analysis_tool* page, select the "data format", "sequencing method", and "the way of construction". Enter the "users' mail address" (**Figure 3B**).
2. Function of *YKPGS_analysis_tool kit*
 1. Upload the data generated from the sequencing system.
NOTE: The NICS CNV analysis is performed automatically by the *YKPGS_analysis_toolkit* software. After the analysis, the result will be sent to the email address the user has provided. The modules of *YKPGS_analysis_tool kit* (**Figure 3C**) are as follows:
1) dir.txt: A parameter file, consists of two lines: data direction and user email. It is required to re-set up parameters each time before running the software. 2) *YKPGS_analysis_toolkit.bat*: After setting up parameters in dir.txt, double click it to upload Fastq data and analysis. 3) reanalysis.bat: In case of an incomplete data upload due to problem like internet misconnection, double click reanalysis.bat to re-upload and analyze data. 4) Program files: Data upload tools.
3. Operate the "*YKPGS_analysis_toolkit*"
 1. To edit "dir.txt" file, open and edit "dir.txt" file with notepad, enter the Miseq default output data direction in the first line. Enter the user's email address in the second line to receive the analysis result. Save the file.
 2. Data upload and analysis (A network > 1MB is advised)
 1. Double click the "*YKPGS_analysis_toolkit*" tool. The data will be uploaded and analyzed synchronously. The result will be sent to the user's email when the analysis completed.
NOTE: If an incomplete report is received, double click Reanalysis.bat tool to restart the process.

Representative Results

We applied the method on a patient with a balanced translocation. IRB approval and informed consents were obtained before applying the NICS assay on the patient. Karyotype analysis of the patient showed a balanced translocation (1; 18) (p13.3; q21). We obtained a total of six blastocysts from the patient and performed NICS on Day 3-Day 5 culture medium of all six embryos. Chromosome abnormalities caused by the parents' balanced translocation were detected in five of them with the NICS assay and therefore could not be used for transfer (**Figure 4A-E**). The NICS results of the two embryos showed the same karyotype 45, XN, -18($\times 1$) are both chromosome 18 deletion (**Figure 4A, B**). The karyotype 46, XN, -1p (pter→p21.1, $\times 1$) is only the short arm of chromosome 1 pter→p21.1 region deletion (**Figure 4D**). The karyotype 46, XN, +1p (pter→p21.2, $\times 3$), -18(q21.32→qter, $\times 1$) means both short arm of chromosome 1 pter→p21.2 region duplication and long arm of chromosome 18 q21.32→qter region deletion caused by the parents' balanced translocation (**Figure 4E**). One out of the six blastocysts showed a normal karyotype with NICS and this was selected for transfer (**Figure 4F**), which resulted in a healthy pregnancy and live birth. Although the karyotype 46, XN, +5q ($\times 4$), -8($\times 1$, mos) is chromosome 5 duplication and 8 mosaic different from the parents' chromosome abnormalities, the NICS assay can screen aneuploidy in all 24 chromosomes. It provides a new method for transferring single normal karyotype blastocyst. We have already performed NICS on IVF embryos from 23 couples with balanced translocation, azoospermia, or recurrent pregnancy loss. 17 of them have achieved successful clinical pregnancies, 9 among them have had healthy live births. The NICS method avoids the need for embryo biopsy and therefore substantially increases the safety of its use.

Figure 1. Diagram of the manipulation pipette. Glass Pasteur pipette was fired and pulled into sealed end manipulation pipette for denuding oocyte. Check the polished degree of the tip under microscope to avoid damage the oocyte. [Please click here to view a larger version of this figure.](#)

Figure 2. Schematic diagram of oocyte denudation to remove the residual granulosa cells. The m-HTF medium without serum is represented in blue, the brown sphere represents the oocyte with residual granulosa cells, the long stick represents the manipulation pipette, which is dipped in SPS. Denudate the oocyte by rolling back and forth. [Please click here to view a larger version of this figure.](#)

Figure 3. Software used for data analysis. (A) The login page for user. Users can login their account on NICS/PGS CNV analysis tool for data analysis at <http://pcat.yikongenomics.cn/>. The PGS CNV analysis tool is also applicable to NICS assay using the same analysis pipeline with PGS assay. (B) Option page for analysis. There are different options that the user can select, such as data format, sequencing method and the way of construction. The users' mail address is used for providing analysis report. (C) The modules of the *YKPGS_analysis_toolkit*, including four elements (e.g. Program files, dir.txt, *YKPGS_analysis_toolkit.bat*, and Reanalysis.bat). [Please click here to view a larger version of this figure.](#)

Figure 4. Embryo screening and selection using NICS from a patient carrying a balanced translocation of chr 1/18. A total of six embryos successfully developed to the blastocyst stage. Day3-Day5 culture medium from these embryos was collected for the NICS assay. (A) and (B) the NICS results of the two blastocyst embryos shows the same karyotype 45, XN, -18($\times 1$); both are chromosome 18 deletion. (C) shows karyotype 46, XN, +5q ($\times 4$), -8($\times 1$, mos) (chromosome 5 duplication and 8 mosaic). (D) shows karyotype 46, XN, -1p (pter→p21.1, $\times 1$) (only the short arm of chromosome 1 pter→p21.1 region deletion). (E) shows karyotype 46, XN, +1p (pter→p21.2, $\times 3$), -18(q21.32→qter, $\times 1$) is short arm of chromosome 1 pter→p21.2 region duplication and long arm of chromosome 18 q21.32→qter region deletion caused by the parents' balanced translocation. (F) shows balanced chromosomal composition and was therefore selected for implantation. The x axis shows 22 autosomes in red and blue and the y axis indicates the copy number of each autosome. The gray dots are the ruler scale of copy number response; each bin window and normal karyotype of copy number must be 2. [Please click here to view a larger version of this figure.](#)

Discussion

If the NICS results are contaminated with parental genetic material, then make sure all cumulus-corona radiata cells are removed and ensure that ICSI is performed for fertilization. Avoid inappropriate storage of the culture medium or template preparation processes that can potentially degrade DNA. Decontaminate the workspace thoroughly by DNase and RNase decontamination reagents. To avoid the contamination from other embryos, always culture one embryo in single droplet of medium to avoid cross-contamination from Day 3. If amplified products show up in the negative control, external DNA materials may have contaminated the reagent or the workspace. Clean the workspace by DNA and RNA removing reagents. Use nuclease-free materials and aliquot the reagents after first use.

The limitations of NICS are as follows: 1) All the cumulus-corona radiata cells (which are of maternal origin and usually with normal chromosomal composition) must be removed before performing ICSI. If the removal is not complete, residual cumulus-corona radiata cells may release DNA during embryo development, and the external DNA would also be amplified thereby potentially contribute to false-negative detection. 2) The NICS procedure is highly recommended to be performed in conjunction with ICSI, due to the difficulty of removal of any supernumerary sperm attached to the zona pellucida. If cleavage culture medium is routinely replaced on Day 3, this may decrease the likelihood of contamination due to residual cumulus cells and supernumerary sperm. All precautions must be taken to reduce such contamination to a minimum if NICS is used routinely in clinical IVF.

The NICS method avoids embryo biopsy and therefore substantially increases the safety of its use. The comparison between NICS and the blastocysts biopsy proves NICS is an easy, timesaving, highly sensitive and reproducible preimplantation screening technique for ART. In comparison to the procedure of performing blastocyst-stage biopsy, which requires considerable training and expertise to perform the sophisticated embryo manipulation, NICS can be easily performed since it involves a simple collection of the spent medium followed by IVF¹⁸. It requires no qualification of PGS/PGD in some countries.

NICS has the potential use in chromosome screening in clinical IVF, not only for ICSI but also IVF embryos. Although ICSI is highly recommended, there must be some methods to remove the sperm attached to the zona pellucida. The influence of sperm can be neglected. Morphological assessment is a traditional way for embryo evaluation, but in most cases, chromosomal abnormality of the embryo is invisible for morphological analysis. Combination of morphological assessment with NICS assay to transfer a ploidy embryo with proper morphology into the uterus, might improve the ongoing pregnancy rates and live birth rate. Randomized clinical trials will be designed and performed using the NICS assay with single embryo transfer to evaluate the clinical effectiveness in different patient groups in the future.

All cumulus-corona radiata cells would need to be removed thoroughly from the oocytes before fertilization. Oocytes should be fertilized by ICSI. Avoid adding human-derived proteins and supplements to the culture medium. The culture medium must be changed at Day 3 and collected at Day 5-Day 6 when blastocysts fully expand. Embryos should be cultured in individual droplets of culture medium from Day 3. When collecting the culture medium, change transfer pipettes between samples to avoid contamination.

Disclosures

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