Takara Bio USA

Embgenix™ ESM Screen Kit User Manual

Cat. No. 634782
(120222)

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I. Introduction

The **Embgenix ESM Screen Kit (Cat. No. 634782)** is a research-use only (RUO) kit designed to evaluate chromosome copy number variation (CNV) using non-invasive sample types collected during an IVF cycle, including embryo spent media (ESM) and/or blastocoel fluid (it is not designed for use in diagnostic testing). All 24 chromosomes can be screened for the identification of whole chromosome and segmental aneuploidies, as well as mosaicism (with limited thresholds).

The kit is optimized for handling larger volume of the input material and comes with a quantification kit for the accurate determination of cell-free DNA (cfDNA) present in non-invasive samples. The kit uses next-generation sequencing (NGS) technology as the detection method and is compatible with Illumina® MiSeq® or NextSeq® systems.

To streamline the generation of sequencing libraries, the kit utilizes PicoPLEX® whole-genome amplification (WGA) and patented library-prep technology with unique dual indexes (UDIs). The simple protocol dramatically speeds up the time to results while reducing handling errors and sequencing noise. The library-prep workflow (fragmentation, repair, ligation, amplification, and indexing) completes in about two hours in a single tube. No intermediate purification steps or sample transfers are necessary, minimizing the potential for loss and mix-up of samples.

After sequencing, data analysis and reporting are performed using our specialized, cloud-based bioinformatics pipeline, Embgenix Analysis Software (RUO) with its advanced ESM workflow, designed for non-invasive samples. The algorithm accurately determines chromosomal copy number variation (CNVs) by using bin counts to calculate copy numbers (CCNs) against an internally configured reference. The software provides options for automated and/or manual calls for whole chromosome, segmental, and mosaic aneuploidies. Data can be conveniently visualized in a chart or an idiogram plot. The software supports downloadable PDF reports, which can be customized for individual laboratory needs.

For a visual overview of the workflow, see Figure 1 on the next page.

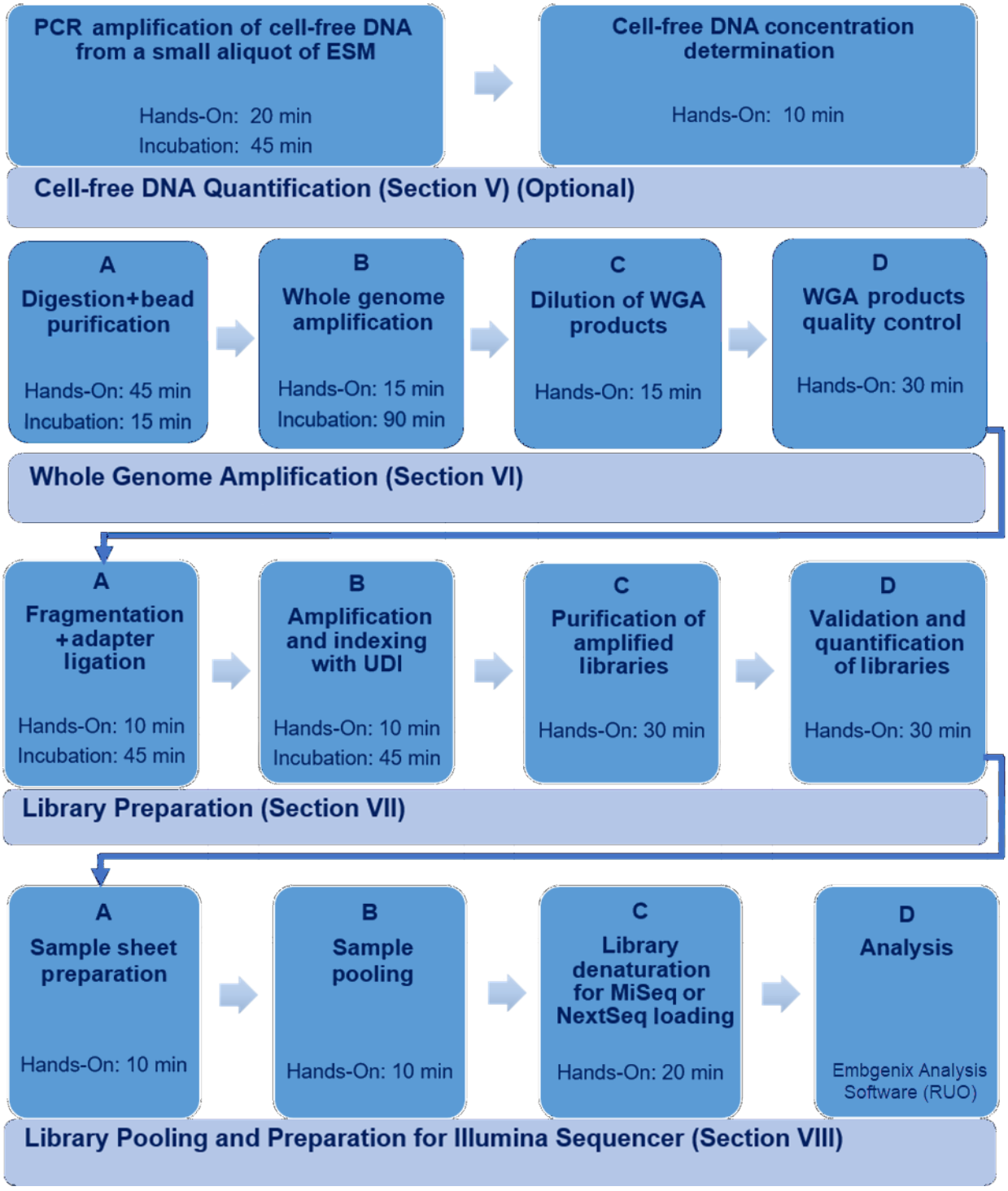


Figure 1. Embgenix ESM Screen Kit Protocol overview.

II. List of Components

The Embgenix ESM Screen Kit 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 ESM Screen Kit components.

Embgenix ESM Screen Kit	Cap color	634782 (96 rxns)
Embgenix ESM Screen Core Kit (Cat. No. 634783*)		
Package 1 (Store at –70°C†)		
TB Green® Advantage® qPCR Premix (2X)	White	4 x 625 µl
ROX Reference Dye LSR (50X)	Yellow	100 µl
ROX Reference Dye LMP (50X)	Brown	100 µl
Package 2 (Store at –20°C)		
Primer Mix (5X)	Clear	1 ml
DNA Standard 1 (100 pg/µl)	Clear	75 µl
EASY Dilution Solution	Clear	2 x 1 ml
qPCR Nuclease-Free Water	Clear	1.5 ml
ESM-WGA Buffer	Red	3 x 1.5 ml
ESM-WGA Enzyme	White	115 µl
ESM-WGA Nuclease-Free Water	Clear	3 x 1.8 ml
Package 3 (Store at 4°C)		
Magnetic Beads‡	N/A	2 x 5 ml
Digestion Enzyme	Orange	250 µl
Digestion Buffer	Blue	1.8 ml
Package 4 (Store at –20°C)		
10X FE	White	60 µl
FE Dilution Buffer	White	1 ml
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	Clear	4 x 1 ml
ESM-WGA Dilution Solution§	N/A	25 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

*Embgenix ESM Screen Core Kit not sold separately.

†After first use, store contents of Package 1 at 4°C.

‡The kit has been specifically validated with Magnetic Beads (Section VI.B) and NucleoMag NGS Clean-up and Size Select (Section VII.C). Do not use substitutions, as it may lead to unexpected results.

- We strongly recommend aliquoting each type of beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots.
- Do not freeze the beads
- 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.

§Upon receipt, remove ESM-WGA Dilution Solution from Package 4 and store at 4°C.

III. Additional Materials Required

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

- Single-channel pipette: 2 µl, 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: 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)
- Vortex mixer

NOTE: For all protocols listed below, select appropriate tubes or plates that are compatible with the thermal cyclers and/or real-time thermal cyclers used.

For DNA quantification (Optional, Section V)

- TempPlate non-skirted 0.2 ml 96-well PCR plate (USA Scientific, Item No. 1402-9596)
- Seal-Rite 1.5 ml Microcentrifuge tubes (USA Scientific, Item. No. 1615-5500)
- Axygen 5 ml Self Standing Screw Cap Transport Tube (Product No. SCT-5ML-S)
- Microseal 'B' PCR Plate Sealing Film, adhesive, optical (Bio-Rad, Cat. No. MSB1001)
- MicroAmp Optical 96-Well Reaction Plate (ThermoFisher Cat. No. N8010560)
- MicroAmp Optical Adhesive Film Kit (ThermoFisher Cat. No. 4313663)
- Real-time PCR instrument (or equivalent)
  - CFX Real-Time PCR Detection System (Bio-Rad)
  - QuantStudio Real-Time PCR System (ThermoFisher)

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

- Single donor human genomic DNA, for use as positive control (e.g., Human Genomic DNA female, Corriel Institute, Cat. No. NA12878)
- 70% Ethanol: freshly made for each experiment. Use a fresh or a pre-PCR-dedicated bottle of molecular biology grade 100% ethanol and nuclease-free water.
- 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, Item No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes
- Thermo Scientific 15 ml Centrifuge Tube (Thermo Fisher, Cat. No. 50-201-997)
- Adhesive PCR Plate Seal (Thermo Fisher, Cat. No. AB0626 or USA Scientific, Item No. 2938-4100) for 96-well plates or cap strips (Thermo Fisher, Cat. No. AB0784/AB0850) for 8-tube strips

- 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
    - Minicentrifuge for 0.2-ml tubes or strips
  - For 24–96 or more samples:
    - Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027)
    - Low-speed benchtop centrifuge for a 96-well plate
- Thermal cycler with a heated lid

For Purification of Amplified Libraries (Section VII.C)

The kit has been specifically validated with NucleoMag NGS Clean-up and Size Select.

NOTES:

- Please do not make any substitutions as it may lead to unexpected results.
- Beads need to come to room temperature before the container is opened. We strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). Aliquoting is also instrumental in decreasing the chances of bead contamination.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

- 80% Ethanol: freshly made for each experiment from molecular-biology-grade 100% ethanol and nuclease-free water
- Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates (similar to above)
- 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR-grade tube strips secured in a PCR rack, or 96-well plates (e.g., Bio-Rad iQ 96-Well PCR Plates, Cat. No. 2239441)

For WGA and Library Quantification Quality Control (Sections VI.E & VII.D) (As Required)

- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854) (may be used in Sections VI.E and VII.D)
- 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, Item 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, Part No. 5067-4626) for BioAnalyzer or equivalent high-sensitivity electrophoresis method (may be used in Sections V.D and VI.D)
  - High Sensitivity D5000 ScreenTape (Agilent, Part No. 5067-5592) with High Sensitivity D5000 Reagents (Agilent, Part No. 5067- 5593)
  - High Sensitivity D1000 ScreenTape (Agilent, Part No. 5067-5584) with High Sensitivity D1000 Reagents (Agilent, Part No. 5067-5585)

For Library Pooling and Denaturation (Section VIII) (As Required)

- 1 N NaOH solution (Fisher Chemical, Cat. No. SS266-1)
- 1 M Tris-HCl solution, pH 7.0 (Fisher BioReagents, Cat. No. BP1756-100)
- 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) or NextSeq® 500/550 Mid Output kit v2.5 (150 cycles) (Illumina, Cat. No. 20024904)

IV. General Considerations

A. Sample Recommendations and Requirements

- The Embgenix ESM Screen Kit is compatible with up to 30 µl of undiluted embryo spent media. The following media have been tested and validated for compatibility.
  - Global plus 10% Quinn's Advantage Serum Protein Substitute (CooperSurgical)
  - SAGE 1-step with HSA (CooperSurgical)
  - G-2 PLUS (Vitrolife)
  - Continuous Single Culture Complete with HSA (Irvine Scientific)
- Our recommendation for the minimum input of cfDNA for the first step in this process (Section VI) is 2 pg. Lower cfDNA input may result in increased calculated copy number (CCN) noise. Fresh, uncultured media for embryo culture can be used as no-template control (NTC).
- Because of the minimum input recommendation above, we also recommend measuring the concentration of cfDNA contained in the spent media (Section V) prior to running the ESM screening process.
  - Plan on no more than 86 samples per 96-well plate, as 10 wells are reserved for the DNA standards dilutions (Section V.A) and NTCs (4 DNA standards + 1 NTC, in duplicate).
  - If more than 86 samples are to be tested, a second plate will be required. Each plate requires a set of standards and NTC (10 wells) to generate a standard curve for the samples on that plate.

B. Requirements for Preventing Contamination

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

- **A PCR clean workstation**, for
  - All DNA quantification reaction preparation, as this assay is highly sensitive to DNA contamination (Section V.A).
  - All pre-PCR experiments that require cleanroom conditions, i.e., sample and WGA reaction preparations (Sections VI.B & C)
- **A second workstation located in the general laboratory** for WGA product dilution and QC (Section VI.D & E), library preparation, purification, and library concentration measurement (Section VII), and library pooling and sequencing (Section VIII).

C. General Requirements

IMPORTANT! We recommend performing a **minimum** of 12 reactions per protocol run to ensure sufficient reagents to perform 96 reactions per kit.

- The assay is very sensitive to variations in pipetting volume. Please make sure all your pipettes are calibrated for reliable delivery.
- Negative and positive control reactions should be performed to verify that the kit components are working properly.
- All lab supplies related to the WGA need to 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.
- Vials and plates containing reagents should be spun down prior to opening to move contents to the bottom.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down or vortex as indicated.
- Do not change the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the Embgenix ESM Screen reagents and protocol.

D. Hazardous Materials

CAUTION:

– Digestion Buffer contains chaotropic salts; wear gloves and goggles.

– Digestion Buffer contains guanidine hydrochloride which can form highly reactive compounds when combined with bleach (sodium hypochlorite). DO NOT add bleach or acidic solutions directly to the sample preparation waste.

E. Beads Handling

Distribution of beads

Magnetic Beads (from Package 3) and NucleoMag NGS Clean-Up and Size Select beads should be equilibrated to room temperature for a minimum of 30 min before use. A homogeneous distribution of the Magnetic Beads and NucleoMag NGS Clean-Up and Size Select beads to the individual tubes is essential for a high sample-to-sample consistency. Therefore, before distributing the beads, make sure that the beads are completely resuspended. Shake the storage bottle well or vortex briefly.

Magnetic separation time

Attraction of the Magnetic Beads and NucleoMag NGS Clean-Up and Size Select beads to the magnetic pins depends on the magnetic strength of the magnetic pins, the selected separation plate, distance of the separation plate from the magnetic pins, and the volume to be processed. The individual times for complete attraction of the beads to the magnetic pins should be checked and adjusted on each system. It is recommended to use the separation plates or tubes specified by the supplier of the magnetic separator.

V. Protocol: Cell-free DNA Quantification (Optional)

We recommend measuring the cfDNA concentration in the spent media samples before processing them for ESM screening. Sample input with less than 2 pg cfDNA is at risk for increased CCN noise using the Embgenix ESM Screen protocol. The following quantification process requires 1 µl of undiluted spent media.

A. Protocol: Real-time PCR

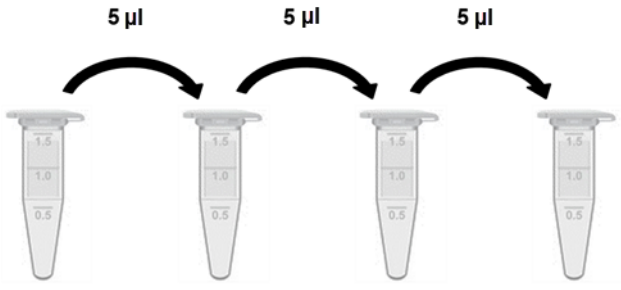
Before you begin

- Please check if your real-time PCR thermal cycler requires an internal ROX reference dye for flourescent signal normalization. If the reference dye has to be incorporated:
  - use ROX Reference Dye LSR with instruments with a 488 nm laser excitation source
  - use ROX Reference Dye LMP with instruments which either have a lamp or an LED as the excitation source
- Thaw the following on ice:
  - Primer Mix
  - DNA Standards 1
  - EASY Dilution solution
  - qPCR Nuclease-Free Water
- If using a new, frozen tube of TB Green Advantage qPCR Premix, thaw it on ice. If using a previously opened tube of TB Green Advantage qPCR Premix, leave at 4°C until needed.
- Once thawed, spin reagent tubes briefly to collect the contents at the bottom.

Procedure

1. Prepare DNA standards by serial dilution
   a. Prepare three 1.5 ml tubes and label them S2, S3 and S4.
   b. Dispense 45 µl of EASY Dilution solution to the tubes.
   c. Add 5 µl of DNA Standard 1 to the S2 tube. Mix by brief vortex and spin down.
   d. Repeat the same process with S2 to S3, then S3 to S4.

   NOTE: S2, S3 and S4 dilutions can be stored for up to three days at 4°C.



Tube label:	DNA Standard1	S2	S3	S4
Final Concentration:	100 pg/µl	10 pg/µl	1 pg/µl	0.1 pg/µl
EASY Dilution solution Volume:	-----	45 µl	45 µl	45 µl

Figure 2. DNA Standard preparation

2. Prepare plate map(s) to test each sample, DNA standards, and NTCs (Figure 3), up to 86 samples per plate.

REMINDER: If more than 86 samples are to be tested, a second plate will be required. Refer to Section IV.A "Sample Recommendations and Requirements" for more details.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1 100 pg/µl	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample39	Sample47	Sample55	Sample63	Sample71	Sample79
B	Std 1 100 pg/µl	NTC	Sample 8	Sample 16	Sample 24	Sample32	Sample40	Sample48	Sample56	Sample64	Sample72	Sample80
C	Std 2 10 pg/µl	Sample 1	Sample 9	Sample 17	Sample 25	Sample33	Sample41	Sample49	Sample57	Sample65	Sample73	Sample81
D	Std 2 10 pg/µl	Sample 2	Sample 10	Sample 18	Sample 26	Sample34	Sample42	Sample50	Sample58	Sample66	Sample74	Sample82
E	Std 3 1 pg/µl	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample43	Sample51	Sample59	Sample67	Sample75	Sample83
F	Std 3 1 pg/µl	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample44	Sample52	Sample60	Sample68	Sample76	Sample84
G	Std 4 0.1 pg/µl	Sample 5	Sample 13	Sample 21	Sample 29	Sample37	Sample45	Sample53	Sample61	Sample69	Sample77	Sample85
H	Std 4 0.1 pg/µl	Sample 6	Sample 14	Sample 22	Sample 30	Sample38	Sample46	Sample54	Sample62	Sample70	Sample78	Sample86

Figure 3. Example Plate Map. Map contains DNA standards, NTC, and 86 spent media samples of unknown cfDNA concentration with the cfDNA Quantification Master Mix for amplification.

3. On ice, assemble the master mix as described below for all reactions plus 10% extra.

REMINDER: The total number of reactions equals: *Number of samples* + 10

cfDNA Quantification Master Mix:

12.5 µl	TB Green Advantage qPCR Premix (2X)
5 µl	Primer Mix (5X)
6 µl	qPCR Nuclease-Free Water
0.5 µl	ROX Reference Dye LSR or LMP (50X)*
24 µl	Total volume per reaction

*Replace with Nuclease-free Water if ROX is not required.

Mix thoroughly with a pipette, spin down to collect the content at the bottom of the tube. Keep on ice.

4. Dispense 24 µl of the cfDNA Quantification Master Mix into wells.
5. Add 1 µl of undiluted sample, each of the four DNA standards, or NTC into appropriate wells. Mix by gently pipetting up and down three times or seal tubes or plates and doing a light vortex.

NOTE: Do NOT mix or vortex intensely. The goal is to mix without creating bubbles.

6. Seal tubes or plates if mixed by pipetting in the previous step. Centrifuge the sealed tubes or plates briefly to collect contents at the bottom of the tube. Visually inspect to ensure no bubbles are in the reaction solution. If bubbles are present, repeat centrifugation until they are removed.
7. Run the following thermocycling conditions:

95°C	120 sec	
40 cycles:		
95°C	5 sec	]
62°C*	30 sec	]

*Measure TB Green fluorescence at the end of each cycle in the SYBR/FAM channel

B. Protocol: Cell-free DNA Concentration Calculation

1. Plot the average Cq values for the four DNA Standards versus their respective concentration in $\log_{10}$ scale to generate a standard curve.

   NOTE: The coefficient of determination (R^2) should be ≥0.98 and the reaction efficiency should be between 90–110%.
2. Use the Cq value for each sample and the standard curve to calculate the cfDNA concentration of each sample.

VI. Protocol: Preparation of Whole Genome Amplification

- This section should be performed in a PCR clean workstation.
- Components of the Embgenix ESM Sample Core Kit from Package 2 and Package 3 are used in Sections VI.A–C.
- Before starting, please read the entire set of protocols in this section as well as Appendix A, which includes additional instructions for reference DNA dilution.

A. Before you Begin

- Bring the Magnetic Beads and the Digestion Buffer to room temperature for at least 30 min.
- If the samples are frozen, thaw them on ice or in a cold chiller rack.
- Place and thaw (if necessary) the following reagents on ice:
  - ESM-WGA buffer
  - ESM-WGA Nuclease-Free Water
  - Digestion Enzyme
- Keep the ESM-WGA enzyme at –20°C until required for use.
- **Positive control:** Create a 15 pg DNA sample diluted to the equivalent volume of the largest spent media sample tested. Refer to Appendix A for more information.
- **Negative control (NTC):** Nuclease-Free Water and/or fresh culture media at a volume equivalent to the largest spent media sample tested.
- Prepare fresh 70% ethanol for each experiment. You will need ~400 µl per sample.
- Preheat thermal cycler lid
- Make a note of the original volume of sample(s) in each sample tube. This value will be used during this protocol to determine volumes of reagents to be added to the sample(s), referred to as "original sample volume".

B. Protocol: Sample Digestion and Purification

IMPORTANT! Do **NOT** create a master mix with the Digestion Buffer and Digestion Enzyme.

1. Spin samples, controls, and reagents briefly to collect the contents at the bottom of the tubes.
2. Prepare new PCR tubes and dispense your positive and negative controls accordingly. If necessary, transfer the sample(s) to a new PCR tube.
3. To each sample or control, add 2 µl of Digestion Enzyme. Mix by flicking the tubes. Spin briefly to collect the contents at the bottom of the tubes.
4. The volume of Digestion Buffer to add is proportional to the original sample volume; add the appropriate volume of Digestion Buffer to each sample or control following the guidelines listed in Table 2.

   NOTE: Digestion Buffer is viscous. Pipette carefully and avoid creating bubbles.

   Table 2. Digestion Buffer volumes.

Sample volume	Digestion Buffer volume
10 µl	5 µl
15 µl	7.5 µl
20 µl	10 µl
25 µl	12.5 µl
30 µl	15 µl

   Vortex briefly to mix. Spin briefly to collect the contents at the bottom of the tubes.

5. Incubate tubes at room temperature for 15 min.
6. Mix the Magnetic Beads well by vortexing; if necessary, refer to Section IV.E for information on beads handling. Briefly spin down, maintaining the homogenous color, and carefully open the tube. The volume of Magnetic Beads to add is proportional to the original sample volume; add the appropriate volume of beads to the digested samples following the guidelines in Table 3.

   Table 3. Magnetic Beads volumes.

Sample volume	Magnetic Beads volume
10 µl	31 µl
15 µl	45 µl
20 µl	58 µl
25 µl	72 µl
30 µl	85 µl

   Vortex to mix.

   NOTES:
   - When vortexing the samples, make sure tube caps or seals are tightly closed.
   - We recommend holding the cap or seal with your fingers while vortexing, as chemicals in the buffers can compromise the seal.

7. Incubate the tubes at room temperature for 5 min.
8. Spin briefly to collect the contents at the bottom of the tubes.

9. Separate the Magnetic Beads against the side of the tube by using a magnetic separator. Once beads are pelleted and the supernatant is clear, discard the supernatant carefully by pipetting.

   NOTE: The supernatant contains Digestion Buffer. See Secction IV.D for additional handling information of this hazardous material.

10. Add 200 µl of freshly prepared 70% ethanol to each sample while on the magnetic separator.
11. Incubate for 1 min.
12. Discard the supernatant carefully by pipetting.
13. Add 200 µl of freshly prepared 70% ethanol while on the magnetic separator.
14. Incubate for 1 min.

    NOTE: We recommend assembling the WGA Master Mix while samples are on the magnetic separator during this time. See Section VI.C, Step 1 for details.

15. Discard the supernatant carefully by pipetting.
16. Spin briefly to collect the liquid from the side of the tubes. Place the samples on the magnetic separation device for 30 sec, then remove any residual liquid with a fine-tip pipette.
17. Air-dry the magnetic beads at room temperature for 2 min or until the pellet is no longer shiny, but before cracks appear. If the pellet is still shiny after 2 min, continue to dry but check at 30 sec intervals to prevent overdrying.

    NOTE: Check the pellet frequently during this time and continue to Step 17 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 WGA product may not elute well from the beads and recovery may be reduced.

18. Immediately proceed to Step 2 of Section VI.C, below.

C. Protocol: Whole Genome Amplification

1. On ice, assemble the WGA Master Mix as indicated below for all reactions plus 10% extra.

   WGA Master Mix:

35 µl	ESM-WGA Buffer
1 µl	ESM-WGA Enzyme
39 µl	ESM-WGA Nuclease-Free Water
75 µl	Total volume per sample

   Mix thoroughly with a pipette, spin briefly to collect the contents at the bottom of the tube. Keep on ice.

2. (continued from Section VI.B step 17) Remove the tubes from the magnetic separator and add 75 µl of the WGA Master Mix to each sample.
3. Set the pipette volume to 65 µl and resuspend the magnetic beads completely by gently pipetting up and down 10 times. Spin briefly to collect the contents at the bottom of the tube.

4. Place the tubes in a thermal cycler with the heated lid set to 100°C–105°C. Perform the WGA reaction using the following cycling conditions:

25°C	5 min
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 the amplification, remove tubes from the thermocycler and 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.

D. Protocol: Dilution of WGA Products for Quality Control and Quantification

For this step, you will need the ESM-WGA Dilution Solution from the Embgenix ESM Sample Core Kit, Package 4.

Dilution of the WGA product 1:10

1. Thaw:
   - ESM-WGA Dilution Solution on the bench top (at room temperature, if needed). Tap gently on the bench before opening.
   - If WGA products from the previous protocol (Section VI.C, Step 5) were stored frozen, thaw them on ice. Once thawed, vortex to mix, and then spin briefly to collect the contents at the bottom of the tubes.
2. Place the WGA products on a magnetic separator.
3. Prepare new PCR tubes for dilution. Pipette 18 µl of ESM-WGA Dilution Solution to each new tube.
4. Add 2 µl of the supernatant after Step 2 (WGA products) into the tubes with ESM-WGA Dilution Solution.
5. Close the tubes, mix by gently vortexing and spin briefly to collect the contents at the bottom.

NOTE: This is not a safe stopping point. Diluted WGA product should be used on the day of dilution. Long term storage is not recommended.

E. Protocol: Determination of WGA Quality and Quantity

WGA product 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 (Cat. No. Q33120).

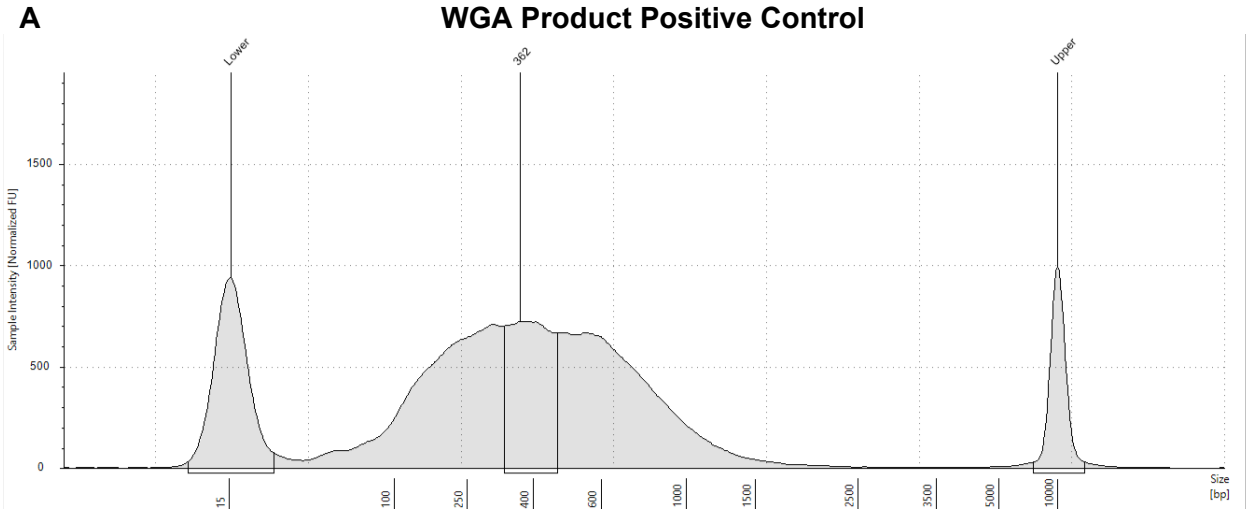
Determination of WGA Quantity: To quantify the WGA product using Qubit Fluorometer, we recommend using 2 µl of the 1:10 diluted product prepared above in Section VI.D. See Qubit dsDNA HS Assay user manual for detailed instructions.

To determine the concentration of the WGA product, multiply the sample measurement by the dilution factor (10). The concentration of the WGA product from the positive control is typically ≥12 ng/µl.

Determination of WGA Quality (Optional): A fragment analyzer, such as the Agilent 2100 BioAnalyzer or the Agilent 4200 TapeStation can optionally be used to assess quality and size of the WGA product.

- We recommend using 2 µl of the 1:10 diluted WGA product prepared in Section VI.D using the Agilent 4200 TapeStation with High Sensitivity D5000 Reagents (Agilent, Part No. 5067-5594). The expected average size of the WGA products of samples and positive controls should range between 200–800 bp in a region between 100–5,000 bp.
- Alternatively, use 1 µl of the 1:10 diluted WGA product for size assessment using Agilent's 2100 BioAnalyzer and High Sensitivity DNA Kit (Agilent, Part No. 5067-4627).

NOTE: See Appendix B for details of other quality control methods such as agarose gel electrophoresis. Additionally, there are other examples of TapeStation and BioAnalyzer electropherogram profiles.



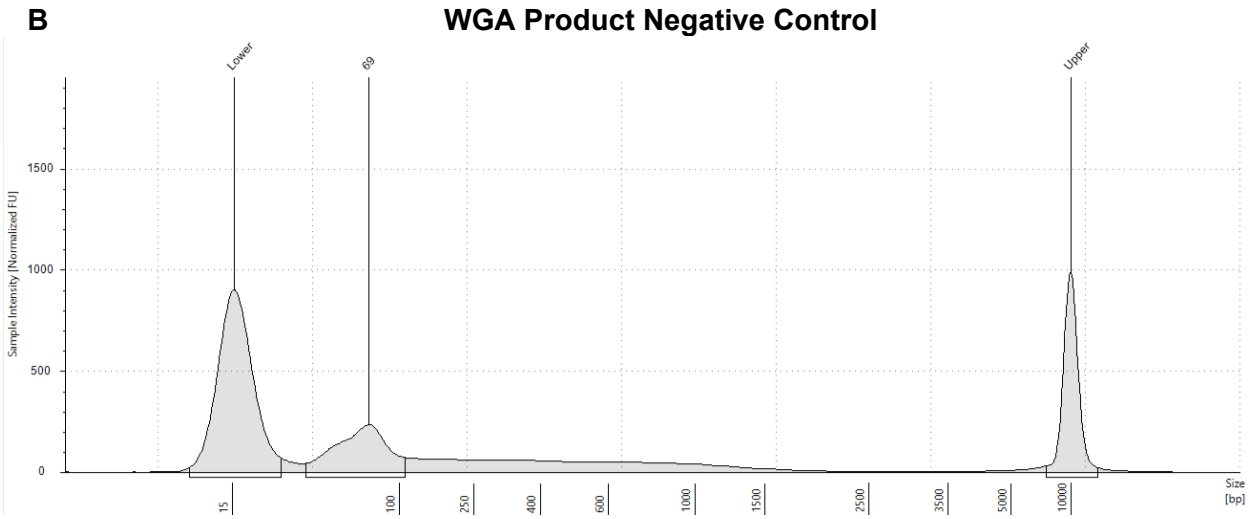


Figure 4. TapeStation analysis of WGA Products. WGA products were prepared using 2 pg sheared gDNA or a negative control (NTC). These products were diluted to 1:10 and 2 µl was loaded on a High Sensitivity D5000 ScreenTape. **Panel A.** WGA product from a 1.5 kb sheared gDNA positive control with a peak of 362 bp and an average size of 486 bp between the range of 100–5,000 bp. **Panel B.** The NTC has a dimer peak at 69 bp and little to no amplified product >150 bp.

VII. Protocol: Library Preparation

- Components of the Embgenix ESM Sample Core Kit from Package 4 are used in Sections VII.A & B
- The Unique Dual Index Kit (1–96) is used in Section VII.B.
- Before starting, please read the entire section as well as Appendix C for information on the Unique Dual Index Kit. Appendix C also contains instructions for use, a plate map, and barcode sequences.

A. Protocol: Fragmentation and Stem-Loop Adapter Ligation

For this protocol, you will need the following components:

From the Embgenix ESM Sample Core Kit, Package 4: ESM-WGA Dilution Solution, FE Dilution Buffer, Lib Prep Buffer, Rxn Enhancer, Stem-Loop Adapters, 10X FE, Library Prep Enzyme

1. Thaw the following on ice:
   - FE Dilution Buffer
   - Lib Prep Buffer
   - Rxn Enhancer
   - Stem-Loop Adapters

   NOTE: Keep the 10X FE and 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.

   (Optional) If performing library amplification and indexing (Section VII.B) on the same day, also thaw the following items on ice:
   - Unique Dual Index Kit (1–96)
   - Amplification Buffer (Core Kit, Package 4)

   Keep them on ice until needed.

2. 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.
3. If WGA products from Section VI.C, Step 5 were stored frozen, thaw them on ice. Once thawed, mix well by vortexing, and then spin briefly to collect the contents at the bottom of the tubes. Place the samples on a magnetic separator until beads are pelleted and solution is clear.
4. The target DNA input of WGA product into library preparation is 2 ng in 8 µl. Using an aliquot of each WGA product from Step 3, create a 0.25 ng/µl dilution in 10 µl using the ESM-WGA Dilution Solution.
   - Serial dilutions should be performed with a minimum of 2 µl at each step to minimize pipetting error.
   - We recommend performing a serial dilution to reach a target of 0.25 ng/µl WGA product:
     a. Make a dilution of 1.25 ng/µl of the WGA product.
     b. Make a second dilution using 2 µl of the 1.25 ng/µl WGA product (a) into 8 µl of ESM-WGA Dilution Solution. This creates a 0.25 ng/µl WGA product dilution in 10 µl.
5. Prepare new PCR tubes. On ice, dispense 4 µl of Stem-Loop Adapters to each tube, according to the number of reactions to be performed.
6. Add 8 µl of freshly diluted WGA product (0.25 ng/µl WGA product) prepared in Step 4 or ESM-WGA Dilution Solution (recommended negative control) to each tube containing the 4 µl of Stem-Loop Adapters (Step 5); the total volume per reaction should be 12 µl.

4 µl	Stem-Loop Adapters
8 µl	Diluted WGA product (0.25 ng/µl) -or- ESM-WGA Dilution Solution (NTC)
12 µl	Total volume per reaction

7. On ice, prepare 1X FE by diluting the 10X FE in cold FE Dilution Buffer (~4°C) 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 8, plus 10% of the total reaction mix volume.

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

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.

8. On ice, prepare the Library Prep Master Mix for all reactions. Combine the following reagents in the order shown below plus 10% extra.

Library Prep Master Mix:

4.0 µl	Library Prep Buffer
3.5 µl	Rxn Enhancer
2.0 µl	Library Prep Enzyme
1.0 µl	1X FE
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.
- Discard leftover 1X FE. Do not reuse.

9. On ice, assemble the library preparation reaction. To each tube containing 12 µl WGA+Stem-Loop Adapters mix or NTC+Stem-Loop Adapters mix from Step 6, add 10.5 µl of the Library Prep Master Mix prepared in Step 8. Mix by vortexing for 5 sec, and then spin the tubes briefly to collect the contents at the bottom of the tubes.

12.0 µl	WGA (or NTC)+Stem-Loop Adapters mix (from Step 6)
10.5 µl	Library Prep Master Mix (from Step 8)
22.5 µl	Total volume 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.

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

20°C	40 min
85°C	10 min
4°C	Hold

11. Once the thermal cycler reaches 4°C, spin briefly to collect the contents at the bottom of the tubes. Proceed to library amplification, Section VII.B.

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

B. Protocol: Library Amplification and Indexing with UDIs

For this protocol, you will need the following components:

- From the Embgenix ESM Sample Core Kit, Package 4: Amplification Buffer and PrimeSTAR HS DNA Polymerase (5 U/µl)
- Unique Dual Index Kit (1–96)

1. If the fragmentation and stem-loop adapter ligation (Section VII.A) was performed on a previous day, thaw the following components on ice:

   – Unique Dual Index Kit (1–96)
   – From the Core Kit: Amplification Buffer

   Also, if the library preparation reactions were stored at –20°C after Section VII.A, Step 11, thaw 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 at the bench.

2. 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.
3. On ice, prepare the Library Amplification Master Mix for all reactions, plus 10% extra.

   Library Amplification Master Mix:

21.5 µl	Amplification Buffer
3.0 µl	Nuclease-Free Water
1.0 µl	PrimeSTAR HS DNA Polymerase (5 U/µl)
25.5 µl	Total volume per reaction

4. Add 25.5 µl of the Library Amplification Master Mix to each reaction from Section VII.A, Step 11.

22.5 µl	Library preparation reaction (from Section VII.A)
25.5 µl	Library Amplification Master Mix (from Step 2)
48.0 µl	Total volume per reaction

5. Add 2 µl of a different index from the Unique Dual Index Kit (1–96) to each reaction. Always use a new pipette tip for each UDI to avoid cross contamination.

48 µl	Library amplification reaction (from Step 4)
2 µl	UDI index
50 µl	Total volume 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. Refer to Appendix C for more information on multiplexing and index pooling.
- Ensure that the orientation of the plate is correct.
- The aluminum foil that covers the plate is pierceable with most pipette tips.
- Every well contains enough material for single use. Do not reuse a well if it was previously pierced.
- Cover the used well with a seal and return the Unique Dual Index Kit (1–96) plate to –20°C after use.

6. Mix by vortexing the solution for 5 sec then spin briefly to collect the contents at the bottom of the tubes/plate.
7. Place in a thermal cycler with a heated lid (100°C–105°C) and perform PCR amplification using the following program:

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

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

C. Protocol: Purification of Amplified Libraries

PCR-amplified libraries are purified by immobilization on magnetic beads provided in the NucleoMag NGS Clean-up and Size Select. The beads are washed with 80% ethanol and the library is eluted with Nuclease-Free Water (Embgenix ESM Screen Core Kit, Package 4).

NOTES:
- Before each use, 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.
- The bead:sample ratio is 0.8:1.
- Prepare fresh 80% ethanol 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 amplified libraries from Section VII.B, Step 7.
2. Mix well by vortexing or gently pipetting the entire mixture up and down 10 times.

   NOTE: The beads are viscous; 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, 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. 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 ~5–10 min, or until the pellet is no longer shiny, but before cracks appear.

   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'd 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 25 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. Incubate at room temperature for ~5 min to rehydrate the beads.
12. Briefly spin the samples to collect the liquid from the side of the tube. Place the tube back on the magnetic separation device for ~2 min or longer, until the solution is completely clear.
13. Transfer the entire volume of clear supernatant containing purified libraries, being careful not to touch the magnetic bead pellet, to a nuclease-free, low-adhesion tube. Label each tube with sample information. The purified libraries can be stored at –20°C.

D. Protocol: Validation and Quantification of Amplified Libraries

1. Quantification

Library quantification can be performed using the following fluorescence-detection-based methods: Qubit dsDNA HS Assay (Thermo Fisher Scientific, Cat. No. Q32851, Q32854) or Quant-iT dsDNA Assay Kit, high sensitivity (Cat. No. Q33120). To quantify the libraries using Qubit Fluorometer, we recommend using 2 µl of the purified libraries (Section VII.C, Step 13). See Qubit dsDNA HS Assay user manual for detailed instructions. The concentration of the libraries or positive control should be equal or greater than 6 ng/µl.

2. Quality and Size Assessment (Optional)

A fragment analyzer, such as an Agilent 2100 BioAnalyzer or Agilent 4200 TapeStation, can optionally be used to assess the quality and size of the libraries. We recommend diluting an aliquot of each library in Nuclease-Free Water to 2–3 ng/µl. Use 2 µl of this diluted sample for size assessment using the Agilent 4200 TapeStation and High Sensitivity D1000 Reagents (Agilent, Part No. 5067-5587). The expected average size of the libraries should be approximately 300–500 bp between 100–1,000 bp on the Agilent 4200 TapeStation. Alternatively, use 1 µl of the diluted sample using Agilent's 2100 BioAnalyzer and High Sensitivity DNA Kit (Agilent, Part No. 5067-4626). For detailed instructions, see Agilent High Sensitivity D1000 ScreenTape System User Manual or Agilent High Sensitivity DNA Kit User Manual.

NOTE: See Appendix B for more examples and instructions about quality control methods.

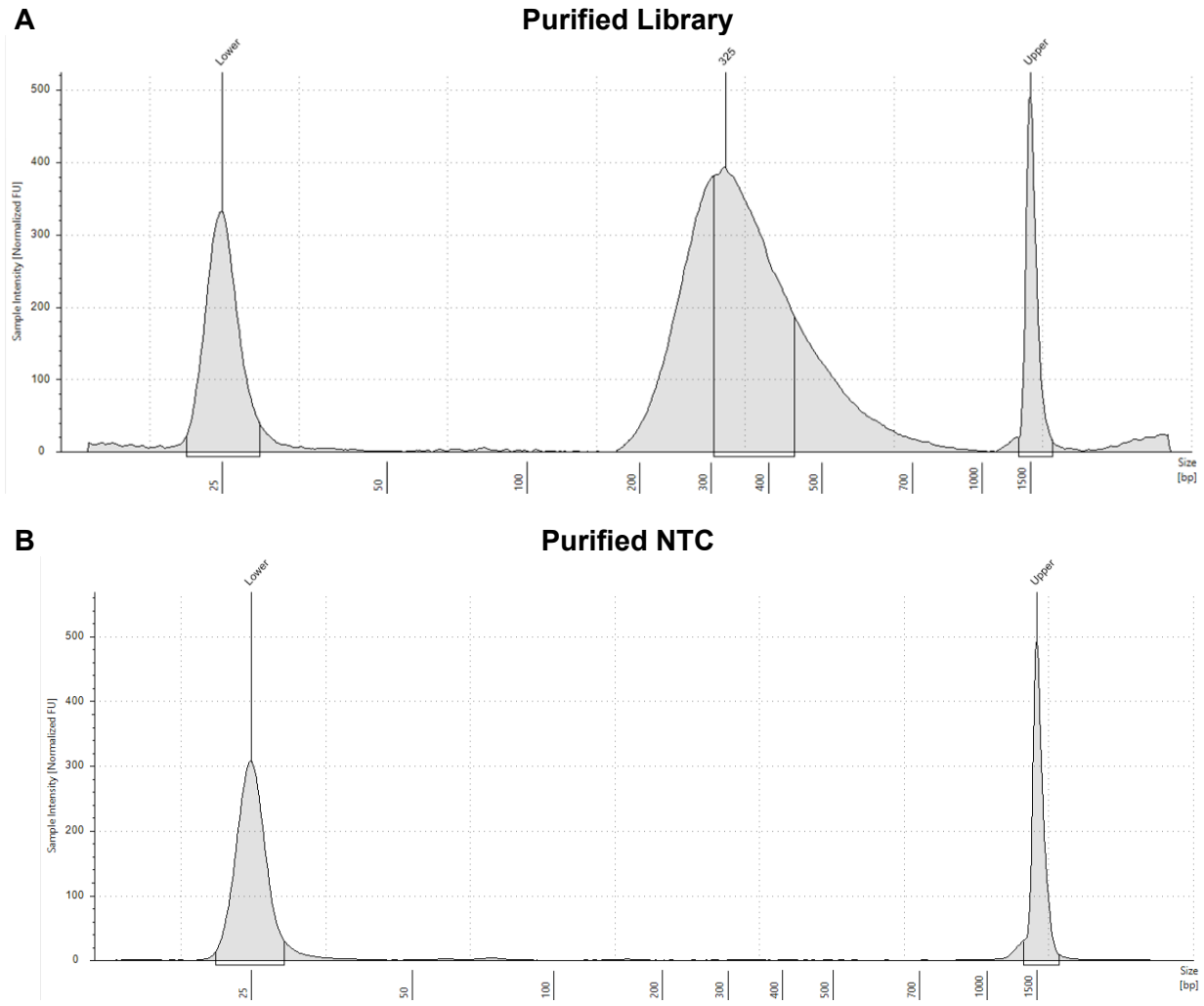


Figure 5. TapeStation Analysis of Libraries. Purified library was diluted to 2 ng/µl and 2 µl was loaded on a High Sensitivity D1000 ScreenTape. **Panel A.** Electropherogram showed a peak of 325 bp and an average size of 370 bp between 100–1,000 bp. 2 µl of purified NTC (undiluted) was loaded on a High Sensitivity D1000 ScreenTape. **Panel B.** Electropherogram of the purified negative control (NTC) showed virtually no product.

VIII. Protocol: Library Pooling and Preparation for Illumina Sequencers

Multiplexed libraries are sequenced simultaneously in a single sequencing run. The different UDIs allow demultiplexing of the sequencing data specific to each sample. See Appendix C for more information.

A. Sample Sheet Preparation—Local Run Manager

If the MiSeq System is running MCS 3.1 (or later), use the sample-sheet template "Embgenix ESM Screen Kit 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 VII.C, Step 13.

Starting at Row 18, fill out the "Sample_ID" field (Column A), by correlating the index added to the sample (Section VII.B, Step 5) 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 4 below contains more information about the data Section columns of the sample sheet.

Table 4. 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

1. Download the SampleSheet File

If using Illumina Experiment Manager with your sequencer, download the appropriate sample sheet file from the takarabio.com website for the system you are using.

– For a MiSeq instrument running MCS 2.0 or older, download the "Embgenix ESM Screen Kit Illumina MiSeq96 SampleSheet"

– For a NextSeq system, download "Embgenix ESM Screen Kit Illumina NextSeq96 SampleSheet"

2. Fill out the SampleSheet for your Experiment

For either file (for MiSeq or NextSeq systems), fill out the SampleSheet with the following guidelines:

- In the [Header] section, add the following information: Investigator Name, Experiment Name, Date (Column B, Rows 3–5).
- In the [Data] Section (MiSeq, Rows 22–117; NextSeq, Rows 24–119), each row corresponds to one of the 96 reaction tubes or purified libraries after Section VII.C, Step 13.

  Starting at Row 22 (MiSeq) or Row 24 (NextSeq), fill out the "Sample_ID" field (Column A) by correlating the index added to the sample (Section VII.B, Step 5) 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 5. [Data] Section header rows in the MiSeq and NextSeq SampleSheet files.

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.

(see Notes on the next page)

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.
- The rest of Sample Sheet should not be edited.
- 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:
  - For MiSeq: select category “Other” and select application “FASTQ Only”
  - Library Prep Workflow: TruSeq Nano DNA
  - IDT-ILMN TruSeq DNA UD Indexes (96 Indexes)
  - Index reads: 2 (Dual)
  - Cycles Read 1: 75
  - Cycles Read 2: 75
  - Use Adapter Trimming
  - Use Adapter Trimming Read 2

C. Sample Pooling

Use the Library Pooling Calculator to determine the volume of each library and Nuclease-Free Water to combine to generate a 4 nM pool.

- For MiSeq Reagent Kit v3, up to 24 libraries can be pooled into a single run.
- For NextSeq Mid Output kit, up to 96 libraries can be pooled into a single run.

1. How to use the Library Pooling Calculator

1. Download the file:

   `Embgenix ESM Screen Kit Library Pooling Calculator.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 VII.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 VII.D). If fragment analyzer results are not available, an average size of 390 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	390	38.9	3.4	ok
22	2	02_Sample	10	390	38.9	3.4	ok

Figure 6. 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 the columns, check Column G ("Volume validation") for error messages; examples are shown in Figure 7. 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	390	3.9	34.3	Volume too high
22	2	02_Sample	100	390	388.5	0.3	Volume too low
23	3	03_Sample	10	390	38.9	3.4	ok

Figure 7. 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 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	390	38.9	3.4	ok
22	2	02_Sample	10	390	38.9	3.4	ok

Figure 8. 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	717.6

Figure 9. 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 10).

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	390	38.9	3.4	ok
22	2	02_Sample	10	390	38.9	3.4	ok

Figure 10. 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 9, 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 Sequencer Loading

Before you begin

- Thaw the Illumina cartridges and 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, then spin briefly to collect the contents at the bottom of the tube.
- If using the NextSeq platform, prepare a solution of 200 mM Tris-HCl, pH 7.0 by adding 20 µl of 1 M Tris-HCl, pH 7.0, 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 VIII.C and 5 µl of freshly diluted 0.2 N NaOH in a 1.5 ml low-adhesion microcentrifuge tube.
2. Vortex the tube briefly. Spin briefly to collect the contents at the bottom of the tube.
3. Incubate at room temperature for 5 minutes.
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 300 µl of the 20 pM library pool (from step 5) with 300 µl of cold HT1 buffer.

   The resulting concentration is 10 pM.
7. Load 600 µl of the 10 pM* libraries in the loading bay of the MiSeq cartridge.

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

NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles)

1. Combine 5 µl of the 4 nM pool of libraries prepared in Section VIII.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 minutes.
4. Add 5 µl of 200 mM Tris-HCl, pH 7.0.
5. Vortex the tube for 5–8 sec and then spin briefly to collect the contents at the bottom of the tube.
6. Add 985 µl of pre-chilled HT1 buffer.
7. Vortex the tube for 5–8 sec and then spin briefly to collect the contents at the bottom of the tube. This results in a denatured library pool of 20 pM.
8. In a new 2 ml low-adhesion microcentrifuge tube, combine 75 µl of the 20 pM library pool (from Step 7) with 1,225 µl of cold HT1 buffer. The resulting concentration is 1.15 pM.
9. Load 1,300 µl of the 1.15 pM* library in the loading bay of the NextSeq cartridge.

   *If necessary, adjust and optimize the loading concentration for your sequencer.
10. Keep the cartridge at 4°C until starting the NextSeq System.

IX. Analysis of Sequencing Data using Embgenix Analysis (ESM) Software

The sequencing data, generated in the form of FASTQ files by the Illumina MiSeq or NextSeq 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 or NextSeq system on to the cloud server and follow the instructions in the "Embgenix Analysis Software (ESM) User Manual" to perform the analysis.

X. 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 (e.g., Human Genomic DNA female, Corriel 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 Guidelines

NOTES:

- All reference DNA dilutions are performed using either fresh culture media or Nuclease-Free Water in 1.5 ml low-binding microcentrifuge tubes.
- Prepare a dilution scheme to create a 15 pg genomic DNA diluted in the largest spent media sample volume (up to 30 µl) tested in the accompanying cohort.
- 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.
- For each dilution step, use at least 2 µl of DNA solution to minimize pipette error.
- Always use freshly diluted DNA for positive control reactions.

1. Using TE buffer, prepare a working stock solution of 1 ng/µl by diluting an aliquot of the original positive control gDNA.
2. Perform serial dilutions to create the final 15 pg sample using either fresh culture media or nuclease-free water.
3. Use the diluted DNA from Step 2 as a positive control in Section VI.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 performed by using a fragment analyzer such as the Agilent 4200 TapeStation (Section VI.E and Section VII.D) or the 2100 BioAnalyzer (Section B, below). An alternative way to confirm the size of the WGA products and libraries is the use of agarose gel electrophoresis (Section C, below).

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

A fragment analyzer, such as the Agilent 2100 BioAnalyzer System, can also be used to assess the quality and size of the WGA product. We recommend using 1 µl on the Agilent 2100 BioAnalyzer and the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4627).

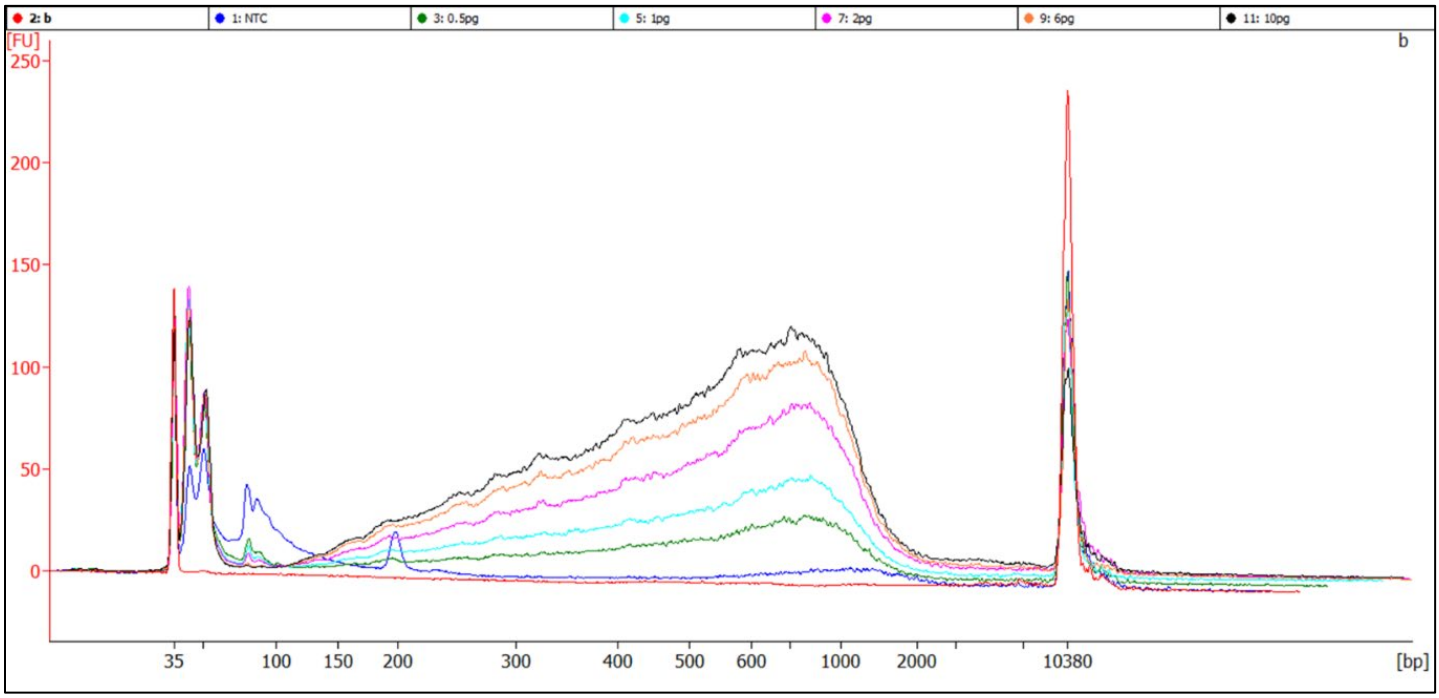
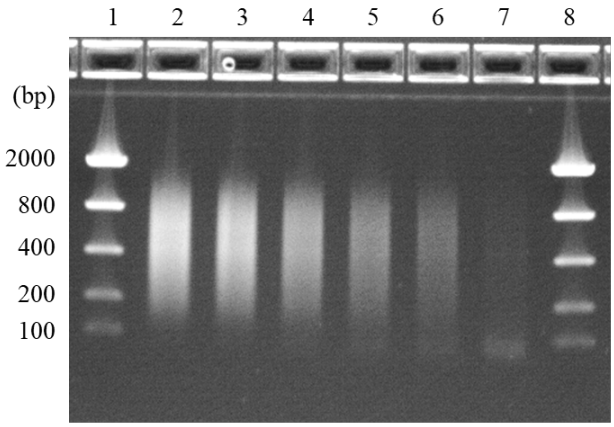


Figure 11. Electropherogram of WGA Products analyzed on the Agilent 2100 Bioanalyzer. WGA products were prepared from one spent media sample diluted to decreasing cfDNA inputs (10 pg, 6 pg, 2 pg, 1 pg, 0.5 pg) as well as a no-template control (NTC). The WGA products and NTC were diluted 1:10, and 1 µl of this diluted WGA product and a blank (b) was run on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. Note the bioanalyzer detects excess oligos (~40 bp) and a dimer peak (~80 bp) in WGA and NTC samples.

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

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 the size of the WGA products. For detailed instructions, see the E-gel Agarose System User Manual.

To check the quality of the WGA product using agarose gel electrophoresis, we recommend using undiluted WGA products from Section VI.C, Step 5. The expected average size of the WGA products or positive control ranges between 200–800 bp.



Lane	Contents
1	Ladder
2	10pg cfDNA spent media
3	6pg cfDNA spent media
4	2pg cfDNA spent media
5	1pg cfDNA spent media
6	0.5pg cfDNA spent media
7	Negative Control
8	Ladder

Figure 12. Agarose gel electrophoresis of WGA Products. WGA products were prepared from one spent media sample diluted to decreasing cfDNA inputs as well as a no-template control (NTC, Negative Control). 10 µl of WGA products or NTC were loaded on the 2% of agarose gel with E-Gel Low Range Quantitative DNA Ladder.

Appendix C. Indexing Recommendations and Sequences

A. Overview

The Embgenix ESM Screen Kit includes the 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 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-v11) and as shown in Table 6 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 6. For example, for a plexity of 9, U001–U009 or U001–U004 + U009–U013 are valid.

Table 6. 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 7. UDI barcode sequences. The table continues over the next three 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

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)
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
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

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)
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
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

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)
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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