Takara Bio USA

Embgenix™ PGT-A Kit (RUO) User Manual for Illumina® MiSeq® System

Cat. No. 634760
(080122)

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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). All 24 chromosomes are screened for whole chromosome aneuploidy and subchromosomal abnormalities utilizing whole genome amplification (WGA) and next-generation sequencing (NGS) technologies.

This kit offers a PGT-A solution that includes WGA, library preparation, unique dual indexes (UDI), NucleoMag NGS Clean-up and Size Select magnetic beads, and the Embgenix Analysis Software (RUO) for the automatic calling of aneuploidy copy number variants (CNV).

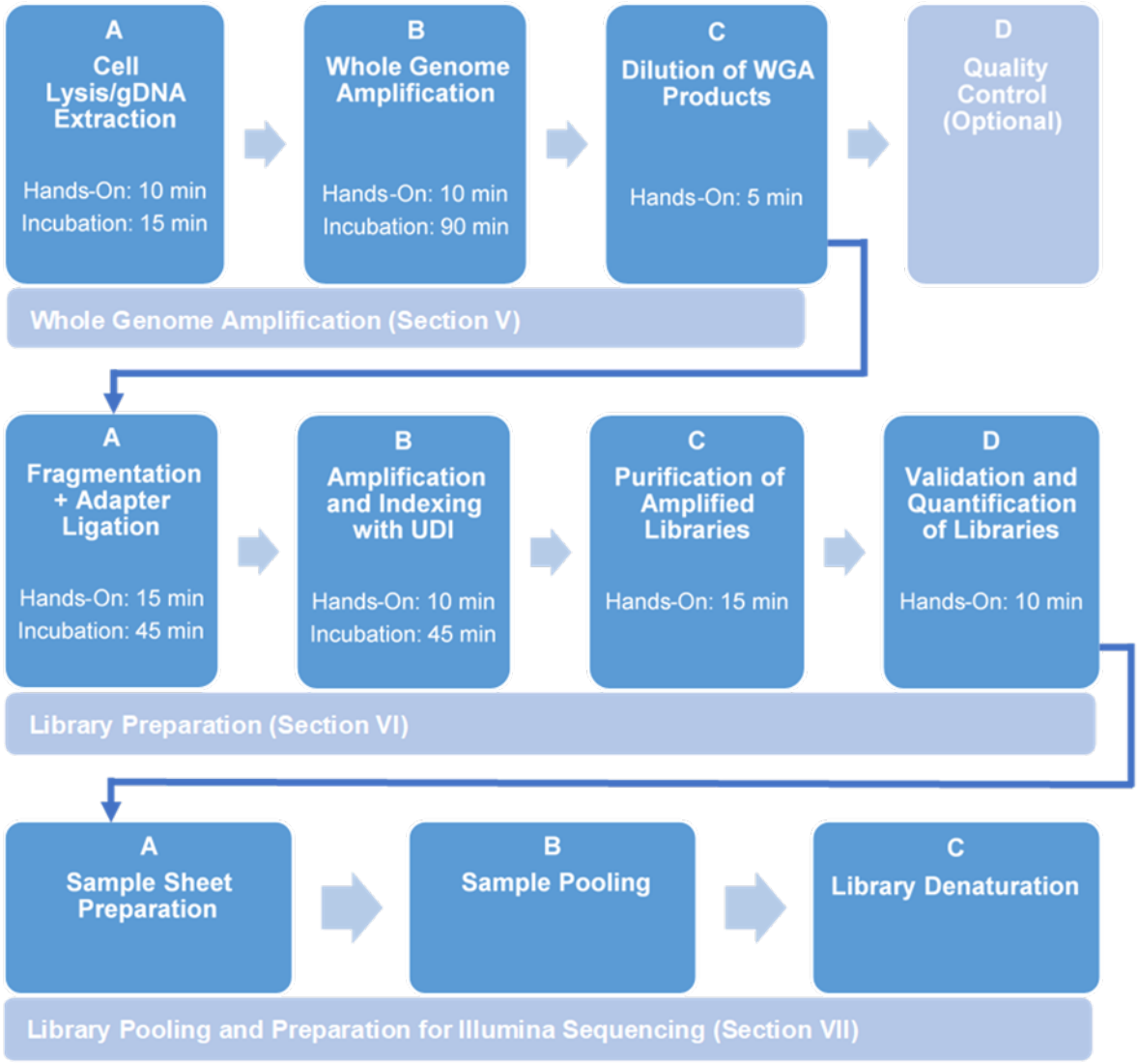

Figure 1. Embgenix PGT-A Kit protocol workflow overview.

II. List of Components

The Embgenix PGT-A Kit (RUO) contains sufficient reagents to prepare up to 96 reactions. The components in this kit have been specifically designed to work together and are optimized for this protocol; please do not make any substitutions. The kit has a shelf life of 18 months, as determined by the component with the shortest shelf life.

NOTE: Do not freeze-thaw reagents more than 10 times.

Table 1. Embgenix PGT-A Kit (RUO) components.

Embgenix PGT-A Kit (RUO)	Cap color	634760 (96 rxns)
Embgenix PGT-A Core Kit (RUO)* (Cat. No. 634761)		
Box 1: WGA Reagents (Store at –20°C)		
Cell Extraction Buffer	Green	1,040 µl
Cell Extraction Enzyme	Yellow	75 µl
WGA Buffer	Red	2 x 1.8 ml
WGA Enzyme	White	115 µl
WGA Nuclease-Free Water	Clear	3 x 1.8 ml
Box 2: WGA Dilution Solution and Library Preparation Solution (Store at –20°C)		
WGA Dilution Solution	—	25 ml
FE Dilution Buffer	White	1 ml
10X FE	White	60 µl
Lib Prep Buffer	Blue	480 µl
Lib Prep Enzyme	Blue	280 µl
Rxn Enhancer	Red	400 µl
Stem-Loop Adapters	Violet	480 µl
Amplification Buffer	Orange	2 x 1.2 ml
PrimeSTAR® HS DNA Polymerase (5 U/µl)	Green	120 µl
Nuclease-Free Water	—	4 x 1 ml
Unique Dual Index Kit (1–96) (Cat. No. 634752; Store at –20°C)		1
Unique Dual Index Kit (1–96) (12.5 µM)		5 µl/well
NucleoMag NGS Clean-up and Size Select (Cat. No. 744970.5, Store at 4°C)†		5 ml

*Throughout this manual, the Embgenix PGT-A Core Kit (RUO) will be referred to as the Embgenix PGT-A Core Kit.

†The kit has been specifically validated with NucleoMag NGS Clean-up and Size Select. We strongly recommend aliquoting the beads into 1.5-ml tubes upon receipt and then refrigerating the aliquots.

- Do not use substitutions, as it may lead to unexpected results.
- Magnetic beads should only be used at room temperature. Individual aliquoted tubes can be removed 30 minutes before an experiment to bring the beads to room temperature.

III. Additional Materials Required

The following reagents and equipment are required but not supplied. The specified brands have been validated to work with this protocol.

- Single-channel pipette: 10 µl, 20 µl, 200 µl, and 1,000 µl
- Eight-channel or twelve-channel pipette (recommended): 20 µl and 200 µl
- 25 ml graduated reservoir (USA Scientific, Cat. No. 1930-2230)

- Filter pipette tips: 20 µl, 200 µl, and 1,000 µl
- Minicentrifuge for 1.5-ml tubes
- Minicentrifuge for 0.2-ml tubes or strips
- Minicentrifuge for 96-well plates
- 96-well PCR chiller rack, such as IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4) or 96-Well Aluminum Block (Light Labs, Cat. No. A-7079)
- Benchtop cooler, such as VWR CryoCoolers (VWR, Cat. No. 414004-286)

For WGA (Section V) and Library Preparation (Section VI. A & B)

- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2-ml PCR 8-tube strip; Thermo Fisher Scientific, Cat. No. AB0264) or similar nuclease-free, PCR-grade thin-wall PCR tubes, strips, or 96-well plates
- Nuclease-free, low-adhesion 1.5-ml tubes (USA Scientific, Cat. No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes

  NOTE: Select appropriate tubes or plates that are compatible with the thermal cyclers and/or real-time thermal cyclers used.

- Axygen 5 ml Self Standing Screw Cap Transport Tube (Corning, Cat No. SCT-5ML-S)
- Adhesive PCR Plate Seal (Thermo Fisher Scientific, Cat. No. AB0626 or USA Scientific 2938-4100) for 96-well plates, or cap strips (Thermo Fisher Scientific, Cat. No. AB0784/AB0850) for 8-tube strips
- Thermal cycler with a heated lid
- Phosphate Buffered Saline, Ca^{2+}- and Mg^{2+}-free (PBS) (Thermo Fisher Scientific, Cat. No. 10010-023)

For Purification of Amplified Libraries by Magnetic Beads (Section VI.C)

- 80% Ethanol: freshly made for each experiment from molecular biology grade 100% ethanol
- Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates, such as:
  - For 12–24 samples:
    - SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011); accommodates two 8-tube or 12-tube strips
    - Nuclease-free, PCR-grade, thin-wall PCR strips (0.2-ml PCR 8-tube strip; Thermo Fisher Scientific, Cat. No. AB0264), or similar nuclease-free, PCR-grade thin-wall PCR strip tubes
    - Minicentrifuge for 0.2 ml tubes or strips
  - For 24–96 or more samples:
    - Magnetic Stand-96 (Thermo Fisher Scientific, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher Scientific, Cat. No. AB0558)
    - Low-speed benchtop centrifuge for 96-well plates

For WGA and Illumina Library Quantification (Sections VI.D & VII.D)

- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854) or Quant-iT dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific, Cat. No. Q33120)
- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2 ml PCR 8-tube strip; Thermo Fisher Scientific, Cat. No. AB0264) or similar nuclease-free, PCR-grade, thin-wall PCR tubes, strips, or 96-well plates

- Nuclease-free, low-adhesion 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes
- Optional (for quality and size assessment):
  - High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) for Bioanalyzer or equivalent high-sensitivity electrophoresis method
  - High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067- 5592) with High Sensitivity D5000 Reagents (Agilent, Cat. No. 5067- 5593)
  - High Sensitivity D1000 ScreenTape (Agilent, Cat. No. 5067- 5584) with High Sensitivity D1000 Reagents (Agilent, Cat. No. 5067- 5585)

For Library Pooling and Denaturation (Section VII.C & D) (As Required)

- 1 N NaOH solution (Fisher Chemical, Cat. No. SS266-1)
- Nuclease-free, low-adhesion, 1.5-ml tubes (USA Scientific, Item No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes
- MiSeq Reagent Kit v3 (150-cycle) (Illumina, Cat. No. MS-102-3001)

IV. General Considerations

A. Sample Recommendations and Requirements

The Embgenix PGT-A Kit (RUO) is compatible with:

- Cells from trophectoderm biopsies
- Low amounts of genomic DNA (gDNA) for positive control

B. Requirements for Preventing Contamination

IMPORTANT: Before you set up the experiment, **make sure you have two physically separated workstations.**

- **A PCR clean workstation** is ideal, if available, for all pre-PCR experiments that require cleanroom conditions, i.e., sample and WGA reaction preparations (Sections VI.A & B).
- **A second workstation located in the general laboratory** for WGA product dilution and QC (Section V.C & D); library preparation, purification, and concentration measurement (Section VI); and library pooling and sequencing (Section VII).

C. General Requirements

- The assay is very sensitive to variations in pipetting volume. Make sure all your pipettes are calibrated for reliable delivery.
- Negative and positive control reactions must be performed to verify that the kit components are working properly.
- All lab supplies related to WGA should be stored in a nucleic-acid-free and nuclease-free closed cabinet.
- Incubate tips, tubes, and pipettes with UV irradiation in the cabinet, as recommended by the cabinet manufacturer, before starting the protocol.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.

- Do not change the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the Embgenix PGT-A Kit (RUO) protocol.

V. Protocol: Preparation of Whole Genome Amplification

IMPORTANT: Before starting, read the entire set of protocols in Section V and Appendix A, "Reference DNA Dilution."

A. Protocol: Cell Lysis/gDNA Extraction

Before you begin

- The WGA Reagents (Box 1) of the Embgenix PGT-A Core Kit are used in both this Section and Section V.B, below.
- **Samples:** Collect the biopsied samples in 2.5 µl of 1X PBS with up to 3% polyvinylpyrrolidone (PVP)
- **Positive control:** Use 30 pg of freshly diluted reference gDNA by adding 2.5 µl of a 12 pg/µl dilution (see Appendix A)
- **No-Template Control (NTC, Negative Control):** 2.5 µl of 1X PBS

Procedure

1. Thaw the following components of the WGA Reagents on ice and briefly spin down before opening tubes:
    - Cell Extraction Buffer
    - WGA Buffer
    - WGA Nuclease-Free Water
2. On ice, assemble the Cell Extraction Master Mix as described below, for all reactions plus 10% overage.

    Cell Extraction Master Mix (CEMM):

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

    Mix thoroughly by gentle vortexing for 5 sec then spin down to collect the contents at the bottom of the tube. Keep on ice.

    NOTE: Sample volumes of up to 5 µl can be processed in the next step (Step 3). For a final reaction volume of 30 µl, the volume of Cell Extraction Master Mix should be 27.5 µl for 2.5 µl of sample or control.
    If the sample volume is larger than 2.5 µl, reduce the volume of Nuclease-Free Water in the Cell Extraction Master Mix to accommodate the final reaction volume of 30 µl. Use the following formula to determine the amount of WGA Nuclease-Free Water to use in the master mix:

    24.6 µl – Sample Volume = Volume of Nuclease-Free Water (per sample)

    The volumes of Cell Extraction Buffer and Cell Extraction Enzyme must remain at 4.8 µl and 0.6 µl, respectively, for each reaction.

3. Calculate the amount of CEMM to add PER sample or control with the formula:

   30.0 µl – Sample (or Control) Volume = Volume of Cell Extraction Master Mix

4. Add the calculated amount of CEMM to each sample or control.

25–27.5 µl	Cell Extraction Master Mix (from Step 3)
2.5–5 µl	Sample or Control
30 µl	Total volume

NOTE: Do not touch the cell or DNA sample with the pipette tip.

5. Spin briefly to collect the contents at the bottom of the tubes

6. 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

7. Once the thermal cycler reaches 4°C, spin briefly to collect the contents at the bottom of the tubes and immediately proceed to Section V.B, "Whole Genome Amplification."

B. Protocol: Whole Genome Amplification

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

WGA Master Mix:

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 the WGA Master Mix to each well/tube of the cell lysis reaction product.

30 µl	Cell lysis product (from Section V.A, Step 7)
45 µl	WGA Master Mix
75 µl	Total volume per reaction

3. Mix by gentle vortexing and then spin briefly to collect the contents at the bottom of the tubes.

4. Return the plate or tubes to the thermal cycler with the heated lid set to 100°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

5. At the end of amplification, spin briefly to collect the contents at the bottom of the plate wells or tubes. If proceeding immediately to dilution of the WGA products (Section V.C), keep the plate or tubes on ice.

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. Protocol: Dilution of Whole Genome Amplified Products

Before you begin

- For this step, you will need the WGA Dilution Solution from Box 2 of the Embgenix PGT-A Core Kit.

Procedure

1. If the samples were stored at –20ºC, remove from the freezer and thaw on ice. Keep on ice for the rest of this protocol.
2. Label two 96-well plates or strip tubes
   - **WD1** for the 1:20 dilution (WGA Dilution 1), and
   - **WD2** for the 1:240 dilution (WGA Dilution 2)

WGA Dilution 1

3. Pipette 76 µl of the WGA Dilution Solution to each well or tube labeled **WD1**.
4. To these wells or tubes, add 4 µl of the WGA product from samples, positive control or NTC from Section V.B, Step 5.
5. Seal the plate with a seal or close the tube and mix by gentle vortexing.
6. Spin briefly to collect the contents at the bottom of the wells or tubes.

The contents of the WD1 are used in the dilution of the WGA to 1:240 (Section V.C, Step 5) and to determine the WGA products quality and quantity (optional) (Section V.D).

WGA Dilution 2

7. Pipette 55 µl of the WGA Dilution Solution to each well or tube labeled **WD2**.
8. To these wells or tubes, add 5 µl of the diluted WGA product from WD1 (Step 5, above).
9. Seal the plate or close the tubes. Mix by gentle vortexing.
10. Spin briefly to collect the contents at the bottom of the wells or tubes.

The contents of the WD2 are used in Library Preparation (Section VI).

NOT A SAFE STOPPING POINT: The diluted WGA product should be used on the day of the dilution. Long-term storage is not recommended.

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

WGA products are quantified using fluorescence-detection-based methods, such as Qubit dsDNA HS Assay (Thermo Fisher Scientific, Cat. No. Q32851, Q32854) or Quant-iT dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific, Cat. No. Q33120).

Determination of WGA Quantity

- See Qubit dsDNA HS Assay user manual for detailed instructions on sample measurement.
- Use 10 µl of WD1 from Section V.C, Step 5 for the sample measurement. To determine the concentration of the WGA product, multiply the sample measurement by the dilution factor of the WD1 content (20).
- The concentration of the WGA products or positive control is greater than 24 ng/µl, or the total yield is greater than 1.8 µg. If the measured concentration is <24 ng/µl please check the dilution or make a fresh dilution and remeasure. If the concentration is still low, check the input material quality, requantify the DNA in the positive control, recheck the dilution scheme, or repeat the WGA reaction.

Determination of WGA Quality and Size

The quality and size of the WGA product can be assessed using either the 4200 TapeStation or the 2100 Bioanalyzer from Agilent; if neither of these methods are available, assessment can be performed by agarose gel electrophoresis.

- Using the Agilent 4200 TapeStation: Use 2 µl of WD1 from Section V.C, Step 5 for size assessment with High Sensitivity D5000 Reagents (Agilent, Cat. No. 5067-5594) (see Figure 2, below, for expected TapeStation electropherogram profiles).
- Using the Agilent 2100 Bioanalyzer: Use 1 µl of WD1 from Section V.C, Step 5 for size assessment using Agilent's 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4627) (see Figure 9 in Appendix B for expected Bioanalyzer electropherogram profiles).
- Using agarose gel electrophoresis: Use approximately 200 ng of the undiluted WGA product from Section V.B, Step 5. Please see Figure 11 in Appendix B for details.

The expected average size of the WGA products should range between 500–900 bp using a region of 100–3,000 bp.

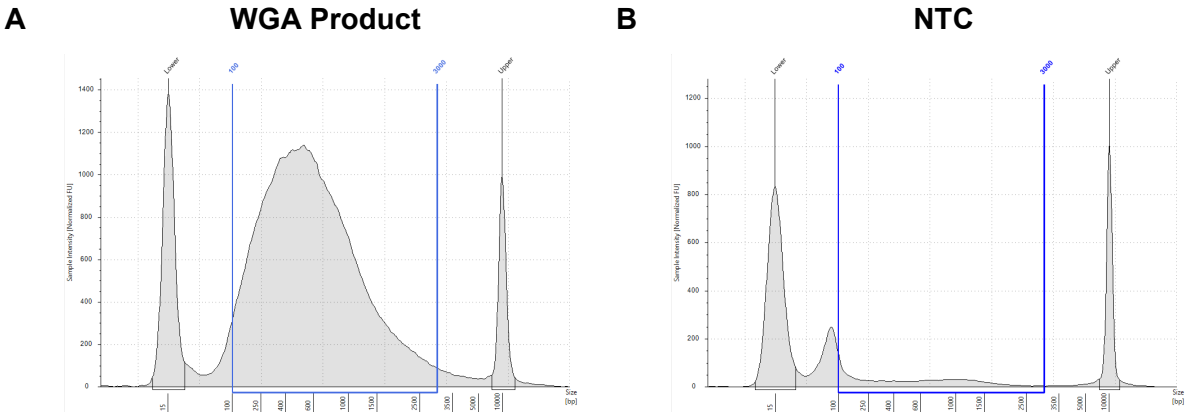

Figure 2. TapeStation analysis of Embgenix WGA products. WGA products were prepared using 5 sorted cells. The products or no-template control (NTC) were diluted 1:20, and 2 µl was loaded on an Agilent High Sensitivity D5000 ScreenTape System. **Panel A**. The electropherogram shows the WGA product with an average size of 700 bp in a region of 100–3,000 bp. **Panel B**. The electropherogram for the NTC shows no or minimal WGA product in a region of 100–3,000 bp. A small peak below 100 bp is a result of primer dimerization in the absence of template.

VI. Protocol: Library Preparation

- The diluted product from Section V.C is used for library preparation without purification: i.e., the WGA DNA is not purified.
- Components from Box 2 of the Embgenix PGT-A Core Kit are used in Sections VI.A & B, and the Unique Dual Index Kit (1–96) is used in Section VI.B.
- Before starting, please read the entire set of protocols in Section VI as well as Appendix C, which contains instructions for use on indexing with UDI, a plate map, and the barcode sequences.

A. Protocol: Fragmentation and Adapter Ligation

Before you begin

- Precool the thermal cycler to 20°C.
- Thaw the following items on ice:
  - FE Dilution Buffer
  - Lib Prep Buffer
  - Rxn Enhancer
  - Stem-Loop Adapters
- If performing library preparation (Section VI.A) and amplification (Section VI.B) on the same day, additionally thaw the following items on ice:
  - Unique Dual Index Kit (1–96)
  - Amplification Buffer

  NOTE: If stopping at the end of Section VI.A, keep the Unique Dual Index Kit (1–96) and Amplification Buffer in the –20°C freezer until ready to begin Section VI.B.
- Keep the 10X FE and Lib 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.

Procedure

1. Once thawed, mix all components by gentle vortexing, and then spin down the tubes briefly to collect the contents at the bottom of the tubes. Keep on ice.
2. On ice, add 4 µl of Stem-Loop Adapters to each tube/well of the PCR tubes, 8-tube PCR strips, or a 96-well PCR plate, according to the number of reactions to be performed.
3. Add a total of 8 µl of freshly diluted WD2 (the 1:240 dilution product from Section V.C, Step 10) or WGA Dilution Solution (as a negative control) to a tube/well containing the 4 µl of Stem-Loop Adapters (Step 2).

4 µl	Stem-Loop Adapters (Step 2)
8 µl	Freshly diluted WD2 or WGA Dilution Solution (NTC)
12 µl	Total volume Stem-Loop Adapter/WD2 dilution per reaction

4. On ice, prepare 1X FE by diluting the 10X FE in cold (4°C) FE Dilution Buffer in 1-part 10X FE to 9-parts FE Dilution Buffer (1:9 ratio). Prepare enough material to accommodate the Library Prep Master Mix in Step 5, plus 10% of the total reaction mix volume.

NOTE: To allow for greater accuracy in pipetting the 10X FE, a minimum of 40 µl of 1X FE Preparation should be assembled, which is enough for 36 rxns.

1X FE Preparation

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

*Volumes include ~10% extra for overage.

Mix gently by gentle vortexing for 5 sec. Spin down and keep on ice. Immediately proceed with assembling the library preparation master mix in Step 5.

5. On ice, calculate and prepare the amount of Library Prep Master Mix needed for all reactions, plus 10% extra for overage. Combine the reagents in the order shown below:

Library Prep Master Mix:

4.0 µl	Lib Prep Buffer
3.5 µl	Rxn Enhancer
2.0 µl	Lib Prep Enzyme
1.0 µl	1X FE (from Step 4)
10.5 µl	Total volume per reaction

NOTES:
- **Library Prep Master Mix is very viscous.** Ensure adequate mixing by vortexing for 5 sec, and then spin the tubes briefly to collect the contents at the bottom of the tubes/plate. If necessary, vortex for an additional 5 sec and spin down again. Keep on ice.
- Always add the 1X FE last. Discard leftover 1X FE. Do not reuse.
- Immediately proceed to the next step to avoid over-fragmentation.

6. On ice, assemble the library preparation reaction. To each tube/well containing 12 µl WGA/Stem-Loop Adapters mix or negative control/Stem-Loop Adapters mix, add 10.5 µl of the Library Prep Master Mix prepared in the previous step (Step 5). Mix by gentle vortexing for 5 sec and then spin the tubes briefly to collect the contents at the bottom of the tubes/wells.

12 µl	Stem-Loop Adapters+WD2 dilution (from Step 3)
10.5 µl	Library Prep Master Mix (from Step 5)
22.5 µl	Total volume library prep solution per reaction

NOTES:
- During this step, keep the samples cold (4°C) until placed in the thermal cycler.
- Do NOT create a master mix with the Stem-Loop Adapters and the Library Prep Master Mix.

7. Perform the library preparation reaction by placing the tubes/plate in a precooled thermal cycler and running the following program:

20°C	40 min
85°C	5 min
4°C	Hold

8. Once the program completes and reaches 4°C, spin briefly to collect the contents at the bottom of the tubes. Proceed to Section VI.B, "Library Amplification and Indexing with UDI."

B. Protocol: Library Amplification and Indexing with UDI

Before you begin

- Thaw the following items on ice:
  - Amplification Buffer
  - Unique Dual Index Kit (1–96)
- Keep the PrimeSTAR HS DNA Polymerase in the –20°C freezer until needed. Use a benchtop cooler to keep the enzyme cold while working with it at the bench.
- Preheat the thermal cycler lid to 100°C–105°C.

Procedure

1. Once the samples and reagents are thawed, mix all components by gentle vortexing and then spin down the tubes briefly to collect the contents at the bottom of the tubes. Keep on ice.
2. On ice, calculate and prepare the Library Amplification Master Mix needed for all reactions, plus 10% extra for overage.

   Library Amplification Master Mix:

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

3. Add 25.5 µl of the Library Amplification Master Mix to the reaction results of each sample well from Section VI.A, Step 8.

22.5 µl	Library prep reaction results (from Section VI.A, Step 8)
25.5 µl	Library Amplification Master Mix (from Step 2)
48 µl	Total volume library amplification solution per reaction

4. Add 2 µl of a different index from the Unique Dual Index Kit (1–96) plate to each sample well.

48 µl	Library amplification solution (from Section VI.A, Step 8)
2 µl	UDI
50 µl	Total volume library amplification solution per reaction

NOTES:

- Each well contains a unique pair of index primers. Make sure that you choose a valid combination of UDI to ensure high sequencing quality.
- Always use a new pipette tip for each UDI/well to avoid cross contamination.
- Ensure the correct orientation of the plate by positioning the notched corner at the bottom left.
- The aluminum foil that covers the plate is pierceable with most pipette tips.
- Every well contains enough material for a single use. Do not reuse a well if its foil was previously pierced.
- Cover the used well with a seal and return the plate to –20°C after use.

Please refer to Appendix C for more information about the use of the Unique Dual Index Kit (1–96).

5. Mix the tubes/plate by gentle vortexing for 5 sec, and then spin briefly to collect the contents at the bottom of the tubes/wells.
6. Place in the preheated thermal cycler 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 program is complete and the thermal cycler reaches 4°C, spin briefly to collect the contents at the bottom of the tubes/wells. Proceed to Section VI.C, "Purification of Amplified Libraries."

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

C. Protocol: Purification of Amplified Libraries

PCR-amplified libraries are purified by immobilization on NucleoMag NGS Clean-up and Size Select beads following the protocol outlined in steps below.

NOTES:
- Before each use, bring the aliquot of NucleoMag NGS Clean-up and Size Select beads and the Nuclease-Free Water to room temperature for at least 30 min. Mix the beads well by vortexing.
- Bead:sample ratio is 0.8:1.
- Prepare 80% ethanol fresh for each experiment. You will need 400 µl per sample.
- You will need a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. Add 40 µl of NucleoMag NGS Clean-up and Size Select beads to the tubes/wells of the amplified libraries from Section VI.B, Step 7.
2. Mix well by vortexing or pipetting the entire mixture up and down 10 times.

   NOTE: The beads are viscous; to mix, pipette the entire volume and push it out slowly.
3. Incubate at room temperature for 5 min to let the libraries bind to the beads.
4. Briefly spin the sample to collect the liquid from the side of the tube. Place the tube on a magnetic stand for 2 min or until the liquid appears completely clear and there are no beads left in the supernatant.
5. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.
6. Keep the samples on the magnetic separation device. 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, again taking care not to disturb the beads. The library remains bound to the beads during washing.
7. Repeat the ethanol wash (Step 6) once more.
8. Briefly centrifuge the samples to collect the liquid from the side of the tube or plate well. Place the samples on the magnetic separation device for 30 sec then remove any residual ethanol with a pipette.

9. Incubate the samples at room temperature for 5 min, or until the pellet is no longer shiny, but before cracks appear. If the pellet is still shiny after 5 min, continue to dry but check at 1 min intervals to prevent overdrying.

   NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you would like additional assistance with this determination.

   https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

   If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

10. Once the beads are dry, elute the purified libraries by adding 27 µl of Nuclease-Free Water to each sample.
11. Remove the samples from the magnetic separation device and vortex the tube for 5–10 sec to mix thoroughly.
12. Incubate at room temperature for 5 min to rehydrate the beads.
13. Briefly spin the samples to collect the liquid from the side of the tube.
14. Place the tube/plate back on the magnetic separation device for 2 min or longer, until the solution is completely clear.
15. Label new nuclease-free, low-adhesion tubes with information for each sample.
16. Transfer 25 µl of clear supernatant containing purified libraries from each tube/well to the new tubes. Take care not to touch or carry over the magnetic bead pellet with your sample during the transfer.

SAFE STOPPING POINT: The samples can be validated (Section VI.D) or stored at –20°C indefinitely. If storing the library, be sure to measure the concentration before reusing it.

D. Protocol: Validation and Quantification of Amplified Libraries

1. Quantification

Library quantification can be performed using fluorescence-detection-based methods, such as Qubit dsDNA HS Assay (Thermo Fisher Scientific, Cat. No. Q32851, Q32854) or Quant-iT dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific, Cat. No. Q33120).

We recommend using 2 µl of the purified libraries from Section VI.C, Step 16. Refer to the appropriate quantification assay user manual for detailed instructions.

The concentration of the libraries or positive control should be greater than 4 ng/µl. These calculated concentrations are used in Section VII.B during sample pooling.

2. Quality and Size Assessment (Optional)

If you choose to perform quality control, a fragment analyzer, such as the 4200 TapeStation or the 2100 Bioanalyzer from Agilent, can be used to assess the quality and size of the libraries. For detailed instructions, see the Agilent High Sensitivity D1000 ScreenTape System User Manual or the Agilent High Sensitivity DNA Kit User Manual.

We recommend diluting an aliquot of each library in Nuclease-Free Water to 3 ng/µl.

- Use 2 µl of this diluted sample for validation using an Agilent 4200 TapeStation and High Sensitivity D1000 Reagents (Agilent, Cat. No. 5067- 5587). Figure 3, below, depicts expected TapeStation electropherogram profiles.
- Alternatively, use 1 µl for validation using an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)

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

NOTE: See Figure 10, Appendix B for example graphs of the Agilent 2100 Bioanalyzer analysis of the purified libraries. Instructions on additional quality control methods, such as agarose gel electrophoresis, can also be found in Figure 11.

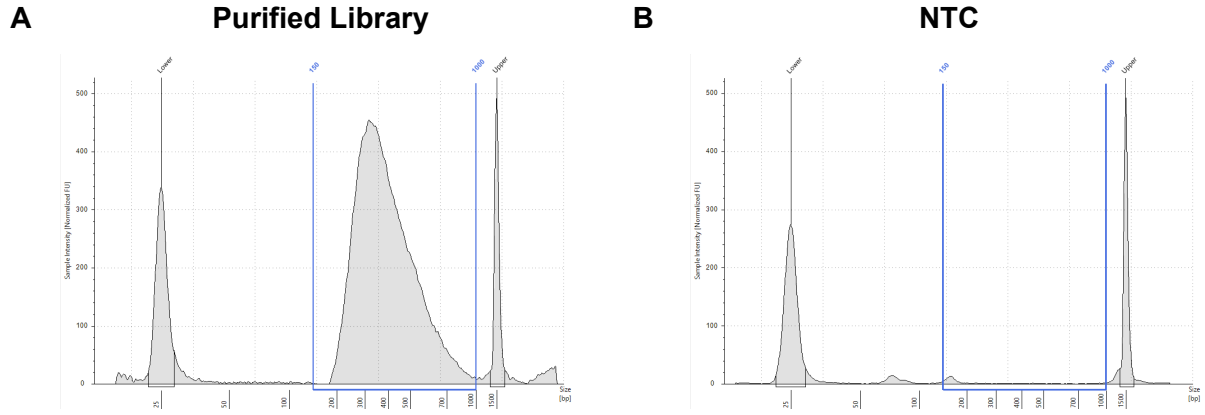

Figure 3. TapeStation analysis of Embgenix libraries. 2 µl of purified, diluted (to 3 ng/µl) library and purified, undiluted NTC were loaded on High Sensitivity D1000 ScreenTapes. **Panel A.** The electropherogram for the library shows an average size of 400 bp using a region of 150–1,000 bp. **Panel B.** The electropherogram for the NTC shows virtually no product.

VII. Protocol: Library Pooling and Preparation for Illumina Sequencing

Multiplexed libraries are sequenced simultaneously in a single sequencing run. A 150-cycle sequence reagent kit is used to perform paired-end sequencing with 75 cycles per read on the MiSeq System. The different UDIs are 8 nucleotides long and allow for the demultiplexing of the sequencing data specific to each sample. See Appendix C for more information.

- If the MiSeq System is running MCS 3.1 (or later), use the Local Run Manager protocol described in Section VII.A
- If the system is running an older version of MCS, use the Illumina Experiment Manager protocol described in Section VII.B

A. Sample Sheet Preparation—Local Run Manager

If the MiSeq System is running MCS 3.1 (or later), use the sample-sheet template "Embgenix PGT-A Illumina MiSeq96 SampleSheet for LocalRunManager" from the takarabio.com website.

- In the [Header] section, update cells B2 and B3 to add your experiment name and the date the sequencing is being run.
- In the [Data] section, (Rows 18–113), each row corresponds to one of the 96 reaction tubes or purified libraries after Section V.C, Step 16.

Starting at Row 18, fill out the "Sample_ID" field (Column A), by correlating the index added to the sample (Section V.B, Step 4) with the index information populated in Columns C–G. If desired, additional experimental information can be recorded in the "Description" (Column B) and "Sample_Project" (Column H) fields.

Information about i7 and i5 values displayed in Columns C–G of this Section can be found in Appendix C; these columns should not be edited.

Table 2 below contains more information about the data Section columns of the sample sheet.

Table 2. Header rows of the [Data] Section in the sample sheet template CSV file for use with LocalRunManager.

Col	Column header	Required?	Field description
A	**Sample_ID**	Required	The name/ID of the sample. Use a unique name for each sample
B	**Description**	Optional	Description of the sample, if different than ID
C	**Index_Plate_Well**	Required	The well position of the UDI used for the sample on the Unique Dual Index Kit (1–96) plate*
D	**I7_Index_ID**	Required	The i7 number ID of the UDI*
E	**index**	Required	The i7 sequence of the UDI*
F	**I5_Index_ID**	Required	The i5 number ID of the UDI*
G	**index2**	Required	The i5 sequence of the UDI*
H	**Sample_Project**	Optional	A custom name assigned to your project

*See Appendix C for more information. Do not edit this field.

NOTES:

- If there are unused rows of the "Sample_ID" and index combination, delete those rows from the file. Rows with no information in the "Sample_ID" field may cause an error while sequencing.
- Do not use spaces or special characters, such as (*.”/\’{};:|=,), in any of the fields.
- The sample sheet can also be created using the Local Run Manager off instrument or web browser version (v2.0 or later) by selecting:
  - GenerateFASTQ
  - Library Prep kit: TruSeq Nano DNA
  - IDT-ILMN TruSeq DNA UD Indexes (96 Indexes)
  - Index reads: 2
  - Read lengths:
    - Read 1: 75
    - Read 2: 75
    - Verify the "Index 1" and "Index 2" values are both '8'
  - Use Adapter Trimming

B. Sample Sheet Preparation— Illumina Experiment Manager

If the MiSeq instrument is running MCS 2.0 or older, prepare a Sample Sheet using the "Embgenix PGT-A Illumina MiSeq96 SampleSheet" template file from the takarabio.com website.

- In the [Header] section, add the following information: Investigator Name, Experiment Name, Date (Column B, Rows 3–5).
- In the [Data] Section (Rows 20–117), each row corresponds to one of the 96 reaction tubes or purified libraries after Section V.C, Step 16.

  Starting at Row 22, fill out the "Sample_ID" and "Sample_Name" fields (Columns A and B), by correlating the index added to the sample (Section VI.B, Step 4) with the index information populated in Columns E–I.
  If desired, additional experimental information can be recorded in the "Sample_Name" (Column B), "Sample_Plate" (Column C), "Sample_Well" (Column D), "Sample_Project" (Column J), and "Description" (Column K) fields.

  Information about the i7 and i5 values displayed in Columns E–I can be found in Appendix C; these columns should not be edited.

Table 3. [Data] Section header rows in the `Embgenix_PGT-A_Illumina_MiSeq96_SampleSheet.csv` file.

Col	Column header	Required?	Field description
A	**Sample_ID**	Required	The name/ID of the sample. Use a unique name for each sample
B	**Sample_Name**	Optional	Name of the sample*
C	**Sample_Plate**	Optional	Name of the plate*
D	**Sample_Well**	Optional	Sample location in the source plate
E	**Index_Plate_Well**	Required	The well position of the UDI used for the sample on the Unique Dual Index Kit (1–96) plate†
F	**I7_Index_ID**	Required	The i7 number ID of the UDI†
G	**index**	Required	The i7 sequence of the UDI†
H	**I5_Index_ID**	Required	The i5 number ID of the UDI†
I	**index2**	Required	The i5 sequence of the UDI†
J	**Sample_Project**	Optional	A custom name assigned to your project
K	**Description**	Optional	A custom description of your project

*If different than the Sample_ID or if additional identifying information is desired.
†See Appendix C for more information. Do not edit this field.

NOTES:

- If there are unused rows of the "Sample_ID" and index combination, delete those rows from the file. Rows with no information in the "Sample_ID" field may cause an error while sequencing.
- Do not use spaces or special characters such as (*.”/\’{};:|=,) when populating the fields.
- The software requires a unique combination of "Sample_ID" and "Sample_Plate".
- The sample sheet can also be created using the Illumina Experiment Manager (v1.18.1 or later) by selecting:
  - IDT-ILMN TruSeq DNA UD Indexes (96 Indexes)
  - Cycles Read 1: 75
  - Cycles Read 2: 75
  - Use Adapter Trimming
  - Use Adapter Trimming Read 2

C. Sample Pooling

Using the MiSeq Reagent Kit v3, up to 24 libraries can be pooled into a single run.

1. How to Use the Library Pooling Calculator

1. Download the file:

   Embgenix_PGT-A_Kit_(RUO)_Library_Pooling_Calculator_for_MiSeq_System.xlsx

   (calculator) from takarabio.com. You will use the calculator to determine the volume of library and Nuclease-Free Water needed to generate a 4 nM pool.

2. In table 2 of the calculator (starting at Row 21), fill the following columns with the specified information:

   a. Column B: Sample ID.

   b. Column C: the library concentration (ng/µl), calculated by the quantification results (Section VI.D). The default is 10 ng/µl; replace with the data for your experiment.

   c. Column D: the library size (bp) calculated by the optional size assessment (Section VI.D). If fragment analyzer results are not available, an average size of 400 bp can be applied to all libraries.

18	Table 2						
19	A	B	C	D	E	F	G
20		Sample ID	Library Concentration (ng/µl)	Library Size (bp)	Calculated Library molarity (nM)	Volume to be pooled (µl)	Volume validation
21	1	01_Sample	10	400	37.9	3.5	ok
22	2	02_Sample	10	400	37.9	3.5	ok

Figure 4. Library pooling calculator: columns B–D of table 2. The information is added to the shaded cells beginning at Row 21.

3. After filling out that the columns, check Column G ("Volume validation") for error messages; examples are shown in Figure 5. This is the result of checking the "Volume to be pooled" value from Column F.

   - If the value in Column G is "ok": no action is needed
   - If the error is "Volume too high" (>20 µl): decrease the total volume of the library pool (table 1, Column 3)
   - If the error is "Volume too low" (<2 µl): increase the total volume of the library pool

18	Table 2						
19	A	B	C	D	E	F	G
20		Sample ID	Library Concentration (ng/µl)	Library Size (bp)	Calculated Library molarity (nM)	Volume to be pooled (µl)	Volume validation
21	1	01_Sample	1	400	3.8	35.2	Volume too high
22	2	02_Sample	100	400	378.8	0.4	Volume too low
23	3	03_Sample	10	400	37.9	3.5	ok

Figure 5. Library pooling calculator: column G validation status messages. The value is automatically displayed based on a check of the volume listed in Column F. The three possible values are shown here.

NOTE: If increasing or decreasing the total library pool volume does not resolve all of the error messages, consider making separate pools for exceptionally high or low concentration libraries.

4. After filling out the columns and addressing any error messages:
   - Column E ("Calculated Library molarity (nM)") will show the calculated nanomolar concentrations of the libraries
   - Column F displays the volumes of each library required for pooling ("Volume to be pooled (µl)")

18	Table 2						
19	A	B	C	D	E	F	G
20		Sample ID	Library Concentration (ng/µl)	Library Size (bp)	Calculated Library molarity (nM)	Volume to be pooled (µl)	Volume validation
21	1	01_Sample	10	400	37.9	3.5	ok
22	2	02_Sample	10	400	37.9	3.5	ok

Figure 6. Library pooling calculator: columns E and F of table 2. The cells in these columns are automatically calculated and should not be overwritten.

   - Table 1, Column 4 of the calculator will show the volume (in µl) of Nuclease-Free Water that will need to be added to the pool to bring it up to the total pool volume displayed in table 1, Column 3.

12	Table 1			
13	1	2	3	4
14	final concentration of the pool (nM)	Number of libraries to be pooled	Total Volume of 4 nM Library Pool (µl)	Volume of Nuclease Free Water (µl)
15	4	24	800	715.5

Figure 7. Library pooling calculator: volume of Nuclease-Free Water to add to the pool, from table 1. This value is automatically calculated and should not be overwritten.

The nanomolar concentration of the libraries can also be calculated manually, using the formula:

$$\frac{\text{Concentration of Library (ng/µl)}}{\text{660 g/mol x Average Library size (bp)}} \times 10^6 = \text{Nanomolar Concentration (nM)}$$

2. How to Pool the Libraries

1. In a fresh 1.5-ml, low-adhesion microcentrifuge tube, add the required volume of each purified library found in table 2, Column F ("Volume to be pooled (µl)") of the calculator (Figure 8).

18	Table 2						
19	A	B	C	D	E	F	G
20		Sample ID	Library Concentration (ng/µl)	Library Size (bp)	Calculated Library molarity (nM)	Volume to be pooled (µl)	Volume validation
21	1	01_Sample	10	400	37.9	3.5	ok
22	2	02_Sample	10	400	37.9	3.5	ok

Figure 8. Library pooling calculator: volume to be pooled, table 2. Use the volumes of this column, starting with Row 21.

2. To the same tube, add the required volume of Nuclease-Free Water, found in table 1, Column 4 ("Volume of Nuclease Free Water (µl)") of the calculator (Figure 7, above).
3. Mix by vortexing for 5 sec and then spin briefly to collect the contents at the bottom of the tube.

SAFE STOPPING POINT: The pooled libraries are stable stored at –20°C for up to 6 months.

D. Library Denaturation for MiSeq Loading

Before you begin

- Thaw the Illumina cartridges and the HT1 buffer. Once completely thawed, keep at 4°C until needed.
- Make fresh 0.2 N NaOH by adding 20 µl of 1 N NaOH to 80 µl of Nuclease-Free Water. Mix by vortexing for 5 sec and then spin briefly to collect the contents at the bottom of the tube.

MiSeq Reagent Kit v3 (150-cycle)

1. Combine 5 µl of the 4 nM pool of libraries prepared in Section VI.C and 5 µl of freshly diluted 0.2 N NaOH in a 1.5-ml low-adhesion microcentrifuge tube.
2. Vortex the tube for 5–8 sec and then spin briefly to collect the contents at the bottom of the tube.
3. Incubate at room temperature for 5 min.
4. Add 990 µl of the cold HT1 buffer.
5. Vortex the tube for 5–8 secs and then spin briefly to collect the contents at the bottom of the tube.

   This results in a denatured library pool of 20 pM.
6. In a new 1.5-ml low-adhesion microcentrifuge tube, combine 330 µl of the 20 pM library pool (from Step 5) with 270 µl of cold HT1 buffer.

   The resulting concentration of the library pool is 11 pM.
7. Load 600 µl of 11 pM* library in the loading bay of the MiSeq cartridge.

   *If necessary, adjust and optimize the loading concentration for your sequencer.
8. Keep at 4°C until starting the MiSeq.

E. Analysis of MiSeq Sequencing Data

The sequencing data, generated in the form of FASTQ files by the Illumina MiSeq System, is analyzed using Embgenix Analysis Software (RUO). Access to the software can be obtained via sign-up at that link on the takarabio.com website. This software is hosted on cloud servers and can be accessed through a web browser using a URL that will be provided to you after sign-up.

Upload the FASTQ files generated by the MiSeq system on to the cloud server and follow the Embgenix Analysis Software (RUO) instructions for use to perform the analysis.

VIII. Technical Assistance

For technical assistance, please contact Takara Bio Technical Support or visit the Takara Bio portal at takarabio.com/.

Appendix A: Reference DNA Dilution

A. Overview

Single donor, human, genomic DNA is ideal for use as positive control DNA (e.g., Human Genomic DNA female, Coriell Institute, Cat. No. NA12878). Follow the steps in Section B to prepare the working dilutions for the reference genomic DNA. At the end of each dilution step, mix the contents gently and

centrifuge briefly before going to the next dilution step. Always use freshly diluted DNA for positive control reactions.

B. Dilution Guideline

NOTES:

- All reference DNA dilutions are carried out using 1X PBS (Phosphate Buffered Saline Ca^{2+}- and Mg^{2+}-free, Thermo Fisher Scientific, Cat. No. 10010-023) in 1.5 ml low-binding microcentrifuge tubes.
- Use ≥40% of the volume required.
- Pipette contents of each tube up and down 10 times gently to mix and pulse centrifuge to collect dilutions before proceeding to the next step.
- Always use freshly diluted DNA for positive control reactions.

1. Prepare a working stock solution of 1 ng/µl by diluting an aliquot of the original positive control gDNA.
2. Pipette 97 µl of 1X PBS buffer into a microcentrifuge tube and add 3 µl of the 1 ng/µl working stock solution from Step 1 to achieve a final concentration of 30 pg/µl.
3. Pipette 30 µl of 1X PBS buffer into a second microcentrifuge tube. Add 20 µl of the 30 pg/µl DNA stock solution from Step 2 to achieve a final concentration of 12 pg/µl.
4. Use 2.5 µl of the 12 pg/µl DNA from Step 3 as a positive control in Section V.A of the protocol.

Appendix B: Quality Control of WGA Products and Libraries

A. Overview

The quality and size of the WGA products and libraries can be measured by using a fragment analyzer such as the 4200 TapeStation or the 2100 Bioanalyzer from Agilent. An alternative way to confirm the size of the WGA products and libraries is the use of agarose gel electrophoresis.

B. Quality Control for WGA Products and Libraries Using an Agilent 2100 Bioanalyzer

The Agilent 2100 Bioanalyzer can optionally be used to assess the quality and size of the WGA products and the libraries.

- We recommend diluting an aliquot of each WGA product from Section V.B in Nuclease-Free Water to 2 ng/µl. Use 1 µl of this diluted WGA sample on Agilent's 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626).
- Alternatively, use 1 µl of the 1:20 dilution of the WGA product (WD1) from Section V.C, Step 3 for validation (See Figure 9 for examples of Bioanalyzer electropherogram profiles).

The expected average size of the WGA products or positive control should range between 500–900 bp using a region of 100–3,000 bp.

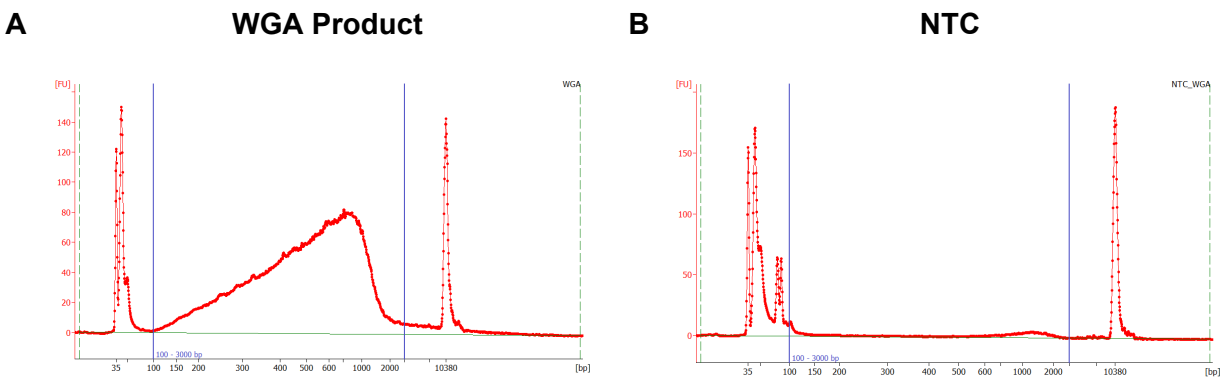

Figure 9. Bioanalyzer analysis of Embgenix WGA products. WGA products were prepared using 5 sorted cells. 1 µl of a 1:20 dilution of the WGA product was analyzed using an Agilent 2100 Bioanalyzer. **Panel A.** The electropherogram shows an average of 700 bp in a region of 100–3,000 bp. **Panel B.** The electropherogram shows the result of a similar analysis of 1 µl of the 1:20 diluted NTC showing no product between 100–3,000 bp. Dimer peaks are present between 50–100 bp.

To assess the quality and size of the libraries from Section VI.C, Step 15, we recommend diluting an aliquot of each library in Nuclease-Free Water to 3 ng/µl. Use 1 µl of this diluted library sample for validation using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (see Figure 10, below, for examples of Bioanalyzer electropherogram profiles). For detailed instructions, see the Agilent High Sensitivity DNA Kit User Manual.

The expected average size of the libraries is approximately 300–600 bp using a region of 150–1,000 bp.

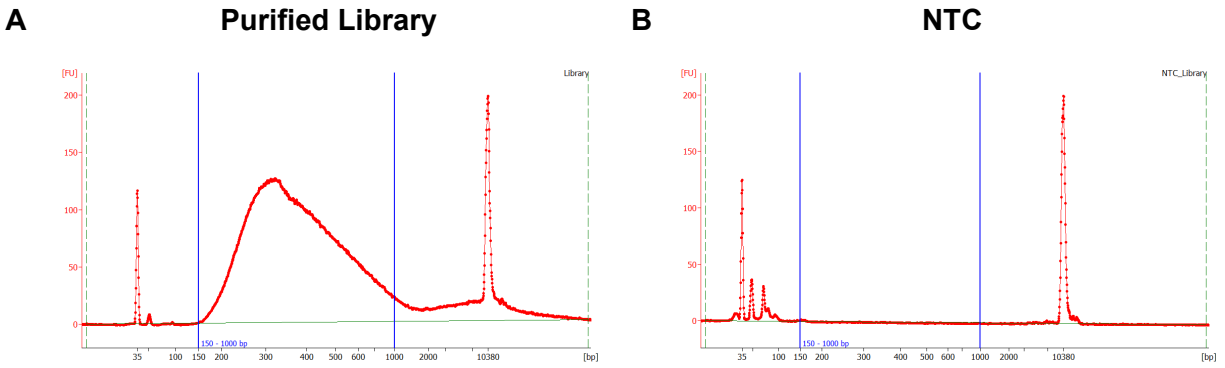

Figure 10. Bioanalyzer analysis of Embgenix libraries. Purified library was diluted to 3 ng/µl and 1 µl was analyzed using an Agilent 2100 Bioanalyzer and Agilent High Sensitivity DNA Kit. **Panel A.** The resulting electropherogram shows an average size of 400 bp in a region of 150–1,000 bp. **Panel B.** A similar analysis of 1 µl of purified NTC (undiluted) results in an electropherogram showing virtually no product.

C. Quality Control for WGA Products and Libraries by Agarose Gel Electrophoresis

Electrophoresis uses an electrical field to move the negatively charged DNA in an agarose gel matrix. Shorter DNA fragments migrate through the gel faster than longer fragments. A DNA size ladder containing nucleic acids of known lengths is used to confirm the size of your WGA product or library.

- To quantify the WGA products using agarose gel electrophoresis, we recommend using 200 ng of the WGA product from Section V.B, Step 5. Adjust the final loading volume, if necessary, following the user guide of the gel loading system. The expected average size of the WGA products or positive control range is between 500–900 bp.

- To quantify the libraries using agarose gel electrophoresis, we recommend using 100 ng of purified libraries from Section V.C, Step 15. The expected average size of the libraries is approximately 400 bp.

An agarose electrophoresis system, such as E-gel Agarose System using E-Gel General Purpose Agarose Gels, 2% (Thermo Fisher Scientific, Cat. No. G501802), or general agarose electrophoresis can be used to assess the quality and size of the libraries. For detailed instructions for the E-gel Agarose System, refer to the vendor's user manual.

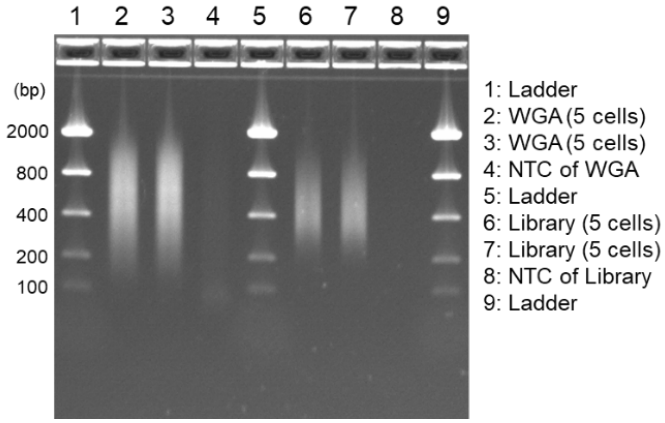

Figure 11. Agarose gel electrophoresis of Embgenix WGA products and libraries. A precast 2% agarose gel was used. Lanes 1, 5, and 9: Low Range Quantitative DNA Ladder (Thermo Fisher Scientific, Cat. No. 12373031). Lanes 2 and 3: 5 µl or 200 ng of WGA products prepared from 5 sorted cells. Lane 4: 5 µl of NTC of WGA. Lanes 6 and 7: 100 ng of purified libraries. Lane 8: 5 µl of undiluted NTC of library. Analysis and interpretation of the electropherogram showed an average peak of 600 bp for the WGA products and 400 bp for the libraries.

Appendix C: Indexing Recommendations and Sequences

A. Overview

The Embgenix PGT-A Kit (RUO) includes Unique Dual Index Kit (1–96), which are 8-nt long and employ "IDT-ILMN TruSeq DNA UD Indexes (96 Indexes)" i5 and i7 dual index sequences. The primers are provided in a 96-well plate format.

B. Multiplexing and Index Pooling

The Unique Dual Index Kit (1–96) contains sufficient volume for a single-use in each well. It is important to select appropriate indexes that are unique and meet the Illumina-recommended compatibility and color balance requirements. The UDIs should be chosen from a minimum number of columns to achieve greater color balance.

Low plexity (2–8 samples)

For low-plex pooling involving fewer than eight samples per sequencing run, follow the guidelines in Illumina's Index Adapters Pooling Guide (Illumina, Document #1000000041074-v8) and as shown in Table 4, below. Pool the libraries with indexes belonging to the same column (e.g., wells A1 to H1). Avoid selecting indexes across rows when pooling less than 8 samples (i.e., A1 to A12).

Higher plexity (>8 samples)

If pooling more than eight samples, use UDIs from multiple columns of Table 4. For example, for a plexity of 9, U001–U009 or U001–U004 + U009–U013 are valid.

Table 4. Unique Dual Index Kit (1–96) index locations on the plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	U001	U009	U017	U025	U033	U041	U049	U057	U065	U073	U081	U089
B	U002	U010	U018	U026	U034	U042	U050	U058	U066	U074	U082	U090
C	U003	U011	U019	U027	U035	U043	U051	U059	U067	U075	U083	U091
D	U004	U012	U020	U028	U036	U044	U052	U060	U068	U076	U084	U092
E	U005	U013	U021	U029	U037	U045	U053	U061	U069	U077	U085	U093
F	U006	U014	U022	U030	U038	U046	U054	U062	U070	U078	U086	U094
G	U007	U015	U023	U031	U039	U047	U055	U063	U071	U079	U087	U095
H	U008	U016	U024	U032	U040	U048	U056	U064	U072	U080	U088	U096

C. Unique Dual Index Kit (1–96) Barcodes

Table 5. UDI barcode sequences. The table continues over the next two pages.

Index Name	i7 Bases for Sample Sheet	i5 Bases for Sample Sheet (MiSeq, NovaSeq™, HiSeq® 2000/2500)	i5 Bases for Sample Sheet (MiniSeq™, NextSeq®, HiSeq 3000/4000)
U001	CCGCGGTT	AGCGCTAG	CTAGCGCT
U002	TTATAACC	GATATCGA	TCGATATC
U003	GGACTTGG	CGCAGACG	CGTCTGCG
U004	AAGTCCAA	TATGAGTA	TACTCATA
U005	ATCCACTG	AGGTGCGT	ACGCACCT
U006	GCTTGTCA	GAACATAC	GTATGTTC
U007	CAAGCTAG	ACATAGCG	CGCTATGT
U008	TGGATCGA	GTGCGATA	TATCGCAC
U009	AGTTCAGG	CCAACAGA	TCTGTTGG
U010	GACCTGAA	TTGGTGAG	CTCACCAA
U011	TCTCTACT	CGCGGTTC	GAACCGCG
U012	CTCTCGTC	TATAACCT	AGGTTATA
U013	CCAAGTCT	AAGGATGA	TCATCCTT
U014	TTGGACTC	GGAAGCAG	CTGCTTCC
U015	GGCTTAAG	TCGTGACC	GGTCACGA
U016	AATCCGGA	CTACAGTT	AACTGTAG
U017	TAATACAG	ATATTCAC	GTGAATAT
U018	CGGCGTGA	GCGCCTGT	ACAGGCGC
U019	ATGTAAGT	ACTCTATG	CATAGAGT
U020	GCACGGAC	GTCTCGCA	TGCGAGAC
U021	GGTACCTT	AAGACGTC	GACGTCTT
U022	AACGTTCC	GGAGTACT	AGTACTCC
U023	GCAGAATT	ACCGGCCA	TGGCCGGT
U024	ATGAGGCC	GTTAATTG	CAATTAAC
U025	ACTAAGAT	AACCGCGG	CCGCGGTT
U026	GTCGGAGC	GGTTATAA	TTATAACC
U027	CTTGGTAT	CCAAGTCC	GGACTTGG
U028	TCCAACGC	TTGGACTT	AAGTCCAA
U029	CCGTGAAG	CAGTGGAT	ATCCACTG

Index Name	i7 Bases for Sample Sheet	i5 Bases for Sample Sheet (MiSeq, NovaSeq™, HiSeq® 2000/2500)	i5 Bases for Sample Sheet (MiniSeq™, NextSeq®, HiSeq 3000/4000)
U030	TTACAGGA	TGACAAGC	GCTTGTCA
U031	GGCATTCT	CTAGCTTG	CAAGCTAG
U032	AATGCCTC	TCGATCCA	TGGATCGA
U033	TACCGAGG	CCTGAACT	AGTTCAGG
U034	CGTTAGAA	TTCAGGTC	GACCTGAA
U035	AGCCTCAT	AGTAGAGA	TCTCTACT
U036	GATTCTGC	GACGAGAG	CTCTCGTC
U037	TCGTAGTG	AGACTTGG	CCAAGTCT
U038	CTACGACA	GAGTCCAA	TTGGACTC
U039	TAAGTGGT	CTTAAGCC	GGCTTAAG
U040	CGGACAAC	TCCGGATT	AATCCGGA
U041	ATATGGAT	CTGTATTA	TAATACAG
U042	GCGCAAGC	TCACGCCG	CGGCGTGA
U043	AAGATACT	ACTTACAT	ATGTAAGT
U044	GGAGCGTC	GTCCGTGC	GCACGGAC
U045	ATGGCATG	AAGGTACC	GGTACCTT
U046	GCAATGCA	GGAACGTT	AACGTTCC
U047	GTTCCAAT	AATTCTGC	GCAGAATT
U048	ACCTTGGC	GGCCTCAT	ATGAGGCC
U049	ATATCTCG	ATCTTAGT	ACTAAGAT
U050	GCGCTCTA	GCTCCGAC	GTCGGAGC
U051	AACAGGTT	ATACCAAG	CTTGGTAT
U052	GGTGAACC	GCGTTGGA	TCCAACGC
U053	CAACAATG	CTTCACGG	CCGTGAAG
U054	TGGTGGCA	TCCTGTAA	TTACAGGA
U055	AGGCAGAG	AGAATGCC	GGCATTCT
U056	GAATGAGA	GAGGCATT	AATGCCTC
U057	TGCGGCGT	CCTCGGTA	TACCGAGG
U058	CATAATAC	TTCTAACG	CGTTAGAA
U059	GATCTATC	ATGAGGCT	AGCCTCAT
U060	AGCTCGCT	GCAGAATC	GATTCTGC
U061	CGGAACTG	CACTACGA	TCGTAGTG
U062	TAAGGTCA	TGTCGTAG	CTACGACA
U063	TTGCCTAG	ACCACTTA	TAAGTGGT
U064	CCATTCGA	GTTGTCCG	CGGACAAC
U065	ACACTAAG	ATCCATAT	ATATGGAT
U066	GTGTCGGA	GCTTGCGC	GCGCAAGC
U067	TTCCTGTT	AGTATCTT	AAGATACT
U068	CCTTCACC	GACGCTCC	GGAGCGTC
U069	GCCACAGG	CATGCCAT	ATGGCATG
U070	ATTGTGAA	TGCATTGC	GCAATGCA

Index Name	i7 Bases for Sample Sheet	i5 Bases for Sample Sheet (MiSeq, NovaSeq™, HiSeq® 2000/2500)	i5 Bases for Sample Sheet (MiniSeq™, NextSeq®, HiSeq 3000/4000)
U071	ACTCGTGT	ATTGGAAC	GTTCCAAT
U072	GTCTACAC	GCCAAGGT	ACCTTGGC
U073	CAATTAAC	CGAGATAT	ATATCTCG
U074	TGGCCGGT	TAGAGCGC	GCGCTCTA
U075	AGTACTCC	AACCTGTT	AACAGGTT
U076	GACGTCTT	GGTTCACC	GGTGAACC
U077	TGCGAGAC	CATTGTTG	CAACAATG
U078	CATAGAGT	TGCCACCA	TGGTGGCA
U079	ACAGGCGC	CTCTGCCT	AGGCAGAG
U080	GTGAATAT	TCTCATTC	GAATGAGA
U081	AACTGTAG	ACGCCGCA	TGCGGCGT
U082	GGTCACGA	GTATTATG	CATAATAC
U083	CTGCTTCC	GATAGATC	GATCTATC
U084	TCATCCTT	AGCGAGCT	AGCTCGCT
U085	AGGTTATA	CAGTTCCG	CGGAACTG
U086	GAACCGCG	TGACCTTA	TAAGGTCA
U087	CTCACCAA	CTAGGCAA	TTGCCTAG
U088	TCTGTTGG	TCGAATGG	CCATTCGA
U089	TATCGCAC	CTTAGTGT	ACACTAAG
U090	CGCTATGT	TCCGACAC	GTGTCGGA
U091	GTATGTTC	AACAGGAA	TTCCTGTT
U092	ACGCACCT	GGTGAAGG	CCTTCACC
U093	TACTCATA	CCTGTGGC	GCCACAGG
U094	CGTCTGCG	TTCACAAT	ATTGTGAA
U095	TCGATATC	ACACGAGT	ACTCGTGT
U096	CTAGCGCT	GTGTAGAC	GTCTACAC

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This document has been reviewed and approved by the Quality Department.