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TITLE:

Semiconductor Sequencing for Preimplantation Genetic Testing for Aneuploidy

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next-generation sequencing, semiconductor sequencing, library construction, preimplantation genetic testing for aneuploidy (PGT-A), whole genome amplification (WGA), blastocyst

SUMMARY:

Here, we introduce a semiconductor sequencing method for preimplantation genetic testing for aneuploidy (PGT-A) with the advantages of short turnaround time, low cost, and high throughput.

ABSTRACT:

Chromosomal aneuploidy, one of the main causes leading to embryonic development arrest, implantation failure, or pregnancy loss, has been well documented in human embryos. Preimplantation genetic testing for aneuploidy (PGT-A) is a genetic test that significantly improves reproductive outcomes by detecting chromosomal abnormalities of embryos. Next-generation sequencing (NGS) provides a high-throughput and cost-effective approach for genetic analysis and has shown clinical applicability in PGT-A. Here, we present a rapid and low-cost semiconductor sequencing-based NGS method for screening of aneuploidy in embryos. The first step of the workflow is whole genome amplification (WGA) of the biopsied embryo specimen, followed by construction of sequencing library, and subsequent sequencing on the semiconductor sequencing system. Generally, for a PGT-A application, 24 samples can be loaded and sequenced on each chip generating 60−80 million reads at an average read length of 150 base pairs. The method provides a refined protocol for performing template amplification and enrichment of sequencing library, making the PGT-A detection reproducible, high-throughput, cost-efficient, and timesaving. The running time of this semiconductor sequencer is only 2−4 hours, shortening the turnaround time from receiving samples to issuing reports into 5 days. All these advantages make this assay an ideal method to detect chromosomal aneuploidies from embryos and thus, facilitate its wide application in PGT-A.

INTRODUCTION:

Choosing good-quality viable embryos with normal chromosome copy numbers (euploid) for transfer in assisted reproduction helps to improve pregnancy outcomes. Traditionally, the well-established morphological grading system is widely used for embryo evaluation due to its easy availability and non-invasive nature. However, it has been shown that morphological assessment can only provide limited information on embryo quality[1] and implantation potential[2]. One fundamental reason is its inability in evaluating the chromosomal composition of the embryos.

Chromosomal aneuploidy (abnormal copy number of chromosomes) is one of the main causes leading to embryonic development arrest, implantation failure or pregnancy loss. The occurrence of aneuploidy has been well documented in human embryos, accounting for 60%−70% in cleavage-stage embryos[3,4] and 50%−60% in blastocysts[5]. This, to some extent, has contributed to the bottleneck in improving the pregnancy rate of in-vitro fertilization (IVF) treatment, which has maintained at around 35%−40%[6,7]. Therefore, selecting euploid embryos for transfer is believed to be beneficial for improving pregnancy outcomes. To this end, preimplantation genetic testing for aneuploidy (PGT-A) has been further developed to investigate embryo viability using genetic approaches. There are increasing numbers of randomized controlled trials and cohort studies supporting the crucial role of PGT-A. It has been proved that the application of PGT-A decreases the miscarriage rate and increases clinical pregnancy rate and implantation rate[8], ongoing pregnancy rate and live birth rate[9].

Historically, different methods have been applied in PGT-A, such as fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), array-CGH, and single nucleotide polymorphism (SNP)-microarray. Previous studies have indicated that PGT-A for

cleavage-stage embryos by FISH yields results that are poorly consistent with those obtained by comprehensive chromosomal screening (CCS) of corresponding blastocysts using array-CGH or SNP-microarray[10]. These discrepancies can be attributed to chromosomal mosaicism, FISH technical artifacts, or embryonic self-correction of chromosomal segregation errors during development[11]. It has been widely recognized that using blastocyst trophectoderm (TE) biopsies for array-based PGT-A such as array-CGH or SNP-microarray is effective for identifying the chromosomal imbalance in embryos[10,12]. Recently, single-cell next-generation sequencing (NGS) provides a high-throughput and cost-effective approach for genetic analysis and has shown clinical applicability in PGT-A[13–15], which make it a promising alternative to currently available methods.

Here, we present a fast, robust, and low-cost semiconductor sequencing-based NGS method for screening of aneuploidy in human embryos. The first step of the workflow is whole genome amplification (WGA) of the biopsied embryo specimen, using a single-cell WGA kit, followed by construction of sequencing library, and subsequent sequencing on the semiconductor sequencing system.

Through detecting the H^+ ions that are released from each deoxyribonucleoside triphosphate incorporation during DNA strand synthesis, the system transfers the chemical signals (pH change) captured by the semiconductor elements to direct digital data, which are further interpreted into DNA sequence information. Eliminating the requirement for expensive optical detection and complex sequencing reactions, this simple sequencing chemistry reduces total reagent cost and shortens the sequencing running time into 2−4 hours[16]. More importantly, based on the manufacturer's performance specifications, the semiconductor sequencing platform can generate up to 15 GB sequencing data (depends on the quality of library) per run, which is significantly higher than some of the other sequencers producing only around 3−4 GB data (with 2 x 75 bp read length)[17]. In clinical applications of PGT-A, this platform can achieve 24 samples per chip generating up to 80 million reads[17] and at least one million unique reads of each sample. The read depth can ensure that each sample has at least 0.05x whole genome coverage. The above advantages of this platform make it an ideal screening method and thus, facilitate its wide applications in PGT-A[18].

PROTOCOL:

Ethical approval was granted by the Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee (Reference Number: 2010.432). Research license was approved by the Council on Human Reproductive Technology of Hong Kong (Number R3004).

1. Whole genome amplification

1.1. Prior to start, check the volume of magnetic beads (**Table of Materials**) to ensure that there is no less than 135 µL (with a 20% excess) for each sample. Keep the magnetic beads at room temperature (RT) for at least 30 min. Prepare 720 µL (with a 20% excess) of 70% ethanol for each sample. Equip a thermal cycler (**Table of Materials**) with heated lid at 105 °C.

NOTE: The newly prepared 70% ethanol (**Table of Materials**) should be used up within 3 days.

1.2. Sample preparation

NOTE: In the routine practice, 5 to 10 trophectoderm cells of the blastocyst are biopsied according to the practice guideline[19].

1.2.1. Suspend biopsies in 2 µL of 1x phosphate-buffered saline (PBS) in a single 0.2 mL polymerase chain reaction (PCR) tube.

1.2.2. Briefly spin the tube on a mini centrifuge for 3 s to collect the droplets.

1.3. Cell lysis and extraction

1.3.1. Thaw the cell extraction buffer (**Table of Materials**) and the extraction enzyme dilution buffer (**Table of Materials**) on ice, vortex and briefly spin on a mini centrifuge for 3 s before use.

1.3.2. Add 3 µL of cell extraction buffer to each tube from step 1.2.2.

1.3.3. Prepare a 5 µL cell lysis master mix for each sample by adding 4.8 µL of extraction enzyme dilution buffer and 0.2 µL cell extraction enzyme (**Table of Materials**). Mix well and aliquot into each tube from step 1.3.2. Flip the tube gently and briefly spin on a mini centrifuge for 3 s.

NOTE: tips should not touch the liquid containing the cell samples when adding the master mix.

1.3.4. Incubate the tube from step 1.3.3 in the thermal cycler with heated lid. Run the program with the following settings: 10 min at 75 °C, 4 min at 95 °C, hold at 4 °C.

1.4. Preamplification

1.4.1. Thaw the preamplification buffer (**Table of Materials**) on ice, vortex and briefly spin on a mini centrifuge for 3 s before use.

1.4.2. Prepare a 5 µL preamplification master mix for each sample by adding 4.8 µL of preamplification buffer and 0.2 µL of preamplification enzyme (**Table of Materials**). Mix well and aliquot into each tube from step 1.3.4. Flip the tube gently and briefly spin on a mini centrifuge for 3 s.

NOTE: Tips should not touch the liquid containing the DNA samples when adding the master mix.

1.4.3. Incubate the tube in the thermal cycler with heated lid. Run the program with the flowing settings: 2 min at 95 °C; 12 cycles for 15 s at 95 °C, 50 s at 15 °C, 40 s at 25 °C, 30 s at 35 °C, 40 s at 65 °C, 40 s at 75 °C; hold at 4 °C.

1.5. Amplification

1.5.1. Thaw the amplification buffer (**Table of Materials**) on ice, vortex and briefly spin on a mini centrifuge for 3 s before use.

1.5.2. Prepare a 60 µL amplification master mix for each sample by adding 25 µL of amplification buffer, 0.8 µL of amplification enzyme (**Table of Materials**), and 34.2 µL of nuclease-free water for WGA (**Table of Materials**). Mix well and aliquot into each tube from step 1.4.3. Flip the tube gently and briefly spin on a mini centrifuge for 3 s.

1.5.3. Incubate the tube in the thermal cycler with heated lid. Run the program with the following settings: 2 min at 95 °C; 14 cycles for 15 s at 95 °C, 1 min at 65 °C, 1 min at 75 °C; hold at 4 °C.

1.6. Purification of the WGA products

1.6.1. Transfer each WGA product from step 1.5.3 into new 1.5 mL tubes. Add 112.5 µL of magnetic beads into each tube. Vortex and incubate at RT for 5 min.

NOTE: Completely mix the magnetic beads before use.

1.6.2. Place the tubes onto a magnetic stand (**Table of Materials**) for 3 min until the supernatant is clear. Discard all the supernatant without disturbing the beads.

1.6.3. Add 300 µL of 70% ethanol to each tube. Rotate each tube 180° to let the beads run through the ethanol and rotate back to the original position. Discard all the supernatant after the beads have settled without disturbing the beads. Repeat this step once.

NOTE: Keep the tube on the magnetic stand while rotating the tube horizontally.

1.6.4. Briefly spin each tube on a mini centrifuge for 3 s. Place the tubes onto the magnetic stand until the residual supernatant is clear. Discard all the residual supernatant without disturbing the beads. Air dry the beads at RT for approximately 3 min.

1.6.5. Remove the tubes from the magnetic stand and resuspend the dried beads by adding 35 µL of low Tris-EDTA (TE) buffer (**Table of Materials**). Incubate at RT for 5 min.

1.6.6. Place the tubes onto the magnetic stand for 3 min until the supernatant is clear. Transfer all the supernatant containing eluted DNA to new 1.5 mL tubes without disturbing the beads.

2. Quality control of the WGA products

2.1. Quantify each purified WGA product from step 1.6.6 by a fluorometer assay (**Table of Materials**) according to the manufacturer's manual using 1 µL of WGA product as starting material.

NOTE: The accepted concentration of the WGA product is ≥ 10 ng/µL. Any product below this threshold is not recommended to proceed to the next steps.

3. Fragmentation of WGA products

3.1. Prior to start, preheat a dry block heater to 37 °C. Prepare 6 µL (with a 20% excess) of 0.5 M EDTA for each sample. Based on the concentration, aliquot 300 ng of DNA from each purified WGA product in step 1.6.6 to new 0.2 mL PCR tubes and bring volume to 16 µL with nuclease-free water for each tube.

3.2. Fragmentation

3.2.1. Prepare a 4 µL double stranded DNA (dsDNA) fragmentation reaction mix for each sample by adding 2 µL of dsDNA fragmentation reaction buffer (**Table of Materials**) and 2 µL of dsDNA fragmentation enzymes (**Table of Materials**). Mix well and aliquot into each tube from step 3.1. Vortex and briefly spin on a mini centrifuge for 3 s. Incubate the tubes for 25 min at 37 °C in a thermal cycler with heated lid.

3.2.2. Add 5 µL of 0.5 M EDTA immediately to each tube. Mix well by vortexing and briefly spin on a mini centrifuge for 3 s.

3.3. Purification and resuspension

3.3.1. Transfer each product from step 3.2.2 into new 1.5 mL tubes. Add 37.5 µL of magnetic beads to each tube. Mix by vertexing and incubate at RT for 5 min.

3.3.2. Purify the products as described from step 1.6.2 to step 1.6.4.

3.3.3. Elute each purified product as described in steps 1.6.5 and 1.6.6 by adding 32 µL of low-TE buffer.

4. Library construction

4.1. Blunt-end repairment, size selection and purification

4.1.1. Prepare a 20 µL blunt-end repairment mix for each sample by adding 9.5 µL of nuclease-free water, 10 µL of 5x end repair buffer (**Table of Materials**), and 0.5 µL of end repair enzyme (**Table of Materials**). Mix well and aliquot into each tube from step 3.3.3. Vortex and briefly

spin on a mini centrifuge for 3 s. Incubate the tubes at RT (20−25 °C) for 30 min.

4.1.2. Add 50 µL of magnetic beads to each tube from step 4.1.1. Vortex and incubate at RT for 5 min.

NOTE: Completely mix the magnetic beads before use.

4.1.3. Place each tube onto the magnetic stand for 3 min until the supernatant is clear. Transfer all the supernatant to new 1.5 mL tubes where 25 µL magnetic beads are added for each. Vortex the tubes with the transferred supernatant and incubate at RT for 5 min.

4.1.4. Purify the products in the incubated tubes as described from step 1.6.2 to step 1.6.4.

4.1.5. Elute each purified product as described in steps 1.6.5 and 1.6.6 by adding 32 µL of low-TE buffer.

NOTE: This is a safe stop point; the purified DNA from this step is stable at 4 °C for no more than 24 h.

4.2. Adaptor ligation and purification

4.2.1. Prepare a 17 µL adapter ligation mix for each sample by adding 10 µL of nuclease-free water, 5 µL of 10x ligase buffer (**Table of Materials**), 1 µL of P1 adapter (**Table of Materials**), and 1 µL of DNA ligase (**Table of Materials**). Mix well by vortexing for 5 s and spin on a mini centrifuge for 15 s, and aliquot into each tube from step 4.1.5.

4.2.2. Add 1 µL of adapters (**Table of Materials**) to each tube from step 4.2.1 according to the sample sheet (**Supplemental File: Sample sheet for adapter ligation**). Vortex and briefly spin on a mini centrifuge for 3 s. Incubate the tubes at RT (20−25 °C) for 20 min.

4.2.3. Add 75 µL of magnetic beads to each tube from step 4.2.2. Mix by vertexing and incubate at RT for 5 min. Then, purify the products as described from step 1.6.2 to step 1.6.4.

4.2.4. Elute each purified product as described in steps 1.6.5 and 1.6.6 by adding 15 µL of low-TE buffer. Transfer all the supernatant containing eluted DNA to new 0.2 mL 8-tube strips.

NOTE: This is a safe stop point; the purified DNA from this step is stable at 4 °C for no more than 24 h.

4.3. Amplification and purification

4.3.1. Prepare a 50 µL amplification master mix for each sample by adding 47.5 µL of super mix (**Table of Materials**) and 2.5 µL of primer mix (**Table of Materials**). Mix well by vortexing and briefly spin on a mini centrifuge, and aliquot into the 0.2 mL 8-tube strips from step 4.2.4.

4.3.2. Vortex the strips for 30 s and briefly spin on a mini centrifuge for 3 s. Incubate the strips in the thermal cycler with heated lid. Run the program with the following settings: 20 min at 72 °C; 5 min at 95 °C; 10 cycles for 15 s at 95 °C, 15 s at 62 °C, 1 min at 70 °C; 5 min at 70 °C; hold at 4 °C.

4.3.3. Transfer each product from step 4.3.2 into new 1.5 mL tubes. Add 97.5 µL of magnetic beads to each tube. Mix by vortexing and incubate at RT for 5 min.

4.3.4. Purify the products as described from step 1.6.2 to step 1.6.4.

4.3.5. Elute each purified product as described in steps 1.6.5 and 1.6.6 by adding 25 µL of low-TE buffer.

5. Quality control and dilution of the DNA library

5.1. Quantify each prepared DNA library from step 4.3.5 by the fluorometer assay according to the manufacturer's manual using 2 µL of DNA library as starting material.

5.2. The accepted concentration of the DNA library is ≥ 0.5 ng/µL and that of the positive control (**Table of Materials**) is ≤ 15 ng/µL. If the concentration of the positive control varies too much from 15 ng/µL, repeat the quantification of the positive control until the concentration is close to 15 ng/µL. If the concentration of the library is below 0.5 ng/µL, restart from fragmentation (section 3).

NOTE: Ensure that the concentration of the positive control reaches the accepted value before quantifying the DNA library.

5.3. Dilute each library to 100 pmol by adding nuclease-free water. Add 1 µL of library to *n* µL of nuclease-free water; calculate *n* using the equation below:

$$n = \left(\frac{\frac{Q}{C} \times 6 \times 10^7}{306} \div 660 \right) - 1$$

where Q is the concentration of each library measured by the fluorometer assay and C is the concentration of the positive control measured by the fluorometer assay.

6. Sequencing

6.1. Prior to start, prepare 48 µL (with a 20% excess) of 1 M NaOH for each sample and one nuclease-free 1.5 mL tube. Thaw the master mix PCR buffer (**Table of Materials**) (2000 µL in volume) at RT. Bring the sphere particles (**Table of Materials**) to RT.

6.2. Library pooling

6.2.1. Vortex each diluted library from step 5.3 and briefly spin 4x on a mini centrifuge for 3 s each time. Take 5 µL of each library to pool into the nuclease-free 1.5 mL tube. Vortex the mixed library and briefly spin on a mini centrifuge for 3 s.

6.3. Emulsion PCR using an emulsion system

6.3.1. Mix by inverting the oil bottle (**Table of Materials**) 3 times. Ensure that both the oil and breaking solution (**Table of Materials**) are at least 2/3 full. Add 150 µL of breaking solution to 2 new recovery tubes (**Table of Materials**).

6.3.2. Install the new recovery tubes, recovery router and amplification plate. Vortex the master mix PCR buffer for 30 s and briefly spin on a mini centrifuge for 3 s.

6.3.3. Vortex the sphere particles and the mixed library from step 6.2.1 for 1 min and briefly spin on a mini centrifuge for 3 s.

6.3.4. Prepare a 2400 µL ligation mix by adding 172 µL of nuclease-free water, 8 µL of mixed library from step 6.3.3, 120 µL of enzyme mix (**Table of Materials**), and 100 µL of sphere particles to the tube containing 2000 µL master mix PCR buffer.

6.3.5. Set a pipette to 800 µL. Load the ligation mix from step 6.3.4 to the reaction filter (**Table of Materials**) through the sample port. Use a 1000P pipette to add 200 µL of reaction oil to the reaction filter.

6.3.6. Select the program **Proton: Ion PI Hi-Q OT2 200 Kit**, and then, select **Assisted** button to ensure that the device has been set up correctly by following the instructions on the monitor. Then, click **Next** to start the program.

6.4. Enrichment by an automatic enrichment system

6.4.1. When the emulsion PCR program is completed, click **Next**, and then click **Final Spin** to spin for 10 min. Take out the 2 recovery tubes after clicking **Open Lid**.

6.4.2. Discard the supernatant from the 2 recovery tubes until 100 µL remains in each tube, and label accordingly. Mix the solution well and transfer to a new 1.5 mL tube.

6.4.3. Add 200 µL of nuclease-free water to each recovery tube, wash by pipetting up and down several times, and transfer all the solution to the 1.5 mL tube in step 6.4.2. Repeat the wash step once.

6.4.4. Add 200 µL of nuclease-free water to one of the recovery tubes and wash by pipetting up and down several times. Transfer all the solution to the other recovery tube and wash by pipetting up and down several times. Then, transfer all the solution to the same 1.5 mL tube from step 6.4.3. Vortex the 1.5 mL tube for 30 s and centrifuge for 8 min at 15,500 x *g*.

NOTE: The final total volume of the emulsion PCR product in this step should be approximately 1200 µL.

6.4.5. Discard the supernatant in the tube and keep 20 µL of the emulsion PCR product. Add 80 µL of resuspension solution (**Table of Materials**) to the tube. Mix by pipetting up and down.

6.4.6. Prepare a 320 µL melt-off solution for each chip by adding 280 µL of polyethylene glycol sorbitan monolaurate solution (**Table of Materials**) and 40 µL of 1 M NaOH.

NOTE: 1 M NaOH should be stored at 4 °C or freshly prepared. Vortex before use.

6.4.7. Vortex the tube containing C1 beads (**Table of Materials**) for 30 s. Take 100 µL of C1 beads to a new 1.5 mL tube. Place the 1.5 mL tube onto the magnetic stand for 2 min at RT. Discard all the supernatant after the beads have settled without disturbing the beads.

6.4.8. Add 1 mL of wash solution C1 (**Table of Materials**) to the tube from step 6.4.7. Vortex for 30 s. Place the tube onto the magnetic stand for 2 min at RT. Discard all the supernatant after the beads have settled without disturbing the beads. Resuspend the beads by adding 130 µL of bead capture solution (**Table of Materials**).

6.4.9. Enrichment system (ES) setup

6.4.9.1. Load the sample (100 µL emulsion PCR product) from step 6.4.5, the washed beads (130 µL) from step 6.4.8, ES wash solution (300 µL) (**Table of Materials**), and melt-off solution (300 µL) from step 6.4.6 into the 8-tube strip. The layout order is: sample (tube 1), washed beads (tube 2), ES wash solution (tubes 3, 4, 5), and melt-off solution (tube 7). Keep tubes 6 and 8 empty.

6.4.9.2. Place the 8-tube strip from step 6.4.9.1 onto the ES. Install a pipette tip and a new 0.2 mL tube and start the program.

NOTE: Ensure that pipetting works normal.

6.4.10. Wash the sphere particles after the enrichment is completed.

6.4.10.1. Centrifuge the 0.2 mL tube from step 6.4.9.2 for 5 min at 15,500 x *g*. Discard the supernatant and keep 10 µL of the enrichment product. Add 200 µL of nuclease-free water to the tube. Mix by vortexing.

6.4.10.2. Centrifuge the 0.2 mL tube from step 6.4.10.1 for 5 min at 15,500 x *g*. Discard the supernatant and keep 10 µL of the enrichment product. Add 90 µL of nuclease-free water to the tube. Mix by vortexing.

6.5. Template preparation

6.5.1. Vortex the positive control and spin briefly. Add 5 µL of positive control to the 100 µL template (the enrichment product from step 6.4.10.2). Vortex and centrifuge for 5 min at 15,500 x *g*. Discard the supernatant and keep 10 µL of the template.

6.5.2. Add 20 µL of sequencing primer (**Table of Materials**) and 15 µL of annealing buffer (**Table of Materials**) to the template tube from step 6.5.1. Vortex the tube and briefly spin on a mini centrifuge for 3 s.

6.5.3. Incubate the tube from step 6.5.2 in the thermal cycler with heated lid. Run the program with the following settings: 2 min at 95 °C, 2 min at 37 °C, hold at 4 °C.

6.5.4. Add 10 µL of loading buffer (**Table of Materials**) to the tube from step 6.5.3. Mix by pipetting up and down.

6.6. Sequencer initialization

6.6.1. Check the tank pressure of nitrogen gas (total pressure ≥ 500 psi, output pressure ≥ 10 psi, optimum 20-30 psi). Top-up 100 mL deionized water (18.2 MΩ) to C1 and C2 tubes (**Table of Materials**) and install them to corresponding C1 and C2 positions on the sequencer.

6.6.2. Prepare W1 (32 µL of 1 M NaOH) and W3 (40−50 mL of W3 buffer [**Table of Materials**]) solutions. Prepare W2 solution by adding 1920 mL of deionized water (18.2 MΩ), a whole bottle of W2 buffer (**Table of Materials**) and 8−12 µL of 1 M NaOH, and invert 4−8 times to mix.

NOTE: Because the water quality varies geologically, adjust the volume of 1 M NaOH as needed. The starting pH of W2 is 5.9−6.1, and the optimal range after adjustment is 7.4−7.6. Change and install new reagent tubes and use lately used chip for washing.

6.6.3. Prepare the 4 new empty tubes from the sequencing supplement kit (**Table of Materials**). Label the 4 tubes as dGTP, dCTP, dATP, and dTTP, and add 70 µL of dGTP, dCTP, dATP, or dTTP (**Table of Materials**) to the corresponding tube (i.e., 70 µL dGTP to the tube labeled as dGTP, etc.). Vortex the tubes before use. Install the tubes to the corresponding positions designated on the sequencer (**Table of Materials**).

6.7. Chip wash

6.7.1. Wash the chip (**Table of Materials**) once by injecting 100 µL of isopropanol into the loading well of the chip. Remove the expelled liquid from the opposite well.

6.7.2. Wash the chip twice by injecting 100 µL of nuclease-free water into the loading well of the chip. Remove the expelled liquid from the opposite well.

6.7.3. Wash the chip once by injecting 100 µL of 0.1 M NaOH into the loading well of the chip. Remove the expelled liquid from the opposite well. Incubate at RT for 1 min.

6.7.4. Wash the chip once by injecting 100 µL of nuclease-free water into the loading well of the chip. Remove the expelled liquid from the opposite well.

6.7.5. Wash the chip twice by injecting 100 µL of isopropanol into the loading well of the chip. Remove the expelled liquid from the opposite well. Dry by blowing nitrogen onto the chip. Keep away from light.

6.8. Sample loading and sequencing

6.8.1. Mix the 55 µL sample from step 6.5.4 by pipetting up and down and load the sample to the loading well of the chip.

NOTE: Keep the pipette tip and the 0.2 mL PCR tube used in this step.

6.8.2. Place the chip onto the chip mini centrifuge (**Table of Materials**) when some sample enters the chip. Check the position and centrifuge the chip on the designated chip mini centrifuge for 10 min.

6.8.3. Prepare two new 1.5 mL tubes for the annealing buffer and flushing solution. Prepare the 50% annealing buffer by adding 500 µL of annealing buffer and 500 µL of nuclease-free water. Prepare the flushing solution by adding 500 µL of annealing buffer and 500 µL of 100 % 2-propanol.

6.8.4. Prepare two new 1.5 mL tubes and prepare the foaming mixture by mixing 49 µL of 50% annealing buffer and 1 µL of foaming solution (**Table of Materials**) in both tubes.

6.8.5. Set a pipette to 100 µL. Make bubbles by pipetting air into the foaming mixture from one of the two tubes from step 6.8.4. Make > 120 µL of bubbles and keep pipetting until no outstanding visible bubbles can be seen. Load 120 µL of bubbles into the loading well.

NOTE: Ensure that there are no outstanding visible bubbles. Otherwise, start it over.

6.8.6. Transfer the excessive expelled liquid from the exit well from step 6.8.5 to the loading well by pipetting. Do not pipette bubbles. Centrifuge the chip for 30 s on the chip mini centrifuge.

6.8.7. Repeat step 6.8.5 by using the second tube containing the foaming mixture from step 6.8.4.

6.8.8. Add 55 µL of the 50% annealing buffer to the 0.2 mL tube kept in step 6.8.1. Use the kept pipette tip in step 6.8.1 to pipette up and down. Load all the 55 µL annealing buffer to the

loading well. Centrifuge the chip for 30 s on the designated chip mini centrifuge.

6.8.9. Load 100 µL of flushing solution into the chip loading well and discard the expelled liquid from exit well. Repeat this loading step once.

NOTE: If there are bubbles in the chip, expel small bubbles by big bubbles and flush by flushing solution. This can be achieved by pipetting 100 µL of flushing solution and leaving 5 µL of air below the flushing solution. Therefore, when pipetting the 105 µL into the chip, air will form a big bubble that can expel the small bubbles, and then, the big bubble can be expelled by the following flushing solution.

6.8.10. Load 100 µL of the 50% annealing buffer into the chip loading well. Repeat this loading step for a total of 3 times.

6.8.11. Add 6 µL of the sequencing enzyme into 60 µL of the 50% annealing buffer into a new 1.5 mL tube. Mix by pipetting up and down. Load 65 µL of this mixed solution into the chip loading well. Pipette slowly to avoid foaming.

6.8.12. Keep the chip away from light and incubate at RT for 5 min.

6.8.13. After the incubation, immediately load the chip onto the sequencer and click **Start the sequencing run** on the screen to start sequencing.

NOTE: The sequencing raw data and quality control files will be uploaded automatically to the company for data analysis.

REPRESENTATIVE RESULTS:

Based on this modified protocol, the semiconductor sequencing platform was for the first time, applied for PGT-A. We tested on biopsies from both cleavage-stage blastomeres and blastocyst-stage embryos. It is suggested that the biopsied cells undergo WGA as soon as possible to prevent any degradation of DNA. A previous study compared the performance of different WGA methods and indicated that the method we described here had the best uniformity at the bin size of 100 KB[20]. Considering the performance of both uniformity and median absolute pairwise difference (MAPD)[21], this WGA method was chosen for PGT-A using the semiconductor sequencer. Through a retrospective statistical analysis on 186 cleavage stage and 1135 blastocyst stage embryos, we observed that the WGA success rates were 95.4% in blastomere samples and 96.9% in blastocyst samples (**Figure 1**). The purification step before library construction as a size selection procedure was crucial for sequencing quality by capturing large DNA fragments. Additionally, it facilitated the input amount of 300 ng for library construction. The enzymatic fragmentation method enabled an efficient shearing of WGA products into approximately 160 bp.

Data analysis was conducted using the Euclidean distance and circular binary segmentation (EDCBS) analysis system. In-house validation was performed to evaluate the robustness of this

bioinformatic algorithm. We established a reference database exclusive for PGT-A through sequencing 379 WGA products from 66 cell lines with known karyotypes by analyzing a bin size of 100 KB. From this database, a reference range was delineated as the threshold for copy number variant (CNV) calling and 10 MB was set as the cutoff for the detection level. Both sensitivity and specificity reached over 99% at this threshold (**Table 1**). In the application for PGT-A on embryo biopsies, the window size was set to 400 KB with a sliding window approach to reach enough reads. Quality control (QC) of each sample was determined by unique reads, MAPD and standard deviation of copy number variant (CNV·SD). Sample beyond one of the three indexes was defined as QC failure (**Figure 2C**). Interpretation of chromosome scatter plots (**Figure 2A**,**B,D)** was conducted by qualified geneticists following a workflow by comparing the identified CNV to DECIPHER, DGV, or ClinGen databases. Individual discrepancies were controlled by an expert curation procedure. Chromosomal abnormalities were grouped into aneuploidy and mosaicism in blastocyst samples. A copy number gain or loss within the range of 30%−70% was classified as carrying mosaic chromosomal composition; otherwise, the result would be interpreted as either euploidy or aneuploidy. In this study, the euploid rates were 45.2% in blastomeres and 52.3% in blastocysts, which echoed to published data[22,23].

FIGURE AND TABLE LEGENDS:

Figure 1: Demographic statistics of 1321 embryo biopsies tested by this method. (**A**) Data from 186 cleavage-stage embryos. (**B**) Data from 1135 blastocyst-stage embryos. WGA success rates are over 95% in both types of specimens. Sequencing quality control failure rates are 3.4% in the cleavage-stage group and only 1.9% in the blastocyst-stage group.

Figure 2: Representative results of PGT-A clinical application of embryo for the 23 pairs of chromosomes. Representative results of (**A**) euploidy; (**B**) aneuploidy (seq[GRCh37] (2)x3, (21)x3); (**C**) sequencing QC failed sample due to CNV·SD at 0.6571 (acceptance ≤ 0.4); (**D**) segmental mosaic deletion (55%) of 4p16.3p15.1 (29.50 MB).

Table 1: Sensitivity and specificity between different Log R ratios by the semiconductor sequencer. A total of 240 samples with known euploid karyotype results were tested by this method and called at different Log R ratios.

DISCUSSION:

Different from other sequencing chemistries, the sequencer described here uses semiconductor for the detection of nucleotides. The chip itself is an electronic device that detects hydrogen ions by polymerase-driven base incorporation[17], which enables 2−4 h sequencing time of the Proton program. Besides, the chip is a microwell chip that allows the localization of one target molecule, which is different from the flow cell sequencing chemistry by other sequencer providers. This protocol is a modified protocol optimized for the application of PGT-A. The optimization includes fragmentation of amplified DNA by the enzymatic method instead of sonication to reduce the chance of contamination as well as the size-selection and the PCR system for better performance with lower costs. Additionally, in the clinical setting, we used a validated in-house pipeline for variant calling.

There are critical steps to be aware of during the practice. Ethanol for purification needs to be freshly prepared before the experiment, as a high concentration of ethanol would cause insufficient wash of contaminated DNA while a low concentration would cause loss of target DNA. The fluorometer has to be calibrated by the positive control with a known concentration to ensure the accuracy. Besides, an adequate loading of the template is crucial for sequencing. Bubbles of the right size help to push the template sphere to fall into microwells, but too large bubbles may cause inadequate loading. Users can modify the number of samples (not necessarily 24) to be sequenced for each run. There are 96 indexes designed for this chip. But sequencing read depth will decrease with increasing samples per chip. One of the most common problems is low library concentration, which can be attributed to a low or poor-quality DNA output from purification due to residual ethanol or magnetic beads, or the cracking of beads as previously mentioned. A suboptimal DNA output may also result from low efficiency of PCR, which can be corrected by cautious preparation of the master mix with accurate sample inputs and temperature check of the thermal cyclers. For sequencing QC-failed samples, such as those with high CNV·SD values (**Figure 2C**), it is recommended to run the samples on a bioanalyzer for size distribution analysis.

One of the limitations of this method is its higher false single-nucleotide calling rate compared to other platforms. The error rate is 1% compared to only 0.1% indel false positive rate by other sequencers[17]. However, this is not a determining factor for CNV calling or PGT-A analysis. Compared to other platforms, application of the emulsion system minimizes operative discrepancies and pipetting errors, increasing the library quality. This is a significant advantage of our method compared to other platforms because the dsDNA concentration is very low and an accurate quantification is required for the following library pooling. Our method introduced the calibration of fluorometer quantification using a standard positive control to control detection error.

As a high throughput platform with short turnaround time, the semiconductor sequencer is ideal for PGT-A and can be widely applied to IVF patients with PGT-A clinical indications such as advanced maternal age[24]. Deleye et al. conducted parallel sequencing of human blastocysts to compare the performance of the semiconductor sequencer with other sequencers[25]. Moreover, this PGT-A kit has obtained the “special approval procedure on innovative medical devices” from the China Food and Drug Administration and has been clinically used for thousands of embryos. In terms of cost, the price of this platform is half the price per giga bases compared to another commonly used platform in the PGT-A market. The trophectoderm cells can reliably represent the genetic constitution of the embryo[26]. This method can potentially be developed for PGT for monogenic/single gene diseases (PGT-M) as Treff et al. have demonstrated[27]. In their model, they facilitated 300 MB to 1 GB throughput on PGT-M of 16 embryo biopsies and compared the results to two conventional PGT-M methods. By capturing and loading 16 samples, their method reached at least 100x read depth of the targeted region and resulted in 100% reliability and reproducibility[27]. The throughput of the semiconductor sequencer by our protocol can reach 15 GB and there are 96 barcodes designed; therefore, if applied to targeted PGT-M, a considerable number of embryo biopsies can be sequenced by one chip at a high read depth.

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DISCLOSURES:

The authors have nothing to disclose.

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738
739

Figure 1

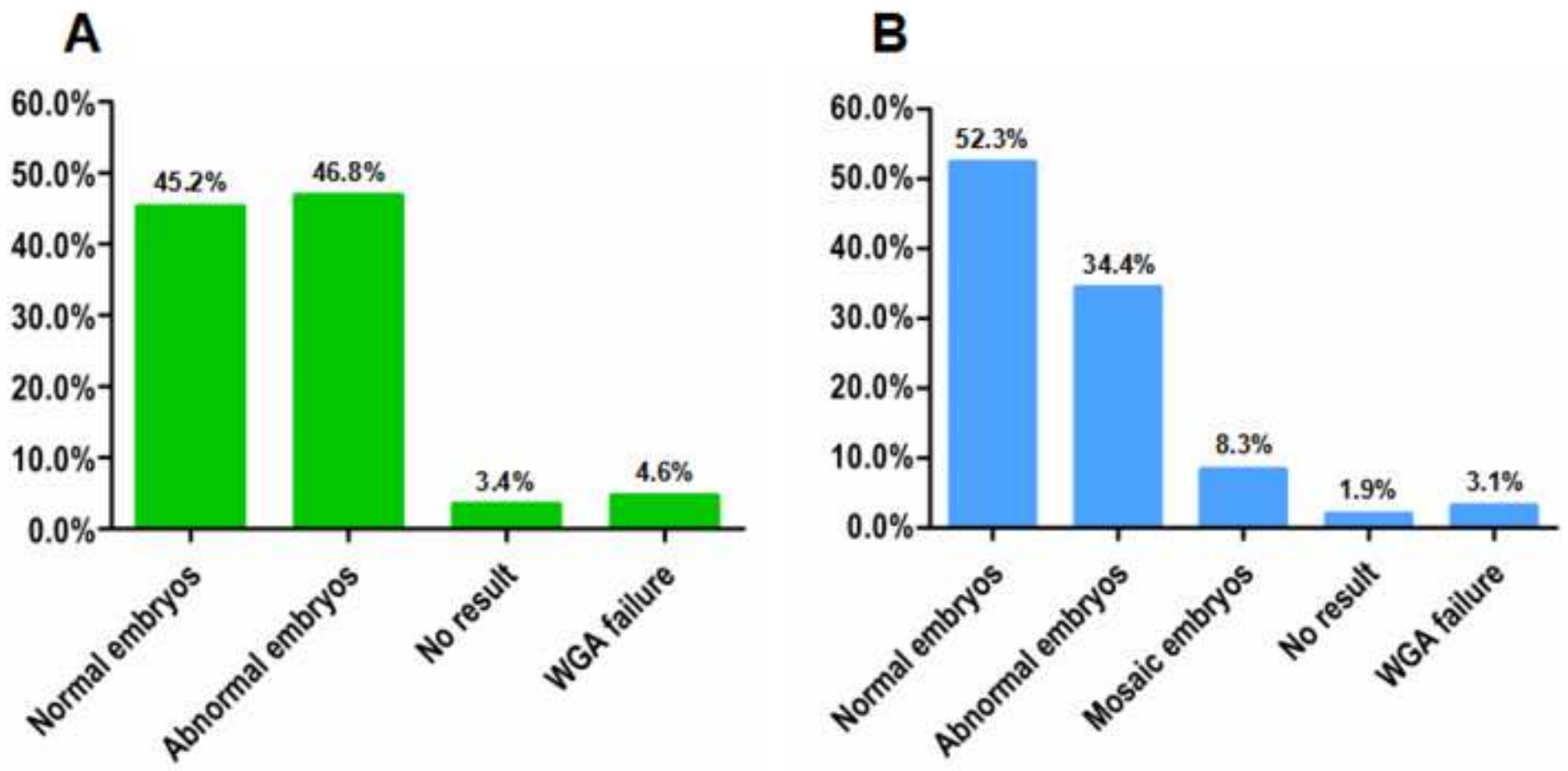
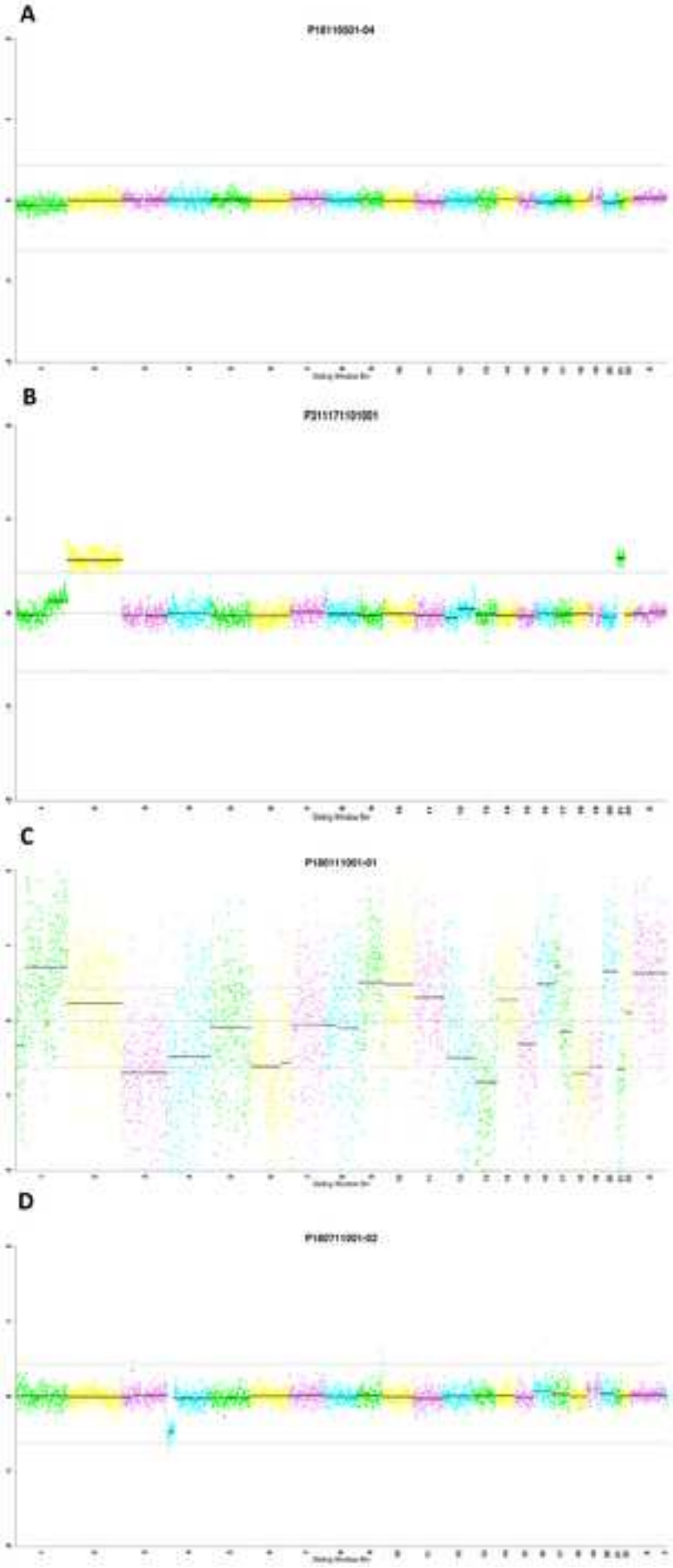


Figure 2

Click here to access/download;Figure;Figure2.tif



Table

	CNV threshold value					
	(-0.23, 0.20)	(-0.32, 0.26)	(-0.41, 0.32)	(-0.51, 0.37)	(-0.62, 0.43)	(-0.73, 0.48)
Sensitivity	100.00%	100.00%	100.00%	100.00%	98.30%	96.02%
Specificity	72.41%	81.03%	91.38%	99.10%	99.54%	100.00%

Generic Term	Commercial Name of the Kit/Material/ Equipment	Company
0.2 mL PCR tube	PCR tubes, 0.2 mL	Axygen
0.5M EDTA	UltraPure 0.5M EDTA, pH 8.0	ThermoFisher
1 x PBS	PBS, pH 7.4, Ca^{2+} and Mg^{2+} free	ThermoFisher
1.0 M NaOH	1.0 M NaOH (1.0N) solution	SIGMA-ALDRICH
1.5 mL tube	Eppendorf LoBind Tubes, 1.5 mL	Fisher Scientific
10 X Ligase Buffer	Ion Plus Fragment Library Kit	ThermoFisher
18 MΩ water system	ELGA PURELAB Flex 3 Water Purification System or Equivalent 18 MΩ water system	ThermoFisher
5 X End Repair Buffer	Ion Plus Fragment Library Kit	ThermoFisher
Amplification buffer	PicoPLEX WGA Amplification buffer	Rubicon Genomics
Amplification enzyme	PicoPLEX WGA Amplification enzyme	Rubicon Genomics
Amplification plate	Ion OneTouch Amplification Plate	
Annealing buffer	Ion PI Annealing Buffer	
Beads capture solution	MyOne Beads Capture Solution	
Bioanalyzer	Agilent 2100 Bioanalyzer instrument	Agilent
Breaking solution	Ion OneTouch Breaking Solution (black cap)	
C1 beads	Dynabeads MyOne Streptavidin C1	ThermoFisher
Cell extraction buffer	PicoPLEX WGA Cell extraction buffer	Rubicon Genomics
Cell extraction enzyme	PicoPLEX WGA Cell extraction enzyme	Rubicon Genomics
Chip	Ion PI Chip Kit v3	ThermoFisher
Chip minifuge	Ion Chip Minifuge, 230 V	ThermoFisher
dATP	Ion PI dATP	ThermoFisher
dCTP	Ion PI dCTP	ThermoFisher
dGTP	Ion PI dGTP	ThermoFisher
DNA Ligase	Ion Plus Fragment Library Kit	ThermoFisher
dNTP Mix	Ion Plus Fragment Library Kit	ThermoFisher
Dry bach	ThermoQ–Temperature Dry Bath	TAMAR
dsDNA fragmentase	NEBNext dsDNA Fragmentase	New England Biolabs
dsDNA fragmentase reaction b	NEBNext dsDNA Fragmentase	New England Biolabs
dTTP	Ion PI dTTP	ThermoFisher
Emulsion system	Ion OneTouch 2 Instrument	ThermoFisher
End Repair Enzyme	Ion Plus Fragment Library Kit	ThermoFisher
Enrichment system	Ion One Touch ES	ThermoFisher

Ethanol	Ethanol	SIGMA-ALDRICH
Extraction enzyme dilution bu	PicoPLEX WGA Extraction enzyme dilution buffer	Rubicon Genomics
Extraction enzyme dilution bu	PicoPLEX WGA Extraction enzyme dilution buffer	Rubicon Genomics
Fluorometer assay	Qubit 3.0 Fluorometer	ThermoFisher
Fluorometer assay	Qubit ds DNA HS Assay kit	ThermoFisher
Fluorometer assay	Qubit Assay Tubes	ThermoFisher
Foaming solution	Ion PI Foaming Solution	ThermoFisher
Index	Index for barcoding of libraries	BaseCare
Loading buffer	Ion PI Loading Buffer	ThermoFisher
Low-TE buffer	Solid(TM) Buffer Kit-1X Low TE Buffer	ThermoFisher
Magnetic beads	Agencour AMPure XP Kit	Beckman Coulte
Magnetic stand	DynaMag-2 magnet (magnetic rack)	ThermoFisher
Master mix PCR buffer	Ion PI Master Mix PCR buffer	
Mini centrifuge	Sorvall Legend Micro 17 Microcentrifuge	Micro 17
Nick Repair Polymerase	Ion Plus Fragment Library Kit	ThermoFisher
Nuclease-free water	Nuclease-free water	ThermoFisher
Nuclease-free water for WGA	PicoPLEX WGA Nuclease-free water	Rubicon Genomics
Oil bottle	Ion OneTouch Oil bottle	
P1 adapter	Ion Plus Fragment Library Kit	ThermoFisher
Positive control	double-strand DNA standard	
Preamplification buffer	PicoPLEX WGA Preamplification buffer	Rubicon Genomics
Preamplification enzyme	PicoPLEX WGA Preamplification enzyme	Rubicon Genomics
Primer mix	Library Amplification Primer Mix	ThermoFisher
Reaction filter	Ion OneTouch Reaction Filter	
Recovery router	Recovery Router	
Recovery tubes	Recovery Tubes	
Resuspension solution	ISP Resuspension Solution	
Sequencer	Ion Proton	ThermoFisher
Sequencing enzyme	Ion PI Hi‑Q Sequencing Polymerase	ThermoFisher
Sequencing primer	Ion PI Sequencing Primer	
Sever	server for sequencer	Lenovo
Sphere particles	Ion PI Sphere Particles	

SuperMix	Platinum PCR SuperMix High Fidelity	ThermoFisher
Syringe filters	Nalgene 25mm Syringe Filters	ThermoFisher
W2 buffer	Ion PI Hi‑Q W2 Solution	ThermoFisher
W3 buffer	Ion PI 1X W3 Solution	ThermoFisher
Wash solution C1	Ion OneTouch Wash Solution C1	
	The Ion PGM Hi‑Q View Sequencing Kit	ThermoFisher
	Ion Plus Fragment Library Kit	ThermoFisher
	Ion PI Hi-Q Sequencing 200 Kit (1 sequencing run per initialization)	ThermoFisher
	Ion PI Hi‑Q OT2 200 Kit	ThermoFisher

Catalog Number	Comments/Description
PCR-02D-C	
15575020	
10010023	
S2567	For Melt-off solution. Molecular grade
13-698-791	
4471252	
4474524	
4471252	
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-03.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-03.
	In kit: Ion OneTouch 2 Supplies (Part No. A26367). Extended kit component in Sheet 5
G2939AA	
	In kit: Ion PI Hi‑Q OT2 Solutions 200 (Part No. A26429). Extended kit component in Sheet 5
65001	
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-00.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-03.
A26771	
4479673	
A26772	
A26772	
A26772	
4471252	
4471252	
HB-T2-A	
M0348L	
M0348L	
A26772	
INS1005527	ThermoFisher Catalog number: 4474778.
4471252	
8441-22	ThermoFisher Catalog number: 4469495. Extended kit component in Sheet 5

51976	This can be replaced by any brand's molecular grade absolute ethanol.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-01.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-02.
Q33216	This model has been replaced by Qubit 4 Fluorometer, Catalog number: Q33226.
M2002-02	
Q32856	
A26772	
	this is a in-house prepared index. Users can buy commercial product from ThermoFisher Ion Xpress Barcode Adapters Kits (Cat. No. 4474517)
A26772	
4389764	
A63880	
12321D	
75002430	
4471252	
AM9922	This can be replaced by other brand.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-03.
	Ion PI Hi‑Q OT2 Solutions 200 (Part No. A26429). Extended kit component in Sheet 5
4471252	Extended kit component in Sheet 3
	This is a in-house prepared DNA standard for calibration of Qubit before quantification of library.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-03.
R30050	This can be replaced by SurePlex DNA Amplification System,catalog number: PR-40-415101-03.
4471252	Extended kit component in Sheet 3
	Extended kit component in Sheet 5
	Extended kit component in Sheet 5
	Extended kit component in Sheet 5
DA8600	This model is imported by Da An Gene Co.,LTD. of Sun Yat-Sen University from ThermoFisher and has been certified by China Food and Drug Administration for clinical application. The catalog number in ThermoFisher is 4476610.
A26772	
T260	

4471252	
724-2045	Pore size: 0.45µm. Specifically for aqueous fluids.
A26772	
A26772	
A30044	Extended kit component in Sheet 2
4471252	Extended kit component in Sheet 3
A26772	Extended kit component in Sheet 4
A26434	Extended kit component in Sheet 5

Name of Material/ Equipment	Company
The Ion PGM Hi-Q View Sequencing Kit	
Wash 1 Bottle w/ label (250 mL)	ThermoFisher
Wash 3 Bottle w/ label (250 mL)	ThermoFisher
Ion PGM Reagent Bottle Sipper Tubes (blue)	ThermoFisher
Ion PGM Wash Bottle Sipper Tubes (gray，for 250-mL bottles)	ThermoFisher
Ion PGM Wash Bottle Sipper Tubes (gray, for 2-L bottles)	ThermoFisher
Reagent Bottles w/ labels (50 mL)	ThermoFisher
Ion PGM Hi-Q View Sequencing Polymerase	ThermoFisher
Ion PGM Hi-Q View Sequencing Primer	ThermoFisher
Ion PGM Hi-Q View Control Ion Sphere Particles	ThermoFisher
Ion PGM Hi-Q View Sequencing W2 Solution	ThermoFisher
Ion Cleaning Tablet	ThermoFisher
Annealing Buffer	ThermoFisher
Ion PGM Hi-Q View Sequencing W3 Solution	ThermoFisher
Ion PGM Hi-Q Sequencing dGTP (black cap)	ThermoFisher
Ion PGM Hi-Q Sequencing dCTP (blue cap)	ThermoFisher
Ion PGM Hi-Q Sequencing dATP (green cap)	ThermoFisher
Ion PGM Hi-Q Sequencing dTTP (red cap)	ThermoFisher
Ion PGM Wash 2 Bottle Kit	ThermoFisher
Wash 2 Bottle w/ label (2 L)	ThermoFisher
Wash 2 Bottle Conditioning Solution	ThermoFisher
Ion 318 Chip v2 BC	ThermoFisher
Ion 316 Chip v2 BC	ThermoFisher
Ion 314 Chip v2 BC	ThermoFisher
Ion PGM System and accessories	ThermoFisher
Ion PGM Torrent Server	ThermoFisher
Ion Chip Minifuge	ThermoFisher
Tank of compressed nitrogen (grade 4.5, 99.995% or better)	
Multistage (dual-stage) gas regulator (0-50 PSI, 2-3 Bar output)	ThermoFisher
ELGA PURELAB Flex 3 Water Purification System	ELGA
0.22-µm or 0.45-µm vacuum filtration system and filters	
Rainin Pipet-Lite LTS Pipette L-100XLS 10–100 µL	Rainin
Rainin Pipet-Lite LTS Pipette L-20XLS 2–20 µL	Rainin
Rainin LTS pipette tips, 200 µL, SR-L200F	Rainin
Rainin LTS pipette tips, 20 µL, SR-L10F	Rainin
PCR tubes, Flat Cap, 0.2-mL	Fisher Scientific
Ion PGM 2.5 L Waste Bottle	ThermoFisher
Ion PGM Controls Kit v3	ThermoFisher
Ion PGM Sequencing Sippers Kit	ThermoFisher

Catalog Number	Comments/Description
A30044	
A25591	
A25591	
A25591	
4488146	
4488145	
4488144	
4462921	
4483643	
4479672	
NC0393866	
4474524	
17014384	
17014392	
17005859	
17005860	
14-222-262	
4482565	
A30046	
4478682	

Name of Material/ Equipment	Company
Ion Plus Fragment Library Adapters Kit	
5X End Repair Buffer - 400 µL	
End Repair Enzyme - 20 µL	
10X Ligase Buffer - 200 µL	
DNA Ligase - 40 µL	
Nick Repair Polymerase - 160 µL	
dNTP Mix - 40 µL	
Adapters - 100 µL	
Platinum PCR SuperMix High Fidelity - 2 × 1000 µL	
Library Amplification Primer Mix - 100 µL	
Low-TE - 2 × 1.5 mL	

Catalog Number	Comments/Description
4471252	

Name of Material/ Equipment	Company
Ion PI Hi-Q Sequencing 200 Kit (1 sequencing run per initialization)	
Ion Proton Wash 2 Bottle w/ label	ThermoFisher
Tank of compressed nitrogen	
Multistage (dual-stage) gas regulator	Fisher Scientific
1/8" x 1/4" stem reducing coupler	McMaster
Non-interruptible Power Supply (UPS)	
NaOH (10 M), molecular biology grade	
0.22-µm or 0.45-µm vacuum filtration system and filters	
25-mL or 50-mL serological pipettes or 100-mL graduated cylinder	
Pipet-Aid XP Pipet Controller	Fisher Scientific
Rainin Pipet-Lite LTS Pipette L‑20XLS 2 to 20 µL	Rainin
Rainin Pipet-Lite LTS Pipette L‑100XLS 10 to 100 µL	Rainin
Rainin LTS pipette tips, 20 µL, SR-L10F	Rainin
Rainin LTS pipette tips, 200 µL, SR-L200F	Rainin
PCR tubes, Flat Cap,0.2-mL	Fisher Scientific
1.5-mL or 1.7-mL microcentrifuge tubes	
Glass bottles (1 L)	
Ice buckets and ice	
Vortex mixer with a rubber platform	
Thermal cycler with a heated lid	
Dry bath incubator	
Standard laboratory vacuum line or vacuum pump	
Liquid trap	
Tygon tubing	
Thermo Scientific Orion Star A111 pH Benchtop Meter Kit with electrode, electrode stand, and calibration buffers	Fisher Scientific
1 N HCl	
Magnetic stirrer (must hold 2-L bottle)	
Magnetic stir bar (4 cm)	
Squirt bottle	
140‑mL Reagent Tubes	
Enriched template‑positive Ion PI ISPs prepared with the Ion PI Hi‑Q OT2 200 Kit	ThermoFisher
Standard laboratory vacuum line or vacuum pump	
Liquid trap	
100% isopropanol	
Thermal cycler with heated lid	
Ion Chip Minifuge (Cat. No. 4479672 or 4479673) equipped with Ion Proton Rotor and Buckets (Cat. No. 4482578)	ThermoFisher

Catalog Number	Comments/Description
A26772	
A24893	
NC0393866	
5779K699	
13-681-06	
17014392	
17014384	
17005860	
17005859	
14-222-262	
13-645-503	
A26434	
4479672 or 4479673; 4482578	

Name of Material/ Equipment	Company	Catalog Number
Ion PI Hi-Q OT2 200 Kit		A26434
Ion OneTouch 2 Supplies		
Ion OneTouch Reagent Tubes	ThermoFisher	A26367
Ion OneTouch Recovery Routers		
Ion OneTouch Recovery Tubes		
Ion OneTouch Sipper Tubes		
Ion OneTouch Amplification Plate		
Ion OneTouch Cleaning Adapter		
Ion OneTouch Reaction Filter		
Ion OneTouch ES Supplies		
Ion PI Hi-Q OT2 Reagents 200		
Ion PI Master Mix (violet cap) 8 × 2 mL	ThermoFisher	A26428
Ion PI Enzyme Mix (brown cap) 960 µL		
Ion PI Ion Sphere Particles (black cap) 800 µL		
Ion PI Hi-Q OT2 Solutions 200		
Ion OneTouch Breaking Solution (black cap) 2 × 1.2 mL	ThermoFisher	A26429
Ion OneTouch Oil 450 mL		
Ion OneTouch Reaction Oil 25 mL		
Nuclease-free Water 30 mL		
Ion OneTouch Recovery Solution 350 mL		
Ion OneTouch Wash Solution 16 mL		
Ion OneTouch ES Wash Solution 7.2 mL		
MyOne Beads Capture Solution (green cap) 2 × 1.5 mL		
ISP Resuspension Solution (red cap) 1.25 mL		
Tween Solution 6 mL		

Comments/Description

Polyethylene glycol sorbitan monolaurate solution for Melt-off solution. Molecular grade

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Author(s):	Baoheng Gui, Yingxin Zhang, Bo Liang, Yvonne Ka Yin Kwok, Wai Ting Lui, Queenie Sum Yee Yeung, Lingyin Kong, Li Ming Xuan, Jacqueline Pui Wah Chung, Kwong Wai Choy

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Responses to the editorial comments

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested. Please email me your revised manuscript at your earliest convenience.

Response: Thanks very much for the editing.

2. Please address specific comments marked in the attached manuscript.

Response: Thanks very much for the suggestions. Changes have been made accordingly.

3. Please provide Figure 3 with higher resolution.

Response: Thank you. We think what you mentioned was Figure 2 (only two figures were included in this paper). Figure 2 was modified by choosing images with higher resolution and was uploaded in the new submission.

4. Please thorough review the Representative Results and Discussion section to ensure that there are no spelling or grammar issues.

Response: Thank you. Changes have been made accordingly.

5. Please turn on Track Changes to mark up new changes made to your manuscript.

Response: Thank you. Changes were marked using the “track changes mode” in MS Word.

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Constituent	Reaction volume (µL) x1	Reaction volume (µL) x _____ No. of reactions
DNA	32	
Nuclease free water	10	
Ligase buffer	5	
DNA Ligase	1	
P1	1	
Index X	1	
Total volume	50	

Sample ID	Index ID