OnePGT Library Preparation for Illumina Sequencing

Protocol

Version C1, December 2020

For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies

Notices

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Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

Technical Support

For support with setup and use of Agilent OnePGT Solution, contact us using the following e-mail address:

onepgt@agilent.com

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This guide describes the optimized workflow for generation of OnePGT libraries compatible with Illumina NextSeq 500/550 and HiSeq 2500 sequencing platforms. NGS data obtained after completing the workflow and subsequent sequencing needs to be analyzed with Agilent's Alissa OnePGT software for reporting of preimplantation genetic testing data.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start the procedure.

2 Whole Genome Amplification of Biopsy Samples using REPLI-g Single Cell Kit

This chapter describes the steps to prepare amplified DNA from a biopsy sample using the REPLI-g Single Cell Kit according to a modified protocol with a two-hour amplification step.

3 Library Preparation

This chapter describes the steps to prepare OnePGT libraries for DNA sequencing.

4 Reference

This chapter contains reference information, including component kit contents, a troubleshooting guide, and abbreviated quick reference protocols for experienced users.

What's New in Version C1

- Updates to thermal cycler recommendations and usage instructions (see Table 2 on page 12, procedural note 13 on page 10, and step 3 on page 18)
- Update to Qubit instrument ordering information in Table 2 on page 12
- Addition of unamplified reference gDNA concentration (≥ 29.4 ng/µl) to page 23
- Update to order of operations in step 13 on page 31
- Updates to support for downstream NGS demultiplexing methods (see step 1 on page 47 and *Troubleshooting* on page 58)

What's New in Version C0

- Updates to Reverse PCR Primer plate orientation information (see *Caution* on page 37 and figure on page 51)
- Updates to p/n and content details for the REPLI-g Single Cell Kit supplied with Agilent OnePGT Solution (see Table 27 on page 49 and Table 30 on page 50)
- Support for 4150 TapeStation (see Table 2 on page 12)
- Update to page 20 to indicate optional sample storage after DNA amplification as a *Stopping Point*
- Update to headings on page 30 and page 38
- Minor updates to 2100 Bioanalyzer, 4200 TapeStation and 4150 TapeStation reference document links (see page 40)
- Updates to instructions for dilution of Custom Read 1 Sequencing Primer during sequencing run setup (see page 44 and page 45)

Content

1 Before You Begin

Make sure you read and understand the information in this section and have the necessary equipment and reagents listed available before you begin the procedure.

Product Description

Agilent OnePGT Solution is a genome-wide, next-generation sequencing (NGS)-based system designed to integrate pre-implantation genetic testing (PGT) for monogenic disorders (PGT-M), translocations (PGT-SR), and aneuploidy screening (PGT-A) in a single workflow. Agilent OnePGT Solution includes the REPLI-g Single Cell Kit for whole genome amplification, the Agilent OnePGT Library Prep Kit for the generation of NGS-ready libraries, and the Agilent Alissa OnePGT software for data analysis and reporting.

Agilent OnePGT Solution is intended for PGT analysis of DNA derived from a blastomere (i.e. a single cell of a human cleavage-stage embryo) or a trophectoderm biopsy (i.e. 3-10 cells of the trophectoderm of a human blastocyst-stage embryo). The protocols are not compatible with DNA derived from polar bodies or other sources.

This publication includes directions for using the REPLI-g Single Cell Kit for whole genome amplification of biopsy samples and for using the Agilent OnePGT Library Prep Kit for sequencing library preparation. The Library Preparation protocol is used to prepare sequencing libraries both from whole genome amplified biopsy samples and from unamplified reference family genomic DNA samples (required only for PGT-M applications).

Use of the Agilent Alissa OnePGT software for data analysis and reporting is described in separate documentation, available through the Agilent Alissa OnePGT software application. Contact onepgt@agilent.com for assistance with setting up your Agilent Alissa environment.

NOTE

If you are using this product for embryo screening please make sure you adhere to your country specific laws and regulations for human assisted reproductive technologies. Your country might have banned sex selection for non-medical purposes, as well as the commercial use of gametes, zygotes, and embryos. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Safety Notes

1. Specimens should be handled as if infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories and in the CLSI Document M29-A. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 70% ethanol in deionized or distilled water.
2. Wear appropriate personal protective equipment (PPE) – including disposable gloves, laboratory coat, and eye protection – when working in the laboratory or when handling specimens and reagents.
3. Material Safety Data Sheets (MSDS) are available from the Agilent website at: www.chem.agilent.com/en-US/Search/Library/Pages/MsdsSearch.aspx.

Procedural Notes

Use Good Laboratory Practice (GLP) principles at all times, including the procedures outlined below.

1. Do not pool reagents from different lots or from different bottles of the same lot.
2. Do not use assay materials after their expiration dates.
3. All volumes stated in the instructions are intended to be used as specified within the tolerance ranges for standard micropipettors. Make sure that all pipettors are calibrated and operating within manufacturer's specifications.
4. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the whole genome amplification or gDNA sample preparation area and moving to the library preparation area.
5. Supplies and equipment for DNA isolation must be dedicated to that activity and not used for other activities or moved between areas.
6. Powder-free gloves must be worn in each area and must be changed before leaving that area.
7. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA.

8. Use best-practices to prevent PCR product contamination of samples throughout the workflow:
   a. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
   b. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
   c. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
   d. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
9. Follow your institution's procedures or common practices for tracking samples throughout the assay.
10. Possible stopping points, where DNA samples may be stored at 4°C or –20°C, are marked in the protocol.
11. Avoid repeated freeze-thaw cycles of solutions containing gDNA or enzymes.
12. When preparing frozen reagent stock solutions not containing gDNA or enzymes for use:
    a. Thaw the aliquot as quickly as possible without heating above room temperature (15°C to 30°C).
    b. Mix briefly on a vortex mixer, then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
    c. Store on ice or in a cold rack until use.
13. For incubation or amplification steps performed using a thermal cycler with heated lid ON, use a lid temperature of 105°C.

Disposal

Dispose of unused reagents, waste, and specimens in accordance with country, federal, state and local regulations.

Required Reagents

Table 1 Reagents Required for OnePGT Library Preparation

Description	Vendor and part number
Agilent OnePGT Solution (see page 49 for list of materials provided)	Agilent p/n G9426AA*
Nuclease-free water	Thermo Fisher Scientific p/n AM9930, or equivalent
Agencourt AMPure XP magnetic particle solution† 5 ml 60 ml 450 ml	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Ethanol, 96%–100%	general laboratory supplier
Qubit dsDNA BR Assay Kit, or equivalent	Thermo Fisher Scientific p/n Q32850
Qubit dsDNA HS Assay Kit, or equivalent	Thermo Fisher Scientific p/n Q32851
Control gDNA (whole genome amplification control)	Agilent OneSeq Reference DNA, Male, p/n 5190-8848, or equivalent

* This Protocol also supports use of Agilent p/n G9427AA (Agilent OnePGT Solution without REPLI-g) plus two REPLI-g Single Cell Kits p/n 5191-4065 (48 reactions/kit).

† Alternatively, SPRIselect Reagent (Beckman Coulter Genomics p/n B23317) may be used for DNA purification steps with the minor protocol modification detailed in the footnote to Table 17 on page 30.

Required Equipment

Table 2 Equipment Required for OnePGT Library Preparation

Description	Vendor and part number
Thermal Cycler with 96-well, 0.2 ml block	Various suppliers
Plasticware compatible with the selected thermal cycler: Polypropylene 96-well PCR plates or 8-well strip tubes 8-well strip tube caps	Consult the thermal cycler manufacturer's recommendations
Magnetic separator	DynaMag-96 Side magnet, Thermo Fisher Scientific p/n 12331D, or equivalent
1.5-mL, PCR clean tubes	Eppendorf p/n 022431021 or equivalent
PippinHT size selection device and consumables PippinHT instrument 1.5% Agarose 300-1500 bp 15C cassette, with electrophoresis buffer and Marker 15 C	Sage Science p/n HTP0001 p/n HTC1510
Qubit Fluorometric Quantitation System, or equivalent	Thermo Fisher Scientific p/n Q33238 and Q32856
DNA Analysis Platform and Consumables Agilent 2100 Bioanalyzer Instrument Agilent 2100 Expert SW Laptop Bundle (optional) DNA 1000 Kit OR Agilent TapeStation Instrument and Consumables 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps D1000 ScreenTape D1000 Reagents	 Agilent p/n G2939BA Agilent p/n G2953CA Agilent p/n 5067-1504 Agilent 4200 TapeStation p/n G2991AA OR Agilent 4150 TapeStation p/n G2992AA Agilent p/n 5042-8502 Agilent p/n 5067-5154 Agilent p/n 401428 Agilent p/n 401425 Agilent p/n 5067-5582 Agilent p/n 5067-5583
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent

Table 2 Equipment Required for OnePGT Library Preparation

Description	Vendor and part number
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
Multichannel pipette	general laboratory supplier
P10, P20, P200 and P1000 pipettes	general laboratory supplier
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier
Freezer, set to –20°C (acceptable range –25°C to –15°C)	general laboratory supplier
Freezer, set to –80°C (acceptable range –84°C to –67°C)	general laboratory supplier
Refrigerator, set to +4°C (acceptable range +2°C to +8°C)	general laboratory supplier

2 Whole Genome Amplification of Biopsy Samples using REPLI-g Single Cell Kit

This section contains instructions for amplification of DNA from human embryo biopsy samples using the REPLI-g Single Cell Kit (Agilent p/n 5191-4065) and using a modified two-hour DNA amplification protocol. The protocol is intended for use with blastomere (i.e. a single cell of a human cleavage-stage embryo) or trophectoderm (i.e. 3–10 cells of the trophectoderm of a human blastocyst-stage embryo) biopsy samples.

CAUTION

Do not use other whole genome amplification (WGA) methods to prepare DNA samples for use in the OnePGT Library Preparation protocol. Use only REPLI-g Single Cell Kits purchased from Agilent and prepare samples according to the two-hour protocol provided in this chapter. Use of REPLI-g Single Cell Kits purchased directly from Qiagen, and use of WGA protocols provided by Qiagen, are not supported and may cause loss of samples or data quality.

Material Preparation

This protocol uses the reagents from the REPLI-g Single Cell Kit listed in Table 3. See Table 1 on page 11 for kit ordering information.

Before starting each protocol step, prepare the reagents as described below.

Table 3 Reagents for whole genome amplification

REPLI-g Single Cell Kit Component	Preparation Steps	Where Used in Protocol
Buffer DLB	Provided lyophilized. For first use, resuspend as directed in "Reconstitution of Buffer DLB" below, then store any unused material at –20°C. For subsequent use, thaw at room temperature then vortex to mix before use.	page 16 (lyophilized), page 18 (reconstituted)
H_2O sc	Thaw at room temperature.	page 16, page 19
DTT, 1 M	Thaw at room temperature, then vortex and centrifuge briefly.	page 18
PBS sc	Thaw at room temperature, then vortex and centrifuge briefly.	page 17
Stop Solution	Thaw at room temperature, then vortex and centrifuge briefly.	page 19
REPLI-g sc Reaction Buffer	Thaw at room temperature, just prior to use. Once thawed, vortex and centrifuge briefly. If a precipitate is present, vortex the tube for an additional 10 seconds to dissolve the material.	page 19
REPLI-g sc DNA Polymerase	Thaw on ice, just prior to use. Once thawed, mix well by inverting the tube and centrifuge briefly.	page 19

Reconstitution of Buffer DLB

During first use of each Buffer DLB vial, reconstitute the lyophilized material by adding 500 µl of H_2O sc to the tube. Mix thoroughly to dissolve and then centrifuge briefly.

NOTE The reconstituted Buffer DLB, which is pH labile, may be stored for 6 months at –20°C.

Whole Genome Amplification Protocol

1 Prepare each biopsy sample to be processed as a cell suspension in PBS solution with a maximum volume of 4 µl, in a microcentrifuge tube compatible with your thermal cycler.

If using <4 µl of cell material, add a sufficient volume of kit-supplied PBS sc to bring the volume to 4 µl.

Keep the samples on ice until they are used in step 5.

CAUTION Due to the small number of cells in the sample, it is important to use the liquid handling methods below to prevent sample loss:

- When adding solutions to tubes containing the cell suspension, pipette the solutions onto the side of the tube. Do not insert the pipette tip into the cell suspension liquid, since cells may adhere to the tip and be removed from the sample.
- Mixtures containing the cell suspension must be mixed as specified in the protocol. Do not mix liquids into the cell suspension by vortexing or by pipetting up and down during any of the protocol steps below. (After amplification, solutions containing the amplified DNA may be mixed using these methods as specified in the library preparation protocol starting on page 23.)

2 Prepare positive and negative control samples in microcentrifuge tubes compatible with your thermal cycler.

a **Positive control:** 4 µl of well-characterized control gDNA (see Table 1 on page 11 for a recommended source) diluted to 15 pg/µl in PBS sc

b **Collection buffer negative control:** 4 µl of the embryo biopsy collection buffer

c **NTC negative control:** 4 µl of PBS sc

3 Preprogram a thermal cycler, with the heated lid ON, using the program in Table 4. Start the program, then immediately pause the program to allow the heated lid to reach temperature while you prepare Buffer D2. Follow the manufacturer's instructions for pausing the PCR program.

Table 4 Thermal cycler program for cell lysis and DNA denaturation

Step	Temperature	Time
Step 1	65°C	10 minutes
Step 2	4°C	Hold

4 Prepare the appropriate volume of Buffer D2 (denaturation buffer) in a 1.5-ml tube, as described in Table 5. Mix by vortexing, then spin the tube briefly to collect the liquid.

Table 5 Preparation of Buffer D2

Reagent	Volume for 12 samples*
DTT, 1 M	3 µl
Reconstituted Buffer DLB (prepared on page 16)	33 µl
Total	**36 µl**

* If processing fewer than 12 samples, store the remaining Buffer D2 at –20°C for up to three months.

5 Add 3 µl Buffer D2 (prepared in Table 5) to each 4-µl cell sample and each control sample. Pipette the Buffer D2 onto the wall of the tube above the liquid surface, then mix by flicking the tubes carefully. Spin the tubes briefly to collect the liquid.

NOTE Before continuing to the next step, verify that the cell material in the tube is suspended in liquid and is not adhering to the tube wall above the liquid surface.

6 Place the samples in the thermal cycler. Close the lid, then resume the cell lysis/DNA denaturation program in Table 4.

7 Once the thermal cycler reaches the 4°C Hold step, remove the samples and add 3 µl of the kit-provided Stop Solution to each tube. Pipette the Stop Solution onto the wall of the tube above the liquid surface, then mix by flicking the tube carefully. Spin the tubes briefly to collect the liquid. Keep the samples on ice.

8 Thaw the REPLI-g sc DNA Polymerase on ice, mix well by inverting the tube, and keep on ice until use in step 10. Thaw the REPLI-g sc Reaction Buffer at room temperature, mix by vortexing, and keep at room temperature until use in step 10. Spin the reagent tubes briefly to collect the liquid before use.

9 Preprogram the thermal cycler, with the heated lid ON, using the program in Table 6. Start the program, then immediately pause the program to allow the heated lid to reach temperature while you set up the reactions.

Table 6 Thermal cycler program for DNA amplification

Step	Temperature	Time
Step 1	30°C	2 hours
Step 2	65°C	3 minutes
Step 3	4°C	Hold

10 Prepare the appropriate volume of amplification master mix in a 1.5-ml tube, as described in Table 7. First combine the kit-supplied H_2O sc and the REPLI-g sc Reaction Buffer, then mix by vortexing, and spin the tube briefly. Just before use of the master mix in step 11, add the REPLI-g sc DNA Polymerase and mix well by pipetting up and down. Keep the master mix on ice and proceed immediately to step 11.

Table 7 Preparation of amplification master mix

Reagent	Volume for 1 sample	Volume for 12 samples (includes excess)
H_2O sc	9 µl	117 µl
REPLI-g sc Reaction Buffer	29 µl	377 µl
REPLI-g sc DNA Polymerase	2 µl	26 µl
Total	**40 µl**	**520 µl**

11 To each 10-µl denatured DNA sample, add 40 µl of the amplification master mix prepared in Table 7. Pipette the master mix onto the wall of the tube above the liquid surface, then mix by flicking the tube carefully. Briefly spin the tubes to collect the liquid.

12 Place the samples in the thermal cycler. Close the lid, then resume the DNA amplification program in Table 6.

NOTE The DNA polymerase is inactivated during incubation at 65°C in Step 2 of this program.

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13 Once the thermal cycler reaches the 4°C Hold step, proceed to the DNA library preparation protocol on page 23.

Stopping Point If the amplified DNA samples will not be used immediately, store the samples at 4°C for up to 3 days or at –20°C for up to 1 year.

3 Library Preparation

This section contains instructions for the preparation of OnePGT libraries compatible with sequencing on Illumina NGS systems.

Overview of the Workflow

The OnePGT library preparation workflow is summarized in Figure 1. The estimated hands-on time (HOT) and estimated total turnaround time (TAT) are shown for each step, when processing 24 samples and using a TapeStation 4200 instrument for QC size profiling steps.

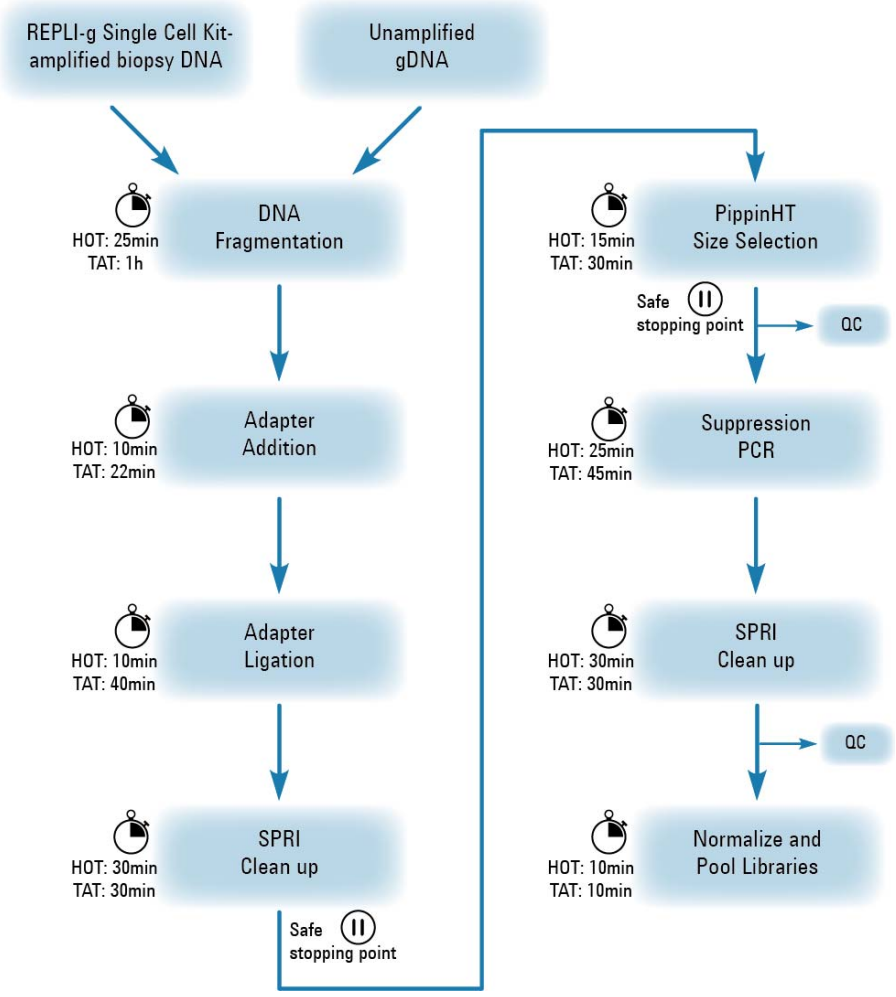

Figure 1 OnePGT library preparation workflow and time requirements.

Protocol

This protocol describes the steps to prepare sequencing libraries from whole genome amplified DNA samples, derived from human blastomere or trophectoderm biopsies. The protocol is also used to prepare sequencing libraries from unamplified family reference gDNA prepared from blood samples with initial concentration ≥ 29.4 ng/µl (required only for PGT-M applications). The protocol is not compatible with DNA derived from polar bodies or other sources.

Biopsy-derived DNA samples must be amplified using the REPLI-g Single Cell Kit supplied by Agilent, using the protocol provided in Chapter 2, "Whole Genome Amplification of Biopsy Samples using REPLI-g Single Cell Kit". The concentration of the amplified product should be ≥200 ng/µl by fluorometric quantitation. **Do not use other whole genome amplification (WGA) methods to prepare DNA samples for use in this protocol.**

Step 1. Prepare DNA samples

The protocol requires 500 ng DNA per sample. Make sure the gDNA samples are of high quality, with OD_{260}/OD_{280} ratio ≥1.8.

1. Mix the DNA sample by vortexing. Spin the sample tube briefly to collect the liquid and keep on ice.
2. Use the Qubit dsDNA BR Assay to determine the initial concentration of each DNA sample. Follow the manufacturer's instructions.
3. Prepare 20 µl of 29.4 ng/µl DNA for each sample by diluting the DNA samples with nuclease-free H_2O. Place the diluted samples in wells of a PCR plate or strip tube and keep on ice.

NOTE Do not use the Nuclease-Free Water provided in Agilent OnePGT Library Prep Kit Box 2 for this step. The kit-supplied water is intended for use only during the ligation step on page 29.

Step 2. Fragment the DNA

In this step, the DNA is enzymatically fragmented. This step uses the components listed in Table 8.

Table 8 Reagents for DNA fragmentation

Kit Component	Storage Location
Restriction Enzyme 1	Agilent OnePGT Library Prep Kit Box 1, –80°C
Restriction Enzyme 2	Agilent OnePGT Library Prep Kit Box 2, –20°C
Restriction Enzyme Buffer	Agilent OnePGT Library Prep Kit Box 2, –20°C

1 Thaw the vial of Restriction Enzyme Buffer completely before use. Mix by vortexing. Spin the vial briefly to collect the liquid and keep on ice.

2 Preprogram a thermal cycler, with the heated lid ON, using the program in Table 9. Start the program, then immediately pause the program to allow the heated lid to reach temperature while you set up the reactions. Follow the manufacturer's instructions for pausing the PCR program.

Table 9 Thermal cycler program for DNA fragmentation

Step	Temperature	Time
Step 1	37°C	15 minutes
Step 2	65°C	20 minutes
Step 3	4°C	1 minute
Step 4	4°C	Hold

3 Place vials of Restriction Enzyme 1 and Restriction Enzyme 2 on ice and verify that the contents are thawed. Spin the tubes briefly to collect the liquid and keep on ice.

NOTE

Return Restriction Enzyme 1 and Restriction Enzyme 2 vials to the storage locations listed in Table 8 as quickly as possible after use in step 4.

4 Prepare the appropriate volume of restriction digest mix in a 1.5-ml tube, as described in Table 10. Mix by pipetting up and down at least 10 times. Do not vortex. Spin the tube briefly to collect the liquid and keep on ice.

Table 10 Preparation of restriction digest mix

Reagent	Volume for 1 sample	Volume for 12 samples (includes excess)
Restriction Enzyme Buffer	2 µl	28 µl
Restriction Enzyme 1	0.5 µl	7 µl
Restriction Enzyme 2	0.5 µl	7 µl
Total	**3 µl**	**42 µl**

5 Set up the digestion reactions using a PCR plate or strip tube.

- **a** To each sample well, add 3 µl of the restriction digest mix prepared in Table 10.
- **b** Using a multichannel pipette, add 17 µl of each 29.4 ng/µl DNA sample to its assigned sample well, for a total reaction volume of 20 µl.
- **c** Mix by pipetting up and down at least 10 times. Do not vortex.

6 Cap the wells, then place the plate or strip tube in the thermal cycler. Close the lid, then resume the thermal cycling program in Table 9.

Step 3. Add adapters to fragmented DNA

In this step, adapters are added to the DNA fragments. The mixture is heated to 65°C in order to increase adapter-fragment interactions and decrease fragment-fragment interactions. This step uses the components listed in Table 11.

Table 11 Reagents for adapter addition

Kit Component	Storage Location
Adapter 1	Agilent OnePGT Library Prep Kit Box 2, –20°C
Adapter 2	Agilent OnePGT Library Prep Kit Box 2, –20°C

1 Thaw vials of Adapter 1 and Adapter 2 on ice.

2 Once the thermal cycler reaches the 4°C Hold step (Step 4 in Table 9), remove the fragmented DNA samples, briefly spin the samples to collect the liquid, then place the samples on ice.

3 Preprogram the thermal cycler (with the heated lid ON) with the program in Table 12. Start the program, then immediately pause the program to allow the heated lid to reach temperature while you set up the reactions.

Table 12 Thermal cycler program for adapter addition

Step	Temperature	Time
Step 1	65°C	10 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

4 Combine appropriate volumes of Adapter 1 and Adapter 2, in a 1.5-ml tube, as described in Table 13. Mix by vortexing for 2 seconds, then spin the tube briefly and keep on ice.

Table 13 Preparation of adapter mixture

Reagent	Volume for 1 sample	Volume for 12 samples (includes excess)
Adapter 1	2.5 µl	32.5 µl
Adapter 2	2.5 µl	32.5 µl
Total	**5 µl**	**65 µl**

5 Add 5 µl of the adapter mixture to each DNA sample well. Mix by pipetting up and down at least 10 times.

6 Cap the wells, then place the plate or strip tube in the thermal cycler. Close the lid, then resume the thermal cycling program in Table 12.

Step 4. Ligate the adapters

In this step, the adapters are ligated to the DNA fragments. This step uses the components listed in Table 14.

Table 14 Reagents for adapter ligation

Kit Component	Storage Location
DNA Ligase	Agilent OnePGT Library Prep Kit Box 2, –20°C
Ligase Buffer	Agilent OnePGT Library Prep Kit Box 2, –20°C
Nuclease-Free Water	Agilent OnePGT Library Prep Kit Box 2, –20°C

1 Once the thermal cycler reaches the 4°C Hold step (Step 3 in Table 12), remove the samples (DNA + adapters), briefly spin the samples to collect the liquid, then place the samples on ice.

2 Thaw vial of Ligase Buffer on ice and place vial DNA Ligase on ice. Thaw vial of Nuclease-Free Water at room temperature and then keep on ice.

3 Preprogram the thermal cycler (with the heated lid ON) with the program in Table 15. Start the program, then immediately pause the program to allow the heated lid to reach temperature while you set up the reactions.

Table 15 Thermal cycler program for adapter ligation

Step	Temperature	Time
Step 1	22°C	15 minutes
Step 2	65°C	10 minutes
Step 3	4°C	1 minute
Step 4	4°C	Hold

4 Prepare the appropriate volume of ligation reagent mix in a 1.5-ml tube, as described in Table 16. Mix well by pipetting up and down at least 10 times. Do not vortex. Spin the tube briefly to collect the liquid and keep on ice.

Table 16 Preparation of ligation reagent mix

Reagent	Volume for 1 sample	Volume for 12 samples (includes excess)
Nuclease-Free Water	1.5 µl	21 µl
Ligase Buffer	3.0 µl	42 µl
DNA Ligase	0.5 µl	7 µl
Total	**5 µl**	**70 µl**

5 Add 5 µl of the ligation reagent mix to each DNA sample well. Mix by pipetting up and down at least 10 times. Do not vortex.

6 Cap the wells, then place the plate or strip tube in the thermal cycler. Close the lid, then resume the thermal cycling program in Table 15.

7 Once the thermal cycler reaches the 4°C Hold step (Step 4 in Table 15), remove the samples, briefly spin the samples to collect the liquid, and place on ice.

Step 5. Purify the DNA using SPRI technology

In this step, the adapter tagged DNA is purified, using a solid-phase reversible immobilization (SPRI) system, to remove excess unligated adapters and adapter-dimers. This step uses the components listed in Table 17.

Table 17 Reagents for SPRI purification

Kit Component	Storage Location
TE	Agilent OnePGT Library Prep Kit Box 2, –20°C
AMPure XP beads*	Consult manufacturer's storage conditions; keep at room temperature for at least 30 minutes before use
Ethanol, 96%–100%	User-determined (component not provided)
Nuclease-free H_2O	User-determined (component not provided)†

* Beckman Coulter's SPRIselect Reagent can be used instead of AMPure XP beads at this step. If using SPRIselect Reagent, replace the 70% ethanol with 85% ethanol in all protocol steps below.

† Do not use the Nuclease-Free Water provided in Agilent OnePGT Library Prep Kit Box 2, which is supplied for use only in the ligation reactions (see page 29).

1 Let the AMPure XP beads come to room temperature for at least 30 minutes before use. *Do not freeze the beads at any time.*

2 Prepare 400 µl of fresh 70% ethanol per sample for use in step 9 and step 11.

NOTE

When the complete library preparation protocol is performed on the same day, you can prepare 0.8 ml of fresh 70% ethanol per sample for use in both SPRI purification procedures in the workflow (steps below and on page 38).

When both SPRI purification procedures are performed on the same day, also retain the AMPure XP bead suspension at room temperature through use on page 38.

3 Add 20 µl of nuclease-free H_2O to each sample well containing adapter-tagged DNA.

4 Mix the room-temperature AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

5 Add 50 µl of the homogeneous bead suspension to each sample well containing the 50-µl DNA samples. Mix by pipetting up and down at least 10 times.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.

6 Incubate samples for 5 minutes at room temperature.

7 Put the plate or strip tube on the magnetic stand at room temperature, and incubate the samples on the stand for 2 minutes.

NOTE The plate or strip tube remains on magnetic stand until step 13.

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8 While keeping the samples on the magnetic stand, carefully remove and discard 90 µl of cleared solution from each well. Do not disturb the beads while removing the solution.

9 Continue to keep the samples on the magnetic stand while you dispense 180 µl of fresh 70% ethanol in each sample well.

10 Wait for up to 30 seconds to allow any disturbed beads to settle, then remove the ethanol.

11 Repeat step 9 and step 10 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.

NOTE Retention of ethanol in the sample wells can cause incomplete elution of DNA from the beads and sample loss. At the end of the final wash step, use a P10 pipette to remove any traces of ethanol from the wells without disturbing the beads.

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12 Allow the beads to air-dry for 8 minutes at room temperature, keeping the plate or strip tube on the magnetic stand.

NOTE Do not exceed 8 minutes. Over-drying the beads can reduce sample recovery.

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13 Add 25 µl of TE to each sample well, then remove the samples from the magnetic stand. Mix by pipetting up and down at least 10 times.

14 Incubate for 2 minutes at room temperature to allow DNA elution.

15 Put the plate or strip tube in the magnetic stand at room temperature and leave for 1 minute or until the solution in each well is clear.

16 Remove 20 µl of each cleared supernatant to wells of a fresh plate or strip tube and keep on ice. You can discard the beads at this time.

CAUTION Take care to avoid transferring any of the beads to the final DNA sample wells.

Stopping Point If you will not continue to the next protocol step within two hours, cap the plate or strip tube wells, and store the samples at 4°C or –20°C for up to one week.

Step 6. Size-select the DNA fragments

In this step, the adapter-tagged DNA fragments are size-selected by agarose gel electrophoresis using the PippinHT system (see supplier information on page 12).

Before you begin, make sure you understand the PippinHT system instructions for use provided by the manufacturer.

1. Transfer the PippinHT system reagents from cold storage to room temperature at least 30 minutes before use.
2. Set up the PippinHT system, using a 1.5% Agarose 300-1500 bp 15C cassette, according to the manufacturer's instructions.

   Make sure to calibrate the PippinHT instrument as directed in the *Optical Calibration* section of the PippinHT *Operations Manual*.
3. Prepare the cassette as described in the *Preparing a Cassette* section of the PippinHT *Operations Manual*.
4. Perform a continuity test as described in the *Continuity Test* section of the PippinHT *Operations Manual*.
5. Program the run using the PippinHT user interface making the selections provided below. Refer to the *Programming a Protocol* section of the PippinHT *Operations Manual* for more information.
   a. Select cassette definition **1.5% Agarose 300-1500 bp 15C**
   b. For each set of lane pairs, select **Range** from the programming mode options (**Tight**/**Range**/**Time**) and enter size range of 335-575 bp
   c. Select **Use Internal Standards**
6. Add 5 µl of Internal Marker 15C to each 20-µl purified DNA sample in the PCR plates or strip tubes. Mix thoroughly by pipetting up and down at least 15 times or vortexing then spin the samples briefly to collect the liquid.

   Refer to the *Sample Preparation* section of the PippinHT *Operations Manual* for more information.

NOTE Insufficient mixing can result in incorrect marker detection in the run and sample loss.

7 For each sample to be run, remove 30 µl of electrophoresis buffer from a loading well of the cassette. Refer to the *Loading Samples* section of the PippinHT *Operations Manual* for more information.

NOTE

Take care to avoid damaging the agarose surrounding the loading well during sample loading steps. Consult the manufacturer's instructions for more information.

Loading using a multi-channel pipette is preferred for greatest efficiency. If loading using a single-channel pipette, process samples in sets of up to four at a time and complete multiple rounds of step 7 and step 8 until all samples have been loaded.

8 Load each 25-µl DNA sample into a separate loading well. Make sure to use the wells from which the buffer was removed in step 7.

9 Press **Start** to start the electrophoresis run.

CAUTION

The run must be started within 10 minutes of loading the samples.

10 Once the run is finished, transfer the 30-µl size-selected DNA samples from the elution wells of the cassette to a fresh PCR plate or strip tube. Keep the samples on ice.

11 Remove 2 µl of each size-selected DNA sample and measure the DNA concentration using the Qubit dsDNA HS assay, according to the manufacturer's instructions (see supplier information on page 12).

The minimum acceptable concentration is 0.2 ng/µl. A sample with concentration <0.2 ng/µl should be discarded, and the preparation steps for the sample repeated.

Stopping Point If you will not continue to the next protocol step within two hours, cap the plate or strip tube wells, and store the samples at 4°C for up to 3 days.

Step 7. Suppression PCR-amplify the size-selected DNA

In this step, the size-selected DNA libraries are amplified in PCR reactions using primers designed for the following purposes:

- Elongation of adapters to full-length adapter sequences required for sequencing on the Illumina platform
- Indexing using sample-specific reverse primers
- Selective amplification of only fragments ligated to both Adapter 1 and Adapter 2

This step uses the components listed in Table 18.

Table 18 Reagents for adapter PCR amplification and indexing

Kit Component	Storage Location
PCR Mix	Agilent OnePGT Library Prep Kit Box 2, –20°C
Forward PCR Primer	Agilent OnePGT Library Prep Kit Box 2, –20°C
Reverse PCR Primers-index 1-96 (96 primers containing sample indexing sequences, supplied in 96-well plate)	Agilent OnePGT Library Prep Kit Box 2, –20°C
Reverse NTC PCR Primer	Agilent OnePGT Library Prep Kit Box 2, –20°C
Nuclease-free H_2O	User-determined (component not provided)

CAUTION To avoid cross-contaminating libraries, set up PCR reactions in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

No-template controls: Each time you run suppression PCR, include a no-template control (NTC), using the same source of nuclease-free H_2O that was used to dilute DNA samples on page 23 (step 3). The reverse PCR primer for NTC reactions is the kit-supplied Reverse NTC PCR Primer, which is supplied in an amount sufficient for 8 PCR reactions.

1 Determine the appropriate index assignments for each sample, using the considerations below, and record the index assignments.

- Use a different index (included in reverse primers used at this step) for each sample to be sequenced in the same lane.
- Refer to "Guidelines for Optimal Index Multiplexing" on page 53 for specific index multiplexing recommendations for OnePGT sequencing data processing.

2 Using the starting DNA concentration determined by fluorometry in step 11 on page 34, dilute each DNA sample to 0.625 ng/µl using nuclease-free H_2O. Prepare at least 25 µl of diluted DNA for each sample. Keep the samples on ice.

Samples with starting concentration between 0.2 ng/µl and 0.625 ng/µl can be added to the PCR reaction (step 8, below) without dilution.

3 Thaw the vial of PCR Mix on ice. Once thawed, mix the reagent well by pipetting up and down at least 10 times.

NOTE The vial of PCR mix may initially contain a precipitate, which should be dissolved by thawing and mixing the vial contents.

4 Preprogram the thermal cycler (with the heated lid ON) with the program in Table 19. Start the program, then immediately pause the program to allow the heated lid to reach temperature while you set up the reactions.

Table 19 Thermal cycler program for suppression PCR

Segment Number	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	7	98°C	15 seconds
		64°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	1 minute
5	1	4°C	Hold

5 Prepare the appropriate volume of PCR reagent mix, as described in Table 20, on ice. Mix by pipetting up and down at least 10 times.

Table 20 Preparation of PCR reagent mix

Reagent	Volume for 1 reaction	Volume for 13 reactions (12 library amplifications + 1 NTC; includes excess*)
PCR Mix	25 µl	375 µl
Forward PCR Primer	2.5 µl	37.5 µl
Total	**27.5 µl**	**412.5 µl**

* When preparing reagent mixtures for <24 samples, include excess reagent volumes equivalent to 2 additional reactions. For 25–48 samples include excess of 4 additional reactions, for 49–72 samples include excess of 6 additional reactions, and for 73–96 samples include excess of 8 additional reactions.

6 Dispense 27.5 µl of the PCR reagent mix prepared in Table 20 into each sample well of a fresh PCR plate or strip tube.

7 To each well add 2.5 µl of the appropriate Reverse PCR Primer (using the specific indexed primer assigned to the sample in step 1 on page 36 or using Reverse NTC PCR Primer for the no-template control). For a map of plate positions for the indexed Reverse PCR primers, see Table 31 on page 51.

CAUTION Check the orientation of the Reverse PCR Primer plate before removing the indexed primer assigned to the sample. **Orient the plate with the notched corner (proximal to well H01) at the bottom, left position**. See page 51 for a plate map and orientation image.

8 Add 20 µl of each DNA sample (0.2–0.625 ng/µl) to the appropriate well. For the no-template control well, add 20 µl of the nuclease-free H_2O that was used to dilute the DNA samples. Mix by pipetting up and down at least 10 times.

9 Cap the wells, then place the plate or strip tube in the thermal cycler. Close the lid, then resume the thermal cycling program in Table 19.

10 Once the thermal cycler reaches the 4°C Hold step (Segment 5 in Table 19), remove the samples, and briefly spin the samples to collect the liquid.

Step 8. Purify the DNA using SPRI technology

In this step, the amplified DNA is SPRI-purified to remove excess primers. Before you begin, verify that the AMPure XP beads have been kept at room temperature for at least 30 minutes and that fresh 70% ethanol has been prepared.

This step uses the components listed in Table 17.

Table 21 Reagents for SPRI purification

Kit Component	Storage Location
AMPure XP beads*	Transferred to room temperature on page 29†
70% ethanol	Prepared on page 30; if samples were stored prior to suppression PCR, prepare fresh 70% ethanol
Nuclease-free H_2O	User-determined (component not provided)

* Beckman Coulter's SPRIselect Reagent can be used instead of AMPure XP beads at this step. If using SPRIselect Reagent, replace the 70% ethanol with 85% ethanol in all protocol steps below.

† Ensure that the AMPure XP beads have been held at room temperature for at least 30 minutes before use if they were returned to cold storage after use on page 30.

1 Mix the room-temperature AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

2 Add 50 µl of the homogeneous bead suspension to each sample well containing the 50-µl amplified DNA samples. Mix by pipetting up and down at least 10 times.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.

3 Incubate samples for 5 minutes at room temperature.

4 Put the plate or strip tube on the magnetic stand at room temperature, and incubate the samples on the stand for 2 minutes.

NOTE The plate or strip tube remains on magnetic stand until step 10.

5 While keeping the samples on the magnetic stand, carefully remove and discard 90 µl of cleared solution from each well. Do not disturb the beads while removing the solution.

6 Continue to keep the samples on the magnetic stand while you dispense 180 µl of fresh 70% ethanol in each sample well.

7 Wait for up to 30 seconds to allow any disturbed beads to settle, then remove the ethanol.

8 Repeat step 6 and step 7 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.

NOTE Retention of ethanol in the sample wells can cause incomplete elution of DNA from the beads and sample loss. At the end of the final wash step, use a P10 pipette to remove any traces of ethanol from the wells without disturbing the beads.

9 Allow the beads to air-dry for 8 minutes at room temperature, keeping the plate or strip tube on the magnetic stand.

NOTE Do not exceed 8 minutes. Over-drying the beads can reduce sample recovery.

10 Remove the samples from the magnetic stand, then add 50 µl of nuclease-free H_2O to each sample well. Mix by pipetting up and down at least 10 times.

11 Incubate for 2 minutes at room temperature.

12 Put the plate or tubes in the magnetic stand at room temperature and leave for 1 minute or until the solution in each well is clear.

13 Remove 45 µl of each cleared supernatant to wells of a fresh plate or strip tube and keep on ice. You can discard the beads at this time.

CAUTION Take care to avoid transferring any of the beads to the final DNA sample wells.

Step 9. Quantify and qualify the OnePGT libraries

In this step, the purified DNA library is quantified by fluorometry and library quality is verified using Agilent's 2100 Bioanalyzer or 4200 TapeStation.

1 Remove 2 µl of each purified library DNA sample and measure the DNA concentration using the Qubit dsDNA HS assay, according to the manufacturer's instructions (see supplier information on page 12).

NOTE

DNA concentration must be determined fluorometrically, using the Qubit dsDNA HS assay. Do not use the concentration reported by the TapeStation system or Bioanalyzer system when pooling samples for sequencing.

2 Remove 1 µl of each purified DNA sample and analyze the fragment size profile of the library using one of the methods below.

- **Option 1: Agilent's 4200 TapeStation or 4150 TapeStation and D1000 ScreenTape**. See the Agilent D1000 Assay Quick Guide for more information.
- **Option 2: Agilent's 2100 Bioanalyzer and DNA 1000 Assay**. See the Agilent DNA 1000 Kit Guide for more information.

3 Analyze the library DNA size profile results using the guidelines below.

- Assess and record the Average Size [bp] of DNA fragments in each library.
- Analyze the distribution of fragments in the library using the electropherogram. A high-quality sample electropherogram is shown in Figure 2 on page 41.

  High-quality libraries should produce an electropherogram showing a major peak with an average size of 500 bp ±40 bp and with a peak width of 285 bp ±50 bp. It is also acceptable for the peak to be bifurcated.

  A peak in the electropherogram at approximately 58 bp or 84 bp indicates incomplete removal of primers or primer-dimers from the sample. When present, the residual primers or primer-dimers should be removed by subjecting the sample to an additional round of SPRI bead purification, using the protocol on page 38.

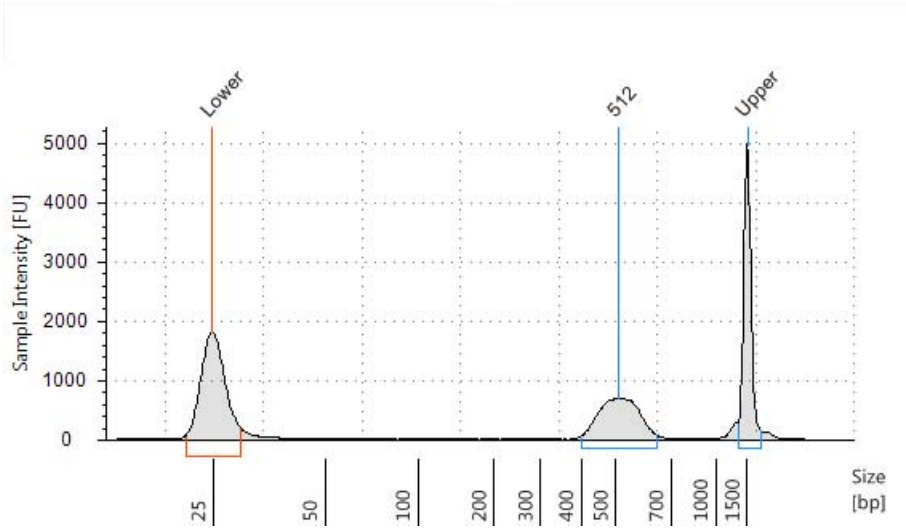


Figure 2 Analysis of the DNA size profile for a representative OnePGT library using the 4200 TapeStation.

Step 10. Pool libraries for multiplexed sequencing

In this step, the molar DNA concentration is calculated for each indexed library, and libraries are pooled for multiplexed sequencing, using equimolar amounts of each index in the pool (see Table 26 on page 45 for pool size guidelines).

1 Determine the molarity of DNA fragments (nM) in each indexed library according to the formula below.

$$\text{Molar concentration (nM)} = \frac{[DNA] \times 1,000,000}{averagesize(bp) \times 650}$$

where *[DNA]* is the DNA concentration in ng/µl determined by fluorometry on page 40 (step 1) and

average size (bp) is the average size determined by Bioanalyzer or TapeStation analysis on page 40 (step 3)

The minimum acceptable concentration is 2 nM. A library with concentration <2 nM should be discarded, and the library preparation for the sample repeated.

2 Determine the appropriate pooling strategy using the following considerations.

a The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample (see Table 26 on page 45 for guidelines).

b Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the following methods:

Method 1: Dilute each sample to be pooled to the same final concentration (typically 2 nM–15 nM, or the concentration of the most dilute sample), then combine equal volumes of all samples to create the final pool. Select the diluent according to the requirements of the sequencing facility.

Method 2: Starting with samples at different concentrations, add the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume. Select the diluent according to the requirements of the sequencing facility. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where ***V(f)*** is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool (typically 2 nM–15 nM or the concentration of the most dilute sample)

is the number of indexes, and

C(i) is the initial concentration of each indexed sample

Table 22 shows an example of amounts of 5 libraries needed for a final volume *V(f)* of 100 µl at final concentration *C(f)* of 10 nM. In this example, the total volume of combined libraries is 69.3 µl, thus 30.7 µl of diluent should be added for a final volume of 100 µl. Select the diluent according to the requirements of the sequencing facility.

Table 22 Example of indexed library volume calculation for total volume of 100 µl

Sample	C(i)	V(f)	C(f)	Volume to add to pool (µl)
Sample 1	14.0 nM	100 µl	10.0 nM	14.3
Sample 2	18.8 nM	100 µl	10.0 nM	10.4
Sample 3	17.7 nM	100 µl	10.0 nM	11.3
Sample 4	15.0 nM	100 µl	10.0 nM	13.3
Sample 5	10.0 nM	100 µl	10.0 nM	20
Diluent	—	—	—	30.7

Step 11. Set up the sequencing run

Supported sequencing platforms include Illumina's NextSeq 500/550 and HiSeq 2500 instruments. Refer to Illumina's protocols to set up a single-indexed, paired-end sequencing run, using the additional guidelines outlined below.

1 The sample-level index (i7) requires an 8-bp index read. See Table 32 on page 52 for index sequences.

2 The OnePGT kit Custom Read 1 Sequencing Primer is provided at 100 µM and must be diluted with Illumina Read 1 Primer to the final concentration shown in Table 23 for each platform. Combine the Read 1 primers using the platform-specific instructions below.

Table 23 Custom Read 1 Sequencing Primer concentration requirements

Platform	Run type	Custom Read 1 Sequencing Primer final concentration
NextSeq 500/550	High and Mid Output	0.3 µM
HiSeq 2500	Rapid Run	0.5 µM

HiSeq 2500 Platform

a Transfer the entire volume of Illumina's Read 1 TruSeq Primer HP10 from position 18 to an empty Eppendorf tube.

b In a fresh Eppendorf tube, combine the amounts of OnePGT kit Custom Read 1 Sequencing Primer and TruSeq Primer HP10 shown in Table 24. Mix well by vortexing.

c Transfer the entire volume of the Read 1 Primer mixture (1.75 ml) prepared in step b back into position 18.

Table 24 **HiSeq 2500** Custom Read 1 sequencing primer dilution

Run Type	Volume of Agilent OnePGT System Primer	Volume of Illumina TruSeq Primer	Total Volume
Rapid Mode	8.8 µl Custom Read Primer 1 Sequencing Primer	1741.2 µl HP10 (PE Rack-position 18)	1.75 ml

NextSeq 500/550 Platform

a Transfer the entire volume of Illumina's Read 1 Primer BP10 from well 20 to an empty Eppendorf tube.

b In a fresh Eppendorf tube, combine the amounts of OnePGT kit Custom Read 1 Sequencing Primer and Primer BP10 shown in Table 25. Mix well by vortexing.

c Transfer the entire volume of the Read 1 Primer mixture prepared in step b back into well 20.

Table 25 **NextSeq 500/550** Custom Read 1 sequencing primer dilution

Run Type	Volume of Agilent OnePGT System Primer	Volume of Illumina Primer	Total Volume
High Output	3.9 µl Custom Read Primer 1 Sequencing Primer	1296.1 µl BP10 (well 20)	1.3 ml
Mid Output	2.7 µl Custom Read Primer 1 Sequencing Primer	897.3 µl BP10 (well 20)	0.9 ml

3 Set up the sequencing run using the parameters in Table 26. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 26 Sequencing run setup guidelines

Application	Platform	Run type	Read length	Number of Samples	Seeding concentration*	%Phi X
PGT-M (with PGT-A and/or PGT-SR)	NextSeq 500/550	High Output (400M PE-reads)	2 × 150 bp	24	1.4 pM	1%
PGT-M (with PGT-A and/or PGT-SR)	HiSeq 2500	Rapid Run Mode, SBS Kit v2 (300M PE-reads per flow cell)	2 × 150 bp	18	10 pM	1%
PGT-SR and PGT-A	NextSeq 500/550	Mid Output (130M PE-reads)	2 × 75 bp	96	1.4 pM	1%
PGT-SR and PGT-A	HiSeq 2500	Rapid Run Mode, SBS Kit v2 (300M PE-reads per flow cell)	2 × 75 bp	96	10 pM	1%

* Seeding concentrations are provided as guidelines and may require optimization.

CAUTION During run setup, leave the *Custom Primer for Read 1* checkbox cleared (default state), since the OnePGT Custom Read 1 Sequencing Primer is spiked into Illumina's Read 1 Primer at the default Read 1 Primer location.

A successful sequencing run is characterized by the following attributes:

- ≥75% bases higher than Q30, averaged across the entire run
- ≥16 x 10^6 paired-end reads per library for PGT-M analysis or ≥1.3 x 10^6 paired-end reads per library for PGT-A/PGT-SR analysis

Step 12. Process sequencing data and upload to Agilent Alissa OnePGT

The sequencing data must be demultiplexed and then uploaded to the Agilent Alissa OnePGT software for PGT-M, PGT-SR, and PGT-A analysis as outlined below. For more information on the Agilent Alissa OnePGT software setup and analysis steps, refer to the Agilent Alissa OnePGT software documentation.

1 Demultiplex the sequencing data using Illumina's bcl2fastq software or similar tool, as appropriate for your NGS pipeline, to generate .fastq formatted files for uploading to Alissa OnePGT. Using the standard Alissa OnePGT upload process, all data for one sample should be captured in one .fastq file per lane for the forward read and one .fastq file per lane for the reverse read. If your pipeline is not compatible with .fastq file merging, see *Troubleshooting* on page 58 for more information.

2 The .fastq files are automatically uploaded to the Agilent Alissa OnePGT platform when the OnePGT Upload Module is installed on your local system, and the .fastq files are placed in the correct directory. Refer to Agilent Alissa OnePGT software documentation topic *Uploading NGS data files* for more information.

3 Once uploaded to the Agilent Alissa OnePGT platform, the sequencing data needs to be associated with an Embryo ID or family member (Mother ID, Father ID or Family member ID) to enable analysis. Refer to Agilent Alissa OnePGT software documentation topic *Associating NGS data to Family Members and Embryos* for more information.

4 Reference

This section contains reference information including kit contents, index assignment and pooling information, troubleshooting information, and abbreviated quick reference protocols for experienced users.

Kit Contents and Supported Configurations

Agilent OnePGT Solution includes the reagent component kits listed in Table 27.

Table 27 OnePGT Solution Component Kits

Component Kit Name	Storage Condition	Component Kit p/n	Quantity Provided
Agilent OnePGT Library Prep Kit Box 1	–80°C	5191-4001	1kit
Agilent OnePGT Library Prep Kit Box 2	–20°C	5191-4002	1kit
REPLI-g Single Cell Kit (48 reactions)	–20°C	5191-4065	2kits

The library preparation reagents are sufficient for 96 libraries, with enough reagents for 8 runs containing 12 samples per run. When processing samples using runs with fewer than 12 samples, some reagents may be depleted before 96 samples are run. Contents of each of the component kits listed in Table 27 are detailed in Table 28 and Table 29.

Table 28 Agilent OnePGT Library Prep Kit Box 1 Content (stored at –80°C)

Kit Component	Configuration
Restriction Enzyme 1	1 vial

Table 29 Agilent OnePGT Library Prep Kit Box 2 Content (stored at –20°C)

Kit component	Configuration
TE	4 vials
Restriction Enzyme 2	1 vial
Restriction Enzyme Buffer	1 vial
Adapter 1	1 vial
Adapter 2	1 vial
DNA Ligase	1 vial
Ligase Buffer	1 vial
PCR Mix	4 vials
Forward PCR Primer	1 vial
Reverse PCR Primers-index 1-96	Plate containing one indexed Reverse PCR Primer per well; see Table 31 for plate map
Reverse NTC PCR Primer	1 vial
Nuclease-Free Water	1 vial
Custom Read 1 Sequencing Primer	1 vial

Each of the two provided REPLI-g Single Cell Kits (p/n 5191-4065) includes reagents for whole genome amplification from 48 samples. Kits contain the components listed in Table 30.

Table 30 REPLI-g Single Cell Kit Content (p/n 5191-4065 stored at –20°C)

Kit Component	Configuration
Buffer DLB	2 vials; provided lyophilized
H_2O sc	2 vials
DTT, 1 M	1 vial
PBS sc	2 vials
Stop Solution	1 vial
REPLI-g sc Reaction Buffer	3 vials
REPLI-g sc DNA Polymerase	2 vials

Reference Information for OnePGT Indexes

When assigning the indexes to samples in the Agilent Alissa OnePGT software for analysis, enter index designations using the well position format of A01 through H12 shown in Table 31 below. Orient the plate as shown in Figure 3 when removing the indexed primers for use.

The nucleotide sequence of the index portion of each Reverse PCR Primer is shown in Table 32 on page 52.

Table 31 Plate map for Reverse PCR Primer indexes

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

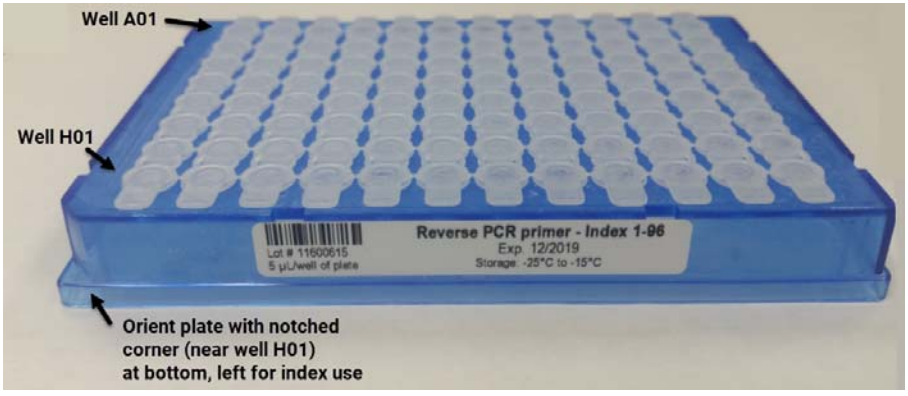


Figure 3 Orientation of Reverse PCR Primer plate

Table 32 Nucleotide sequences of OnePGT Indexes in Reverse PCR Primers

Index	Ref #	Sequence	Index	Ref #	Sequence	Index	Ref #	Sequence	Index	Ref #	Sequence
A01	1	ATGCCTAA	A04	25	AACTCACC	A07	49	ACGTATCA	A10	73	AATGTTGC
B01	2	GAATCTGA	B04	26	GCTAACGA	B07	50	GTCTGTCA	B10	74	TGAAGAGA
C01	3	AACGTGAT	C04	27	CAGATCTG	C07	51	CTAAGGTC	C10	75	AGATCGCA
D01	4	CACTTCGA	D04	28	ATCCTGTA	D07	52	CGACACAC	D10	76	AAGAGATC
E01	5	GCCAAGAC	E04	29	CTGTAGCC	E07	53	CCGTGAGA	E10	77	CAACCACA
F01	6	GACTAGTA	F04	30	GCTCGGTA	F07	54	GTGTTCTA	F10	78	TGGAACAA
G01	7	ATTGGCTC	G04	31	ACACGACC	G07	55	CAATGGAA	G10	79	CCTCTATC
H01	8	GATGAATC	H04	32	AGTCACTA	H07	56	AGCACCTC	H10	80	ACAGATTC
A02	9	AGCAGGAA	A05	33	AACGCTTA	A08	57	CAGCGTTA	A11	81	CCAGTTCA
B02	10	GAGCTGAA	B05	34	GGAGAACA	B08	58	TAGGATGA	B11	82	TGGCTTCA
C02	11	AAACATCG	C05	35	CATCAAGT	C08	59	AGTGGTCA	C11	83	CGACTGGA
D02	12	GAGTTAGC	D05	36	AAGGTACA	D08	60	ACAGCAGA	D11	84	CAAGACTA
E02	13	CGAACTTA	E05	37	CGCTGATC	E08	61	CATACCAA	E11	85	CCTCCTGA
F02	14	GATAGACA	F05	38	GGTGCGAA	F08	62	TATCAGCA	F11	86	TGGTGGTA
G02	15	AAGGACAC	G05	39	CCTAATCC	G08	63	ATAGCGAC	G11	87	AACAACCA
H02	16	GACAGTGC	H05	40	CTGAGCCA	H08	64	ACGCTCGA	H11	88	AATCCGTC
A03	17	ATCATTCC	A06	41	AGCCATGC	A09	65	CTCAATGA	A12	89	CAAGGAGC
B03	18	GCCACATA	B06	42	GTACGCAA	B09	66	TCCGTCTA	B12	90	TTCACGCA
C03	19	ACCACTGT	C06	43	AGTACAAG	C09	67	AGGCTAAC	C12	91	CACCTTAC
D03	20	CTGGCATA	D06	44	ACATTGGC	D09	68	CCATCCTC	D12	92	AAGACGGA
E03	21	ACCTCCAA	E06	45	ATTGAGGA	E09	69	AGATGTAC	E12	93	ACACAGAA
F03	22	GCGAGTAA	F06	46	GTCGTAGA	F09	70	TCTTCACA	F12	94	GAACAGGC
G03	23	ACTATGCA	G06	47	AGAGTCAA	G09	71	CCGAAGTA	G12	95	AACCGAGA
H03	24	CGGATTGC	H06	48	CCGACAAC	H09	72	CGCATACA	H12	96	ACAAGCTA

Guidelines for Optimal Index Multiplexing

The provided 96-well plate contains 96 reverse primers, each with a unique index, for suppression PCR (see page 35). Use the considerations below when determining which reverse primer to include in the suppression PCR reaction for each sample.

- Each reverse primer in the 96-well plate should only be used once.
- Design the sample indexing and pooling strategies so that indexes are combined in the ordered combinations specified below. Pool indexes column-wise (from A to H), and then from left to right (from 1 to 12). Table 33 shows example index pooling configurations for pools containing 12 libraries. A similar strategy, using indexes pooled in order by column, should be applied to pools of different sizes.

Table 33 Recommended index pooling order for 12-library pools

Libraries in pool	Number of pools from single primer plate	Indexes to combine in each pool
12	8	A01 to H01 and A02 to D02 (full column 1 + first half column 2)
		E02 to H02 and A03 to H03 (second half column 2 + full column 3)
		A04 to H04 and A05 to D05 (full column 4 + first half column 5)
		E05 to H05 and A06 to H06 (second half column 5 + full column 6)
		A07 to H07 and A08 to D08 (full column 7 + first half column 8)
		E08 to H08 and A09 to H09 (second half column 8 + full column 9)
		A10 to H10 and A11 to D11 (full column 10 + first half column 11)
		E08 to H08 and A09 to H09 (second half column 11 + full column 12)

Troubleshooting Guide

If sample yield after whole genome amplification is low (<200 ng/µl)

- ✔ Ensure that at least 1 embryo cell is collected during the biopsy.
- ✔ Use only REPLI-g Single Cell Kits purchased from Agilent and prepare samples using the two-hour incubation step shown in Table 6 on page 19. Do not use WGA protocols provided by Qiagen.
- ✔ Ensure reconstituted buffer DLB has not been stored longer than the recommended 6 months at –20°C, or prepare fresh buffer DLB.
- ✔ Ensure WGA reagents are stored properly, including thawing the amplification REPLI-g sc DNA polymerase on ice and keeping samples and reagents on ice during use.
- ✔ Ensure all vials containing biopsies are spun down so that all material is at the bottom of the vial.
- ✔ Dispense all WGA reagents onto the side of the vial and spin down. Never make direct contact with the sample by the pipette tip.
- ✔ Ensure precise adherence to all sample mixing instructions. Mix samples containing single cells (i.e. during WGA steps) by flicking the tube, not by pipetting up and down.

If lane failure occurs during the PippinHT continuity test

- ✔ Buffer levels inside the cassette may be too low. Refer to the *Continuity test failure and troubleshooting* section of the PippinHT manual. If the issue persists, do not use the affected lane.
- ✔ Ensure that the cassettes have been stored at the temperature specified by the supplier.
- ✔ Contact the PippinHT manufacturer, Sage Science, for additional lane failure troubleshooting.

If lane failure occurs during the PippinHT run (indicated by Elution Timer box turning red) with no marker peaks visible

- ✔ Ensure that 5 µl of marker mix 15C is added to each sample before loading into the PippinHT cassette. Never use the Loading Solution for External Marker Size Selection.
- ✔ Contact the PippinHT manufacturer, Sage Science, for additional lane failure troubleshooting.

If lane failure occurs during the PippinHT run (indicated by Elution Timer box turning red) with one or two rounded or merged marker peaks visible instead of two individual sharp marker peaks

- ✔ Ensure that the time between sample loading into the PippinHT cassette and start of the run is <10 minutes. Pre-program the PippinHT run before loading the samples and use a multichannel pipette for sample loading.
- ✔ Ensure that the marker mix 15C solution is brought to room temperature for at least 15 minutes before use. Ensure that the sample and marker are mixed thoroughly either by vortexing or by pipetting at least 15 times.
- ✔ Contact the PippinHT manufacturer, Sage Science, for additional lane failure troubleshooting.

If the barcode of the cassette is not correctly identified

- ✔ The PippinHT system automatically registers the barcode of the cassette. When the barcode is damaged, however, no unique cassette barcode number is registered. Failure to register the barcode does not affect sample separation and no action is required.

If sample yield is low after PippinHT size selection

- ✔ Ensure that the protocol is executed with 500 ng of input DNA as measured by Qubit. Do not use an absorbance-based method to determine input DNA concentration.
- ✔ Ensure that the plasticware specified in Table 2 on page 12 is used in all protocol steps.
- ✔ Ensure that all reagents, including both kit-supplied and user-supplied materials, are used prior to the expiry date and are stored in adherence with the manufacturer-specified requirements.
- ✔ Ensure that the amplified DNA samples and AMPure XP beads are mixed to homogeneity during SPRI purification (see step 2 on page 38).
- ✔ Ensure that all ethanol is removed after each wash step during SPRI purification (see "Step 5. Purify the DNA using SPRI technology" on page 30). Any residual ethanol may reduce sample recovery.
- ✔ Ensure that freshly-prepared 70% ethanol is used during the SPRI purification step.

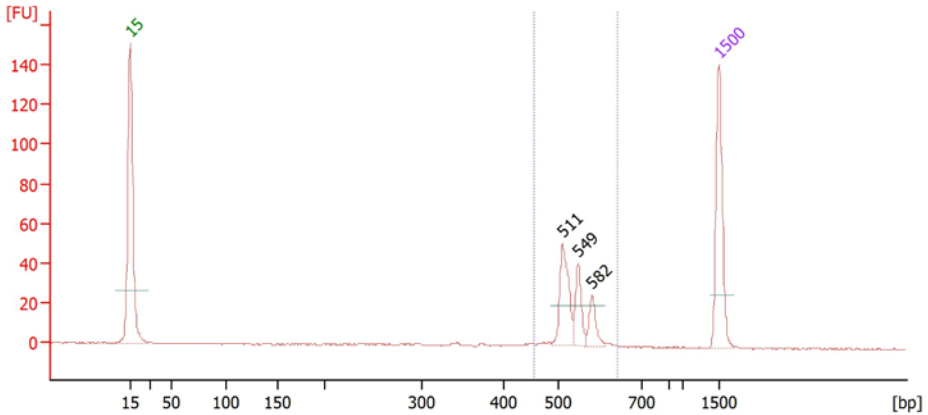
- ✔ Ensure that the AMPure XP beads are not over-dried prior to sample elution during the SPRI purification step.
- ✔ Ensure that the sample volume is 20 µl after SPRI purification. Adjust the volume with TE buffer if <20 µl is recovered from purification. Never use electrophoresis buffer or another high-salt buffer for sample elution or volume adjustments.

If concentration of the final library is low (< 2 nM)

- ✔ Ensure that the correct volume of indexed Reverse PCR Primer is added to each reaction by briefly spinning down the Reverse PCR Primer plate to remove any air bubbles before removing an aliquot for use.
- ✔ Ensure that the PCR components are stored at the correct temperature.
- ✔ Ensure that all ethanol is removed after each wash step during SPRI purification (see "Step 8. Purify the DNA using SPRI technology" on page 38). Any residual ethanol may reduce sample recovery.

If the size profile of the final library is different than expected

- ✔ A size profile similar to Figure 2 on page 41 should be observed in the final library after PCR. Slight shifts in peak position (either leftward or rightward) within the error range of the instrument are also acceptable.
- ✔ A size profile similar to the electropherogram below may indicate low quality starting material, resulting in a sequencing library containing primarily mitochondrial DNA. It is unlikely that samples with this type of profile will provide valid PGT conclusions.



If an additional low molecular weight peak is present in the final library electropherogram

✔ The presence of a low molecular weight peak indicates the presence of adaptor-dimers in the library. A low abundance (<10% of total library DNA fragments) of adaptor-dimers is acceptable. If excessive adaptor-dimers are observed, adjust the sample volume to 50 µl with nuclease-free water, then repeat the SPRI purification procedure detailed on page 38.

If cluster density is too low in sequencing results

✔ Ensure that Illumina's denaturation protocol is performed exactly as directed. In particular, verify that the NaOH reagent was freshly prepared at the correct concentration and was stored as directed.

✔ The library concentration may be too low. For each final library remeasure the concentration, using Qubit dsDNA HS Assay, and the average DNA fragment size (see page 40). Recalculate the molar DNA concentration for each indexed library, and then pool using equimolar amounts of each index in the pool as directed on page 42.

✔ Consider using a higher seeding concentration. Seeding concentrations provided in Table 26 on page 45 are guidelines and may require optimization.

If cluster density is too high in sequencing results

✔ The library concentration may be too high. For each final library remeasure the concentration, using Qubit dsDNA HS Assay, and the average DNA fragment size (see page 40). Recalculate the molar DNA concentration for each indexed library, and then pool using equimolar amounts of each index in the pool as directed on page 42.

✔ Consider using a lower seeding concentration. Seeding concentrations provided in Table 26 on page 45 are guidelines and may require optimization.

If your NGS demultiplexing pipeline produces unmerged FASTQ files

✔ Alissa OnePGT allows the upload of either unmerged or merged FASTQ files. Use the appropriate supported FASTQ file naming convention shown below, which allows the software to automatically assign the lane when required. Refer to Agilent Alissa OnePGT software documentation topic *Uploading NGS data files* for more information.

- **Example 1–Unmerged Files**

  Read group R1:

  <Sample ID>_<***>_<Lane1>_<R1>_001.fastq

  <Sample ID>_<***>_<Lane2>_<R1>_001.fastq

  Read group R2:

  <Sample ID>_<***>_<Lane1>_<R2>_001.fastq

  <Sample ID>_<***>_<Lane2>_<R2>_001.fastq

- **Example 2–Merged Files**

  Forward read:

  <Sample ID>_<***>_<R1>_001.fastq

  Reverse read:

  <Sample ID>_<***>_<R2>_001.fastq

If demultiplexing in BaseSpace trims the OnePGT adapters

✔ Downstream analysis in Alissa OnePGT is compatible with sequences demultiplexed in BaseSpace either with or without adapter trimming. Adapter trimming during demultiplexing in BaseSpace is optional but is switched on by default. If you wish to disable adapter trimming during demultiplexing in BaseSpace, go to the *Settings* section of the index sheet and clear (leave blank) both *AdapterSequenceRead1* and *AdapterSequenceRead2* fields.

Quick Reference Protocols

The Quick Reference Protocols listed below, showing abbreviated summaries of the protocol steps, are provided on the following pages for experienced users.

- Whole Genome Amplification Protocol (page 60). Use the complete protocol on page 16 to page 20 until you are familiar with all protocol details.
- Library Preparation Protocol (page 61 to page 63). Use the complete protocol on page 21 to page 46 until you are familiar with all protocol details such as reagent mixing instructions and instrument settings.

NOTE

Most Library Preparation protocol steps use customer-supplied nuclease-free H_20. The vial of Nuclease-Free Water supplied with Agilent OnePGT Library Prep Kit Box 2 is intended for use only during the ligation step, as specified in the full protocol.

Quick Reference Protocol: Whole-Genome Amplification

Step 1: Prepare Buffer DLB

Reconstitute lyophilized Buffer DLB by adding 500 µl of kit-supplied H_2O sc.

Step 2. Prepare controls

- Positive control: 4 µl of gDNA diluted to 15 pg/µl in PBS sc
- Collection buffer negative control: 4 µl of the embryo biopsy collection buffer
- NTC negative control: 4 µl of PBS sc

Step 3. Prepare Buffer D2

For 12 samples:

- 3 µl of 1M DTT
- 33 µl of reconstituted Buffer DLB

Step 4. Add Buffer D2 to samples

Add 3 µl of Buffer D2 to each 4-µl sample.

Step 5. Lyse cells and denature DNA

1. Place in thermal cycler and run program below with heated lid:
   - 65°C for 10 min
   - Hold at 4°C
2. Add 3 µl of Stop Solution to each sample.

Step 6. PCR-amplify the sample DNA

1. Prepare PCR reagent mix (per rxn):
   - 9 µl of H_2O sc
   - 29 µl of REPLI-g sc Reaction Buffer
   - 2 µl of REPLI-g sc DNA Polymerase
2. Add 40 µl of the PCR reagent mix to each sample.
3. Place in thermal cycler and run program below with heated lid:
   - 30°C for 2 h
   - 65°C for 3 min
   - Hold at 4°C
4. Store amplified DNA at -20°C until ready to proceed with OnePGT workflow.

Quick Reference Protocol: Library Preparation

Step 1: Prepare DNA samples

1. Measure the concentration with Qubit dsDNA BR assay.
2. Dilute the samples to 29.4 ng/µl with nuclease-free H_2O.

Step 2. Digest the DNA

1. Prepare digestion mix (per rxn):
   - 2 µl of Restriction Enzyme Buffer
   - 0.5 µl of Restriction Enzyme 1
   - 0.5 µl of Restriction Enzyme 2
2. Mix 17 µl of diluted DNA sample with 3 µl of digestion mix.
3. Run thermal cycler with heated lid:
   - 37°C for 15 min
   - 65°C for 20 min
   - 4°C for 1 min
   - Hold at 4°C

Step 3. Add adapters

1. Prepare adapter mix (per rxn):
   - 2.5 µl of Adapter 1
   - 2.5 µl of Adapter 2
2. Add 5 µl of adapter mix to sample.
3. Run thermal cycler with heated lid:
   - 65°C for 10 min
   - 4°C for 1 min
   - Hold at 4°C

Step 4. Ligate the adapters

1. Prepare ligation mix (per rxn):
   - 1.5 µl of Nuclease-Free Water (from kit)
   - 3.0 µl of Ligase Buffer
   - 0.5 µl of DNA Ligase
2. Add 5 µl of ligation mix to sample.
3. Run thermal cycler with heated lid:
   - 22°C for 15 min
   - 65°C for 10 min
   - 4°C for 1 min
   - Hold at 4°C

Step 5. SPRI-purify the DNA

1. Prepare 70% ethanol.
2. Add 20 µl of nuclease-free H_2O to sample.
3. Add 50 µl of premixed AMPure XP beads to sample.
4. Incubate:
   - 5 min off-magnet at room temperature (RT)
   - 2 min on-magnet at RT
5. Remove 90 µl of supernatant.
6. Wash 2 times on-magnet with 180 µl of 70% ethanol.
7. Remove all residual ethanol.
8. Air dry on-magnet for 8 min at RT.
9. Resuspend in 25 µl of TE.
10. Incubate:
    - 2 minutes off-magnet at RT
    - 1 minute on-magnet at RT
11. Transfer 20 µl of sample to a fresh PCR plate or strip tube well.

SAFE STOPPING POINT: UP TO 7 DAYS AT +4˚C OR -20˚C

Quick Reference Protocol: Library Preparation

Step 6. Size-select the DNA fragments using PippinHT

1. Set up PippinHT instrument per supplier's instructions:
   - Calibrate the instrument
   - Prepare the cassette
   - Perform a continuity test
2. Program the PippinHT instrument:
   - Select cassette type:
     1.5% Agarose 300-1500 bp 15 C
   - Select *Use Internal Standards*
   - Select *Range* and enter range 335-575 bp
3. Add 5 µl of internal marker to sample. **Mix thoroughly**.
4. Remove 30 µl of buffer from the loading well.
5. Load 25 µl of sample into the loading well.
6. Press *Start* to begin the electrophoresis run.
7. At end of run, transfer 30 µl of size-selected sample to a fresh PCR plate or strip tube well.

SAFE STOPPING POINT: UP TO 3 DAYS AT +4˚C

Step 7. Suppression PCR-amplify the size-selected DNA

1. Dilute size-selected DNA if required:
   - For samples >0.625 ng/µl, dilute using nuclease-free H_2O to 0.625 ng/µl
   - For samples 0.2–0.625 ng/µl, use undiluted
2. Prepare PCR reagent mix (per rxn):
   - 25 µl of PCR mix
   - 2.5 µl of Forward PCR primer
3. Put 27.5 µl of PCR reagent mix in a fresh PCR plate or strip tube well.
4. For each sample, add 2.5 µl of unique reverse primer (index A01-H12) from the Reverse PCR primer plate.
5. Add 20 µl of size-selected DNA.
6. Run thermal cycler with heated lid:
   - 98°C for 45 sec
   - 7 cycles of:
     98°C for 15 sec
     64°C for 30 sec
     72°C for 30 sec
   - 72°C for 1 min
   - 4°C for 1 min
   - Hold at 4°C

Quick Reference Protocol: Library Preparation

Step 8. SPRI-purify the DNA

1. Add 50 µl of premixed AMPure XP beads to sample.
2. Incubate:
   - 5 min off-magnet at RT
   - 2 min on-magnet at RT
3. Remove 90 µl of supernatant.
4. Wash 2 times on-magnet with 180 µl of 70% ethanol.
5. Remove all residual ethanol.
6. Air dry on-magnet for 8 min at RT.
7. Resuspend in 50 µl of nuclease-free H_2O.
8. Incubate:
   - 2 minutes off-magnet at RT
   - 1 minute on-magnet at RT
11. Transfer 45 µl of sample to a fresh PCR plate or strip tube well.

Step 9. Quantify and qualify the library DNA

1. Analyze 2 µl of library DNA with Qubit dsDNA HS assay.
2. Analyze 1 µl of library DNA with Agilent's 4200 TapeStation or 2100 Bioanalyzer instrument.

Step 10. Pool libraries for multiplexed sequencing

1. Calculate the molarity of each sample.
2. Pool equimolar amounts of each library (see table below).

Step 11. Set up sequencing

1. Choose the read length according to your desired application:
   - PGT-M (with PGT-A/SR) 2 × 150 bp
   - PGT-SR or PGT-A 2 × 75 bp
2. Spike in the Custom Read 1 Sequencing Primer (provided at 100 µM) into Illumina's Read 1 primer and set up sequencing reactions for the run as shown in table below.

Sequencing Parameter Guidelines

Application	Platform & Run Type	Number of Samples Pooled	Seeding Conc.	%Phi X	Final Concentration of Custom Read 1 Sequencing Primer
PGT-M (with PGT-A/SR)	NextSeq 500/550 High Output	24	1.4 pM	1%	0.3 µM
PGT-M (with PGT-A/SR)	HiSeq 2500 Rapid Run	18	10 pM	1%	0.5 µM
PGT-SR or PGT-A only	NextSeq 500/550 Mid Output	96	1.4 pM	1%	0.3 µM
PGT-SR or PGT-A only	HiSeq 2500 Rapid Run	96	10 pM	1%	0.5 µM

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In This Book

This guide contains information to run the OnePGT Library Preparation for Illumina Sequencing protocol.

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