Embgenix™ PGT-A Kit (RUO) Protocol-At-A-Glance for Illumina® MiSeq® System

I. Introduction

The Embgenix™ PGT-A Kit (RUO) (Cat. No. 634760) has been developed to analyze DNA obtained from biopsies of single or multiple embryonic cells for preimplantation genetic testing for aneuploidies (PGT-A).

NOTE: This document provides brief instructions for the protocol steps and should only be used by personnel who are experienced with performing this experiment. The Embgenix PGT-A (RUO) User Manual for Illumina® MiSeq System should be referred to by newer users or for additional protocol details.

II. General Considerations

Refer to the user manual for more detailed information about the points below.

- **Requirements for Preventing Contamination:** Make sure you have two physically separated workstations. Refer to the user manual for more detailed information.
- **Sample Recommendations and Requirements:** Cells from trophectoderm (TE) biopsies and low amounts of Genomic DNA (gDNA).
- **Positive and Negative Controls:** Use 30 pg of freshly diluted reference gDNA as a positive control by adding 2.5 µl of a 12 pg/µl dilution. Use 2.5 µl of 1X PBS (Ca^{2+}- and Mg^{2+}-free) as a negative control.
- During the protocol, thaw components on ice and briefly mix and quick-spin component tubes before use.

III. Protocol

A. Cell Lysis/gDNA Extraction

1. Thaw the Cell Extraction Buffer, WGA Buffer, and WGA Nuclease-Free Water on ice.
2. Calculate and prepare the **Cell Extraction Master Mix** (CEMM) on ice as described below for all reactions, plus 10% extra. Mix thoroughly with a pipette and then spin down to collect the contents at the bottom of the tube.

4.8 µl	Cell Extraction Buffer
0.6 µl	Cell Extraction Enzyme
22.1 µl	WGA Nuclease-Free Water
27.5 µl	Total volume per reaction

NOTE: The CEMM volume should be 27.5 µl for each 2.5 µl of sample in a final reaction volume of 30 µl (Step 3). If the sample volume is larger than 2.5 µl, refer to the user manual.

3. To each sample or control, add [30.0 µl – Sample Volume] of CEMM from Step 2.
4. Spin briefly to collect the contents at the bottom of the tubes.
5. Place the samples in a thermal cycler with a heated lid set to 100°C–105°C. Perform the Cell Lysis Reaction using the following program:

75°C	10 min
95°C	4 min
4°C	Hold

6. Once the thermal cycler reaches 4°C, spin briefly to collect the contents at the bottom of the tubes and immediately proceed to the next section.

B. Whole Genome Amplification

1. On ice, assemble the **WGA Master Mix** for all reactions, plus 10% extra. Mix thoroughly by gentle vortexing for 5 sec, and then spin briefly to collect the contents at the bottom of the tubes.

29.8 µl	WGA Buffer
1.0 µl	WGA Enzyme
14.2 µl	WGA Nuclease-Free Water
45.0 µl	Total Volume per reaction

2. Add 45 µl of WGA Master Mix to each well/tube of the Cell Lysis reaction (Section III.A, Step 6). Mix by gentle vortexing for 5 sec and then spin briefly to collect the contents at the bottom of the tubes.
3. Return the tubes to the thermal cycler with the heated lid set to 101°C–105°C. Perform the WGA Reaction using the following cycling conditions:

95°C	2 min
12 cycles:	
95°C	15 sec
15°C	50 sec
25°C	40 sec
35°C	30 sec
65°C	40 sec
75°C	40 sec
14 cycles:	
95°C	15 sec
65°C	1 min
75°C	1 min
75°C	5 min
4°C	Hold

4. At the end of the amplification, spin briefly to collect the contents at the bottom of the tubes.

SAFE STOPPING POINT: The samples can be stored in the thermal cycler at 4°C overnight or transferred to –20°C for up to a week.

C. Dilution of Whole-Genome-Amplified Products

Dilution of the WGA product 1:20 (WD1)

1. Pipette 76 µl of Embgenix WGA Dilution Solution to each well or tube labeled WD1.
2. To this, add 4 µl of the WGA product (from previous section) from samples, positive control, or no-template control.
3. Seal the plate or close the tubes, mix by gentle vortexing and spin briefly to collect the contents at the bottom of the well or tubes.

Dilution of the WGA product 1:240 (WD2)

4. Pipette 55 µl of Embgenix WGA Dilution Solution to each well or tube labeled WD2.
5. To this, add 5 µl of the contents of WD1 from Step 3.
6. Seal the plate or close the tubes, mix by gentle vortexing, and spin briefly to collect the contents at the bottom of the well or tube.

D. Determination of WGA Product Quality and Quantity (Optional)

Quantification of WGA product (Optional):

- WGA products can be quantified using a fluorescence-detection-based method such as Qubit dsDNA HS Assay on the Qubit Fluorometer. See Qubit dsDNA HS Assay user manual for detailed instructions.
- Use 10 µl of the WD1 from Section III.C, Step 3 for sample measurement.

The concentration of the WGA product or positive control is determined by multiplying the sample concentration measurement with the dilution factor 20. The result is usually greater than 24 ng/µl.

Determination of WGA Quality and Size (Optional): The quality and size of the WGA product can be assessed using either the 4200 TapeStation or the 2100 BioAnalyzer from Agilent.

- Use 2 µl of WD1 from Section III.C, Step 3 for size assessment using the 4200 TapeStation with High Sensitivity D5000 Reagents from Agilent.
- Alternatively, you can use 1 µl of WD1 for validation using the Agilent 2100 Bioanalyzer with Agilent's High Sensitivity DNA Kit.

The expected average size of the WGA products or positive control should range between 500–900 bp.

E. Library Preparation: Fragmentation and Adapter Ligation

1. Thaw the following items from Embgenix Library Preparation Reagents (Box 2) on ice: FE Dilution Buffer, Lib Prep Buffer, Rxn Enhancer, and Stem-Loop Adapters.

   NOTE: Keep the 10X FE and the Library Prep Enzyme in the –20°C freezer until needed. Use a benchtop cooler to keep the enzymes cold while working with them at the bench.

2. Once thawed, gently vortex each of the components and then spin down the tubes briefly to collect the contents at the bottom of the tubes. Keep on ice.

3. On ice, add 4 µl of Stem-Loop Adapters to each tube/well of PCR tubes, 8-tube PCR strips, or a 96-well PCR plate, according to the number of reactions to be performed.

4. Add 8 µl of freshly diluted WD2 (1:240 dilution) from Section III.C, Step 6 or 8 µl WGA Dilution Solution as a negative control to a tube/well containing the 4 µl of Stem-Loop Adapters.

5. On ice, prepare 1X FE by diluting the 10X FE in thawed but cold FE Dilution Buffer in a 1:9 ratio (1 part 10X FE to 9 parts FE Dilution Buffer).
   - Prepare enough material to accommodate the Library Prep Master Mix in Step 6, plus 10% of the total reaction mix volume.
   - A minimum of 40 µl of 1X FE Preparation should be assembled.
   - Mix gently by pipetting up and down 10 times.
   - Spin down and keep on ice.

Component	1 rxn	1–36 rxns*
FE Dilution Buffer	0.9 µl	36.0 µl
10X FE	0.1 µl	4.0 µl
Total volume (1X FE)	1.0 µl	40.0 µl

*Volumes in µl include 10% overage

After preparing, immediately proceed to the next step.

6. Calculate and prepare the **Library Prep Master Mix** on ice for all reactions plus 10% of the total reaction mix volume. Combine the following reagents in the order shown:

4.0 µl	Lib Prep Buffer
3.5 µl	Rxn Enhancer
2.0 µl	Lib Prep Enzyme
1.0 µl	1X FE (from Step 5)
10.5 µl	Total volume per reaction

7. Assemble the library preparation reaction on ice. To each tube/well from Step 4, add 10.5 µl of the Library Prep Master Mix prepared in Step 5.
8. Mix by gentle vortexing for 5 sec, and then spin the tubes briefly to collect the contents at the bottom of the tubes/wells. Keep the samples cold (4°C) until placed in the thermal cycler.
9. Perform the library preparation reaction by placing the tube/plate in a precooled thermal cycler and running the following program:

20°C	40 min
85°C	5 min
4°C	Hold

10. Once the thermal cycler reaches 4°C, spin briefly.

SAFE STOPPING POINT: Samples can be stored in the thermal cycler at 4°C overnight or at –20°C for up to a week .

F. Library Amplification and Indexing with UDI

1. Thaw the library samples and the following items on ice: Unique Dual Index (1–96) and Amplification Buffer. Once thawed, mix each component by gentle vortexing, and then spin down the tubes briefly to collect the contents at the bottom of the tubes. Keep on ice.

   NOTE: Keep the PrimeSTAR® HS DNA Polymerase (5 U/µl) in the –20°C freezer until needed. Use a benchtop cooler to keep the enzyme cold while working with it on the bench.

2. Calculate and prepare on ice the **Library Amplification Master Mix** for all reactions plus 10% of the total reaction mix volume by combining the following reagents:

21.5 µl	Amplification Buffer
3 µl	Nuclease-Free Water
1.0 µl	PrimeSTAR HS DNA Polymerase
25.5 µl	Total volume per reaction

3. Add 25.5 µl of the Library Amplification Master Mix to each reaction from Section III.E, Step 8.
4. Add 2 µl of a different index from the Unique Dual Index (1–96) to each reaction. Use a new pipette tip for each UDI.
5. Mix by vortexing for 5 sec and then spin briefly to collect the contents at the bottom of the tubes/plate.

6. Place in a thermal cycler with a heated lid (105°C) and perform PCR amplification using the following program:

72°C	3 min
85°C	2 min
98°C	2 min
15 cycles	
98°C	20 sec
60°C	75 sec
68°C	5 min
4°C	Hold

7. Once the thermal cycler reaches 4°C, spin briefly to collect the contents at the bottom of the tubes.

SAFE STOPPING POINT: The samples can be stored in the thermal cycler at 4°C overnight or at –20°C for up to a week.

G. Purification of Amplified Libraries

1. Bring the NucleoMag NGS Clean-up and Size Select beads and the Nuclease-Free Water to room temperature for at least 30 min and mix well by vortexing.
2. Prepare fresh 80% ethanol. You will need ~400 µl per sample.
3. Add 40 µl of NucleoMag NGS Clean-up and Size Select beads to the amplified libraries from Section III.B, Step 7. Mix well by vortexing or pipetting the entire mixture up and down 10 times.
4. Incubate at room temperature for 5 min to let the libraries bind to the beads.
5. Briefly spin the samples to collect the liquid to the bottom of the tubes.
6. Place the tubes on a magnetic stand until the liquid appears to be completely clear and there are no beads left in the supernatant (~2 min).
7. Remove and discard the supernatant while samples are on the magnetic stand without disturbing the beads.
8. Keep the samples on the magnetic stand. Add 200 µl of fresh 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec, and then remove and discard the supernatant, taking care not to disturb the beads.
9. Repeat the ethanol wash (Step 8).
10. Briefly centrifuge the samples to collect the liquid at the bottom of the tubes.
11. Place the samples on the magnetic stand for 30 sec, then remove any residual ethanol with a pipette.
12. Incubate the samples at room temperature until the pellet is no longer shiny, but before cracks appear (~5–10 mins). Check the pellet frequently during this time and continue to Step 13 when it is dry enough.
13. Once the beads are dry, add 27 µl of Nuclease-Free Water to each sample.
14. Remove the samples from the magnetic stand and vortex the tubes for 5–10 sec to resuspend the beads.
15. Incubate at room temperature for ~5 min to rehydrate the beads.
16. Briefly spin the samples to collect the liquid from the side of the tubes.
17. Place the tubes back on the magnetic stand until the solution is completely clear (~2 min or longer).
18. Transfer 25 µl of supernatant containing purified libraries to a nuclease-free, low-adhesion tube taking care to not touch the magnetic bead pellet. Label each tube with sample information.

SAFE STOPPING POINT: The purified libraries can be immediately validated or stored at –20°C.

H. Validation and Quantification of Amplified Libraries

Quantification:

- Libraries are quantified using fluorescence-detection-based methods such as Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay kit or Quant-iT dsDNA Assay kit. See assay user manuals for more details
- Use 2 µl of the purified libraries from Section III.G, Step 18 for the measurement.
- The measured concentrations of the libraries or positive control should be greater than 4 ng/µl.

Quality and Size Assessment (optional):

- A fragment analyzer such as the Agilent 4200 TapeStation or the 2100 Bioanalyzer is used to assess the quality and size of the libraries. For detailed instructions, see the Agilent High Sensitivity D1000 ScreenTape System or Agilent High Sensitivity DNA Kit User Manual.
- Dilute aliquots of the purified libraries from Section III.G, Step 18 in Nuclease-Free Water to 3 ng/µl.
    - For validation using the 4200 TapeStation, use 2 µl of these diluted samples.
    - Alternatively, use 1 µl of these diluted samples for size assessment using the 2100 Bioanalyzer.

The libraries should have an average fragment size between 300–600 bp using a region of 150–1,000 bp.

I. Library Pooling and Illumina Sequencing

Multiplexed libraries are sequenced simultaneously in a single sequencing run. The different UDIs allow the demultiplexing of the sequencing data specific to each sample. Refer to the Embgenix PGT-A Kit (RUO) User Manual for MiSeq System for details.

Sample Pooling and loading concentration of pooled libraries:

When using the MiSeq Reagent Kit v3, up to 24 libraries can be pooled into a single run. We recommend a loading concentration of 11 pM for the denatured pooled libraries.

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