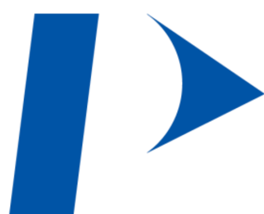

PerkinElmer®
For the Better

PG-Seq™

Whole Genome Amplification Library Preparation

TECHNICAL DATA SHEET

Version 1.2

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PG-Seq™ WGA & Library Preparation Technical Data Sheet Version 1.2 September 2018

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Revision History

Document	Date	Description of Change
PG-Seq WGA and Library Prep TDS V1.2	July 2018	• Adapter format change from 2x48 to 1x96. • Adjusted Adapter ligation mastermix to suit new adapter format. • Additional information added to Section 1 - Kit Contents. • Purification beads now included in the kit, adjusted workflow to compensate. • Addition mixing information added throughout the protocol.
PG-Seq WGA and Library Prep TDS V1.0	April 2018	Initial Release

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1 Kit Contents

PG-Seq™ WGA Reagents	Component	Cap colour
RHS5096-I-A 96 reactions (2 x 48 reaction format) Storage – Frozen at -20°C	PCR-grade H_2O	White
	Cell Lysis Enzyme	Yellow
	Cell Lysis Buffer	Yellow
	WGA Polymerase	Red
	WGA PCR Buffer	Red
	Primer	Red

PG-Seq™ Library Preparation Reagents	Component	Cap colour
RHS5096-I-B 96 reactions (2 x 48 reaction format) Storage – Frozen at -20°C	Fragmentation Buffer	Red
	Fragmentation Enzyme	Red
	ERAT Buffer	Blue
	ERAT Enzyme	Blue
Each library preparation reagent tube contains a sufficient volume to prepare 48 samples according to the recommended master mix volumes specified below with generous reagent excess.	Ligation Buffer	Yellow
	Ligation Enzyme	Yellow
	PCR Readymix	White
	Library Amplification Primer	White

PG-Seq™ Adapters
RHS5096-I-C 1 x 96 reaction format Storage – Frozen at -20°C

PG-Seq™ Purification Beads
RHS5096-I-D Storage – Refridgerated at 4°C

The PG-Seq kit contains a 96 well adapter plate containing 96 uniquely indexed adapters. Each well contains sufficient volume for single use. Avoid repeated freezing and thawing of the plate. An excel spreadsheet of Index sequences can be supplied in either row (A1, A2, A3, A4, …) or column (A1, B1, C1, D1…) format.

To minimise potential adapter cross-contamination:

- Regularly clean pipettes and work surfaces
- Use aerosol barrier pipette tips
- Use a plate centrifuge to spin down the plates before each use and after removal from a thermal cycler
- Gently peel off the plate seal to prevent splashing
- Re-seal used wells
- Never mix adapter plates using a vortex

2 Storage Information

PG-Seq™ WGA reagents, **Library Preparation Reagents** and **Adapters** are shipped on dry ice. Upon receipt, store the kit at -20°C in a constant temperature freezer (not frost free).

Please note that reagents stored at temperatures above -20°C are more prone to degradation and contamination; therefore storage at other temperatures is not recommended. It is not recommended to store reagents at -80°C. When stored under the correct conditions and handled correctly, full activity of the kit is retained until the expiry date indicated on the kit label.

The PG-Seq™ WGA Reagents are designed to amplify picogram quantities of DNA; therefore extreme caution must be exercised to prevent the introduction of foreign DNA contaminants. WGA Reagents should be stored in a clean laboratory away from potential PCR contaminants. Library Preparation Reagents should be stored in a separate general laboratory. Good laboratory practices for performing PCR should be followed when storing and handling reagents, as well as during the setup of reactions.

PG-Seq™ Purification beads are shipped seperately at room temperature. Upon receipt, store the beads at 2-8ºC.

3 Overview of Workflow

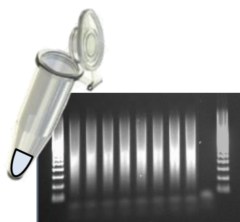
Degenerate Oligonucleotide Primed PCR (DOP-PCR)-based Whole Genome Amplification (WGA) generates representative amplification of total DNA from small numbers of cells or their DNA equivalent. The WGA reproducibly amplifies total DNA from single cells to produce microgram quantities of amplified DNA in less than 3 hours. The kit can be used on both cellular and purified genomic DNA inputs.

Sample collection

- Place the sample into a PCR tube in <2 µl of buffer
- Mark the sample location on the tube

Lysis

- Add 3 µl of lysis solution above the sample
- Tap the PCR tube to allow the lysis solution to roll over the sample
- Incubate for 15 minutes according to the Lysis Program



Whole Genome Amplification (WGA)

- Add 22 µl of PCR master mix to the lysed sample
- Amplify for 2.5 hours using the WGA PCR program
- Assess WGA quality by agarose gel electrophoresis

Library Preparation

- Perform enzymatic fragmentation
- End repair and A-tailing
- Adapter ligation
- Library Amplification

NGS

- Pool 48 uniquely indexed samples
- Denature and dilute sample pool
- Load on MiSeq 1x75bp (Miseq v3 150bp Reagent Kit)
- Analyse using PG-Seq Software

4 Space, Equipment and Materials to be Supplied by User

NOTE: When working with chemicals, always wear a suitable lab coat and disposable gloves. For more information, please consult the appropriate safety data sheets (SDS), available from PKI(A) upon request or reagent supplier.

4.1 Laboratory Set Up

Clean Laboratory

Cell lysis and master mix set-up for Whole Genome Amplification (WGA) should be performed in a contained and dedicated clean laboratory equipped with a laminar flow hood, dedicated pipettes and a PCR thermocycler with a programmable ramp rate.

It is imperative to maintain a clean, tidy work space with regular decontamination, limiting possible opportunities for DNA contamination.

All PG-Seq™ WGA Reagents should be stored in a -20°C freezer located in the clean laboratory.

General Laboratory

Agarose gel electrophoresis, handling of amplified DNA and all library preparation steps should be performed away from any WGA master mix set-up.

All PG-Seq™ Library Preparation Reagents should be stored in -20°C freezer located in a general laboratory.

4.2 Other Laboratory Items

The cell lysis and WGA steps are recommended to be performed in 0.2 or 0.5mL PCR tubes (supplied by user). A cold block at a temperature of approximately 4°C should be used when preparing all master mixes and for storing samples during set up.

4.3 Input Specifications

Cell Types

Single blastomeres, polar bodies, trophoblastic cells, amniocytes, lymphocytes, as well as cultured cells, are suitable for amplification using the PG-Seq™ kit.

Number of Cells

The **PG-Seq**™ kit is suitable for single cells or its DNA equivalent, as well as small numbers of cells (e.g. <10 cells).

Cell Collection Method

Flow sorting, dilution and micromanipulation are collection methods that are compatible with the PG-Seq™ kit. Single cells should be transferred to a PCR tube with minimal transfer buffer (<2 µl). The location of the cell in the tube should be marked with a dot on the outside of the tube using a permanent marker pen so as to enable easy cell location for the lysis step.

DNA Dilution

It is recommended that DNA is diluted to a final concentration of 30pg/µl in 10mM Tris-HCl (pH 8.0) (no EDTA).

Compatible Buffers

Recommended cell transfer buffers include 10 mM Tris-HCl (pH 8.0) (no EDTA) and PBS (Mg^{2+}, Ca^{2+} free and BSA free).

5 Protocols: Whole Genome Amplification

5.1 Cell Lysis

Estimated Time for 48 samples + 1 No Template Control (NTC)

- Hands on: 20 minutes
- Thermocycler: 15 minutes
- Total duration: 35 minutes

In this step, cells are lysed and DNA is made soluble with the addition of a Cell Lysis Mix and a short incubation in a PCR thermocycler.

Consumables:

- 0.2 mL or 0.5 mL sterile PCR tubes (user supplied)
- PCR-grade H_2O
- Cell Lysis Enzyme
- Cell Lysis Buffer
- Cell or DNA samples (user supplied)

Preparation:

- Remove PCR-grade H_2O and Cell Lysis Buffer from freezer and thaw to room temperature.
- Remove Cell Lysis Enzyme from storage and store in a cold block at 4°C.
- Mix reagents well then briefly centrifuge to collect contents at the bottom of the tube.
- Calculate volumes of reagents required for the Cell Lysis Mix. Enough Cell Lysis Mix should be prepared for all samples and one No Template Control (NTC) plus 1-2 reactions extra to allow for pipetting loss. If you are preparing a small number of samples, it is recommended that a minimum of 6-7 reactions are prepared per batch to minimise error from small pipetting volumes.

Procedure:

NOTE: The following steps are to be performed in a **Clean Laboratory**.

1. Prepare Cell Lysis Enzyme Dilution 1 in a 4°C cold block by combining the following reagents:

Component	Volume	Cap Colour
PCR-grade H_2O	6.5 µl	White
Cell Lysis Enzyme	1.0 µl	Yellow
Total volume	**7.5 µl**	

2. Mix well then briefly centrifuge.
3. Prepare Cell Lysis Mix in a 4°C cold block for the required number of reactions by combining the following reagents:

Component	Volume for 1 lysis reaction	Recommended minimum volume (6.7x master mix)	Cap colour
PCR-grade H_2O	2.7 µl	18 µl	White
Cell Lysis Buffer	0.15 µl	1.0 µl	Yellow
Cell Lysis Enzyme Dilution 1	0.15 µl	1.0 µl	-
Total volume	**3.0 µl**	**20.0 µl**	

Steps for Lysing Single Cells/Multi Cell samples

a. Add 3 µl of Cell Lysis Mix above the cell sample located in a PCR tube. Make sure that the lysis mix rolls over the sample location as marked on the tube by gently tapping the tube on the benchtop. **Do not mix or vortex.**

b. Briefly spin in a mini centrifuge if required to collect contents at the bottom of the tube.

c. Repeat with other samples.

Steps for NTC Preparation

a. Add 3 µl of Cell Lysis Mix to 1 sterile PCR tube labelled NTC.

b. Add 1 µl of PCR-grade H_2O to the tube labelled NTC.

Recommended: Lyse cell samples and NTC in a **Clean Laboratory** thermocycler to avoid DNA contamination (proceed to Step 4).

Steps for DNA Sample Preparation prior to WGA PCR (if required)

a. Add 3 µl of Cell Lysis Mix to the required number of sterile empty PCR tubes.

NOTE: The following steps are to be performed in a **General Laboratory**.

b. Add 1µl of 30pg/µl DNA sample to each tube containing Cell Lysis Mix.

c. Proceed to Step 4.

4. Incubate all samples and NTC in a thermocycler programmed as follows:

Cell Lysis:

Process	Temperature	Duration	Cycles	Hotlid
Lysis	75°C	10 minutes	1	105°C
Heat inactivation	95°C	5 minutes		
Cooling	4°C	Hold		

5. Place the lysed samples in a cold block.
6. Proceed immediately to Whole Genome Amplification.

5.2 Whole Genome Amplification

Estimated Time for 48 samples + 1 NTC

- Hands on: 30 minutes
- Thermocycler: 2 hours 20 minutes
- Total duration: 2 hours 50 minutes

In this step a master mix is created and added to the lysed samples before Degenerate Oligonucleotide Primer PCR (DOP-PCR) based Whole Genome Amplification (WGA). This generates representative amplification of total DNA from cells or their DNA equivalent.

Consumables

- 1.5 mL tube (user supplied)
- PCR-grade H_2O
- WGA PCR Buffer
- Primer
- WGA Polymerase
- Lysed samples and NTC (from 5.1 Cell Lysis)

Preparation:

- Remove PCR-grade H_2O, WGA PCR Buffer and Primer from freezer and thaw to room temperature.
- Remove WGA Polymerase from storage and store in a cold block at 4°C.
- Mix reagents well then briefly centrifuge to collect contents at the bottom of the tube.
- Calculate volumes of reagents required for the WGA master mix. Enough WGA master mix should be prepared for all samples and 1 NTC plus 1-2 reactions extra.

Procedure:

NOTE: The following steps are to be performed in a **Clean Laboratory**.

1. Prepare WGA master mix for the required number of reactions by combining the following reagents in a 1.5 mL tube in the order they are listed below:

Component	Volume for 1 WGA reaction	Cap colour
PCR-grade H_2O	6.5 µl	White
WGA PCR Buffer	12.5 µl	Red
Primer	2.5 µl	Red
WGA Polymerase	0.5 µl	Red
Total volume	**22 µl**	

2. Mix very well then briefly spin down in a mini centrifuge.

3. Transfer 22 µl of PCR master mix to the individual tubes containing lysed template (sample or NTC in Cell Lysis Mix). To prevent removal of any DNA from the sample, do not insert the pipette tip into lysed sample mix. **Do not mix or vortex the PCR tubes.** Briefly centrifuge or spin in mini centrifuge to collect contents at the bottom of the tube. Proceed to step 5.

Steps for DNA Sample Preparation prior to WGA PCR (if required)

NOTE: The following steps are to be performed in a **General Laboratory**.

4. Transfer 22 µl of PCR master mix to the individual tubes containing DNA template in Cell Lysis Mix. To prevent removal of any DNA from the sample, do not insert the pipette tip into lysed sample. Mix well then briefly centrifuge to collect contents at the bottom of the tube.

5. Incubate samples and NTC in a thermocycler programmed as follows:

Whole Genome Amplification:

Step	Temperature	Duration	Cycles	Hotlid
Initial denaturation	95°C	5 minutes	1	105°C
Denaturation	98°C	20 seconds	8	
Annealing	25°C	1 minute 30 seconds		
Extension	Ramp to 72°C	0.25°C/1 second		
	72°C	1 minute		
Denaturation	98°C	20 seconds	21	
Annealing	58°C	1 minute		
Extension	72°C	1 minute		
Final extension	72°C	1 minute	1	
Cooling	15°C	Hold		

SAFE STOPPING POINT: If you are stopping, store at -25°C to -15°C.

5.3 WGA Quality Control

To confirm amplification of the DNA sample and to check for contamination in the NTC, it is highly recommended that an agarose gel is run.

Consumables (user supplied):

- Gel Loading Buffer
- DNA Ladder 100-3000+bp (e.g. Geneworks DMW-100M)
- Agarose
- 0.5x TBE
- WGA products
- Gel Red (Biotium 41003)

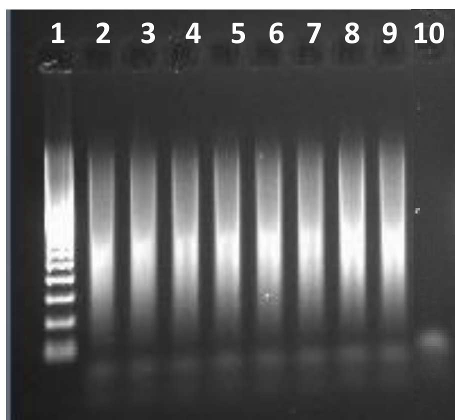
Preparation:

- Create and pour a 1% agarose gel by combining 1 g agarose, 100 mL 0.5x TBE and 5 µL Gel Red. Adjust the volumes to suit the size of your electrophoresis system.

Procedure:

1. Load 2 µl of PCR product in 4 µl gel loading buffer into the gel.
2. Load DNA Ladder.
3. Electrophorese for ~30 minutes at 100 volts.

> Quality Control
>
> The WGA DNA products should appear as a smear, ranging in size from 200 bp - 2000 bp (see WGA Example 1). The NTC should appear clean, with the presence of primer dimers.



WGA Example 1:

Lane 1: DNA Ladder (100bp)

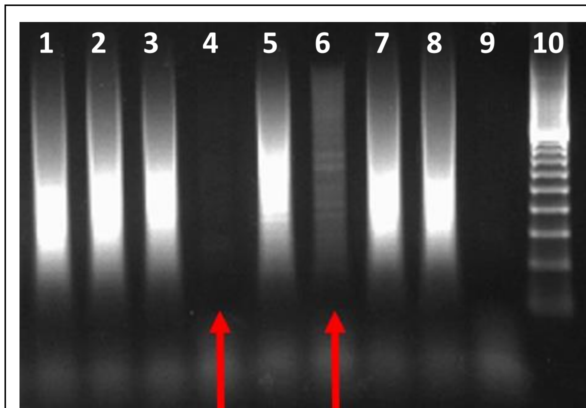
Lanes 2 - 5: Amplified single cells

Lanes 6 - 9: Amplified 30 pg genomic DNA reference

Lane 10: PCR no template control (NTC)

A **failed** WGA amplification is indicated by the presence of primer dimers, but no evidence of the smear of amplification products (see WGA Example 2, lane 4). Possible causes are that the sample was not successfully transferred to the PCR tube or that the sample was located in the PCR tube above the lysis and PCR reagents. Failed samples should be discarded.

Poor WGA amplification is indicated by smears with lower intensity or with PCR products that are notably larger or smaller than the expected size range observed for the other samples on the same agarose gel (see WGA Example 2, lane 6). The results from these samples should be interpreted with caution and it is recommended that these samples are discarded.



WGA Example 2:

Lanes 1, 2, 3, 5, 7, 8: Amplified single cells

Lane 4: Failed WGA reaction

Lane 6: Poor WGA reaction

Lane 9: PCR No Template Control (NTC)

Lane 10: DNA Ladder (100bp)

6 Protocols: Library Preparation

6.1 WGA DNA Purification

Estimated Time for 48 samples

- Hands on: 30 minutes
- Total duration: 30 minutes

In this step the WGA DNA is purified and excess PCR master mix components are removed (e.g. primer and salts)

Consumables:

- Sample WGA DNA from previous step 5.2
- Purification Beads
- 80% Ethanol prepared fresh each use (user supplied)
- 10 mM Tris-HCl pH 8.0-8.5 (user supplied)
- 96 well plate (user supplied)
- Magnetic bead plate (user supplied)

Preparation:

- Prepare fresh 80% Ethanol.
- Warm Purification beads to room temperature.
- Vortex the Purification beads for 30 seconds to ensure even dispersion.

Procedure:

1. In a 96 well plate add 20 µl of WGA PCR product to individual wells.
2. Add 16 µl of Purification beads to each sample.

Note: Mix the Purification Beads between each aliquot to ensure even dispersion.

3. Mix thoroughly by gentle vortexing and/or pipetting up and down at least 15-20 times.
4. Incubate the plate at room temperature for 5 minutes.
5. Place the plate on a magnet to capture the beads, incubate for 2-5 minutes or until the liquid is clear.
6. Remove the supernatant with a pipette.
7. Add 200 µl of 80% Ethanol to each sample well.
8. Incubate at room temperature for 30 seconds.
9. Carefully remove the Ethanol using a pipette.
10. Wash a second time by adding 200 µL of 80% Ethanol to each sample well.
11. Incubate at room temperature for 30 seconds.
12. Carefully remove the Ethanol using a pipette. Remove all residual Ethanol without disturbing the beads.
13. Dry the beads by placing at room temperature for 3-5 minutes.
14. Remove the plate from the magnet.
15. Add 25 µl of 10mM Tris-HCl to each well.
16. Resuspend the beads by gentle vortexing and/or pipetting up and down at least 15-20 times.
17. Incubate at room temperature for 2 minutes.
18. Place the plate on a magnet to capture the beads, incubate for 2-5 minutes or until the liquid is clear.
19. Transfer 23 µl of supernatant to the wells of a new plate.

SAFE STOPPING POINT: If you are stopping, seal the plate with an adhesive seal and store at -25°C to -15°C.

6.2 Quantification and Sample Dilution

In order to achieve homogeneous library preparation, it is important that the amount of DNA is accurately quantified. It is recommended that Qubit dsDNA High Sensitivity Assay kit (tube format) or Quant-iT HS Assay Kit (96-well plate format) is used for quantification as other methods which measure total nucleic acid content may over represent the amount of DNA available for NGS. In this guide the tube format only is outlined, for details on the 96-well plate format please refer to the manufacturers instructions.

Estimated Time: 48 samples

- Hands on: 30 minutes
- Total duration: 30 minutes

Consumables:

- Qubit dsDNA HS Assay Kit (user supplied)
- Qubit Assay tubes (user supplied)
- Qubit Fluorometer (user supplied)
- 10mM Tris-HCl pH 8.0-8.5 (user supplied)
- 96 well plate suitable for PCR (user supplied)

Preparation:

- Ensure all quantification reagents are at room temperature before use.
- If required, perform standard calibration measurements according to the manufactures instructions.
- Prepare working solution for the required number of samples by diluting the dsDNA HS reagent 1:200 in the dsDNA HS Buffer.

Qubit Procedure:

1. Add 2 µl of the purified sample to 198 µl of qubit working solution in a Qubit Assay tube.
2. Vortex to mix for 3-4 seconds then briefly spin down to collect contents in the bottom of the tube.
3. Incubate sample at room temperature for 2 minutes.
4. Quantify each sample according to manufacturers instructions and convert the units to ng/µl if required.

Quality Control

The purified sample DNA yield should be greater than 10 ng/ul to ensure a high quality NGS result. If this yield is not achieved, exclude the sample from the NGS run and contact support@rhsc.com.au for further assistance.

5. Add 200 ng of each sample in a final volume of 35 µl to individual wells of a new 96 well plate (suitable for PCR) by diluting in 10 mM Tris-HCL pH 8.0-8.5.
6. Proceed immediately to Enzymatic Fragmentation.

6.3 Enzymatic Fragmentation

Estimated Time for 48 samples

- Hands on: 5 minutes
- Incubation: 15 minutes
- Total duration: 20 minutes

In this step the purified WGA DNA is fragmented into smaller sequencable fragments of approximately 200-300 base pairs.

Consumables:

- Purified WGA DNA from the previous step 6.1
- Fragmentation Buffer
- Fragmentation Enzyme
- Ice (user supplied)

Preparation:

- Remove Fragmentation Buffer from the freezer and thaw.
- Transfer Fragmentation Enzyme from the freezer to ice.
- Mix reagents well then briefly centrifuge to collect contents at the bottom of each tube.

Note: The Fragmentation Enzyme is a viscous solution, ensure it is mixed well before use.

Procedure:

1. Prepare the Fragmentation master mix on ice by adding the components according to the table below:

Component	Volume for 1x fragmentation reaction	Volume for 55x fragmentation reactions
200ng purified WGA DNA	35 µL	
Fragmentation Buffer	5 µL	275 µL
Fragmentation Enzyme	10 µL	550 µL
Total Volume	**50 µL**	
It is recommended that a 55x master mix is made when processing 48 samples to allow for pipetting loss due to the viscous nature of the master mix.		

2. Mix the master mix well by inverting and flick mixing then briefly centrifuge to collect contents at the bottom of the tube.
3. Keeping the sample plate on ice or in a cool block, dispense 15 µl of master mix into each sample well.
4. Briefly centrifuge sample plate to ensure master mix collects in the bottom of the wells
5. Set the multichannel pipette to 30 µL and mix the master mix into the sample very well by pipetting up and down at least 15-20 times with a multichannel pipette. Use a new pipette tip for each sample.

Note: For optimal fragmentation, it is vital that the fragmentation mastermix is mixed well with the sample DNA. Ensure you are diligent with pipette mixing for this step.

6. Briefly centrifuge to collect contents at the bottom of the plate then return the plate to ice.
7. Transfer the plate from ice to a thermocycler once it reaches 37°C of the following program:

Fragmentation:

Process	Temperature	Duration	Hotlid
Have samples on ice and transfer to PCR machine when reaches 37°C			
Fragmentation	37°C	15 minutes	45°C
Cooling	4°C	Hold	

8. Transfer the plate to ice then proceed immediately to End Repair and A-Tailing.

6.4 End Repair and A-tailing

Estimated Time for 48 samples

- Hands on: 5 minutes
- Incubation: 30 minutes
- Total duration: 35 minutes

In this step the ends of the DNA fragments are repaired to create blunt ends and then an A-tail is added to allow adapters to bind.

Consumables:

- Fragmented DNA from previous step 6.3
- ERAT Buffer
- ERAT Enzyme

Preparation:

- Remove ERAT Buffer from the freezer and thaw.
- Trasfer ERAT Enzyme from the freezer to ice.
- Mix ERAT Buffer and ERAT Enzyme well then briefly centrifuge to collect contents at the bottom of the tube.

Procedure:

1. Prepare the ERAT master mix on ice by adding the components according to the below table:

Component	Volume for 1x End repair and A-tailing reaction	Volume for 55x End repair and A-tailing reactions
Fragmented DNA	50 µL	
ERAT Buffer	7 µL	385 µL
ERAT Enzyme	3 µL	165 µL
Total Volume	60 µL	
It is recommended that a 55x master mix is made when processing 48 samples to allow for pipetting loss due to the viscous nature of the master mix.		

2. Mix well by flick mixing then briefly centrifuge to collect the contents at the bottom of the tube.
3. Keeping the sample plate on ice or in a cool block, dispense 10 µl of ERAT mastermix into each sample well containing fragmented sample.
4. Use a multichannel pipette set on 40 µL to pipette mix up and down at least 15-20 times. Use a new tip for each sample.
5. Briefly spin down the sample plate.
6. Incubate the sample plate in a thermocycler programmed as outlined below. Add the sample plate to the thermocycler once the temperature reaches 65°C.

End Repair and A-tailing:

Process	Temperature	Duration	Hotlid
Have samples on ice and transfer to PCR machine when reaches 65°C			
End repair and A-tailing	65°C	30 minutes	85°C
Cooling	4°C	Hold	-

7. Proceed immediately to Adapter Ligation.

6.5 Adapter Ligation

Estimated Time for 48 samples

- Hands on: 5 minutes
- Incubation: 15 minutes
- Total duration: 20 minutes

In this step the uniquely indexed adapters are ligated onto the sample DNA fragments.

Consumables:

- Fragmented, End repair and A-tailed DNA from previous step 6.4
- Ligation Buffer
- Ligation Enzyme
- Adapters

Preparation:

- Remove Ligation Buffer and Adapters from the freezer and thaw.
- Transfer Ligation Enzyme from the freezer to ice.
- Mix reagents well then briefly centrifuge to collect contents at the bottom of the tube.
- Briefly centrifuge the Adapter plate to collect contents at the bottom of the plate.

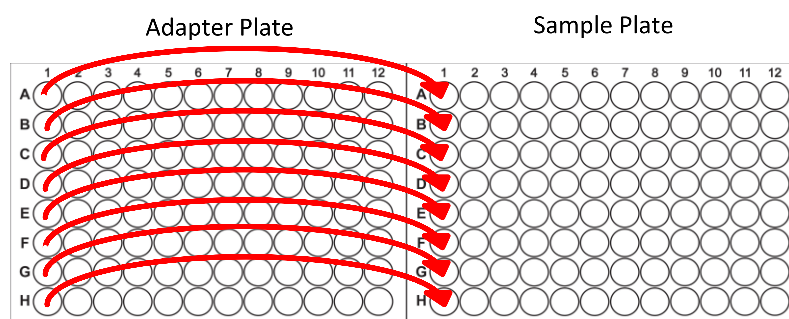
Procedure:

1. Prepare the Ligation master mix by adding the components according to the following table:

Component	Volume for 1x Adapter Ligation reaction	Volume for 55x Adapter Ligation reactions
Fragmented, End repaired and A-tailed DNA	60 µL	
Ligation Buffer	30 µL	1650 µL
Ligation Enzyme	10 µL	550 µL
Total Volume	**100 µL**	
It is recommended that a 55x master mix is made when processing 48 samples to allow for pipetting loss due to the viscous nature of the master mix.		

2. Mix the master mix very well by flick mixing then dispense 40 µL into each sample well containing fragmented, end-repaired and A-tailed DNA from the previous step.
3. Add 10 µL of an individual adapter into a corresponding sample well. For example add 10 µL of the adapter located in position A1 of the adapter plate to location A1 of the sample plate (see figure below). Be sure to track the correspondance of each sample and the adapter used to prevent sample misidentification.

PG-Seq Adapters	10 µL
Total Volume	**110 µL**



4. Using a multichannel pipette set to 80 µL, mix the samples well by pipetting up and down at least 15-20 times. Use a new tip for each sample.
5. Briefly spin down sample plate.

6. Incubate the sample plate in a thermocycler as outlined below. Add the sample plate once the thermocycler reaches 20°C. **Do not use a hot lid for this step.**

Adapter Ligation:

Process	Temperature	Duration	Hotlid
Have samples on ice and transfer to PCR machine when reaches 20°C			
Adapter Ligation	20°C	15 minutes	Off
Cooling	4°C	hold	

7. Proceed immediately to Adapter Purification.

6.6 Adapter Purification

Estimated Time for 48 samples

- Hands on: 30 minutes
- Total duration: 30 minutes

In this step the adapter ligated DNA is purified to remove left-over adapter stocks, adapter dimers plus other reagents which may affect the following library amplification PCR reaction.

Consumables:

- Adapter ligated library from the previous step 6.5
- Purification beads
- 80% Ethanol (user supplied)
- 10mM Tris-HCl pH 8.0-8.5 (user supplied)
- 96 well plate (user supplied)
- Magnetic bead plate (user supplied)

Preparation:

- Prepare fresh 80% Ethanol.
- Warm Purification beads to room temperature.
- Vortex the Purification beads for 30 seconds to ensure even dispersion.

Procedure:

1. In a 96 well plate add 110 µl of Adapter ligated DNA to individual wells of a 96 well plate.
2. Add 88 µl of Purification beads to each sample.

Note: Mix the Purification Beads between each aliquot to ensure even dispersion

3. Mix thoroughly by gentle vortexing and/or pipetting up and down at least 15-20 times.
4. Incubate the plate at room temperature for 5 minutes.
5. Place the plate on a magnet to capture the beads, incubate for 2-5 minutes or until the liquid is clear.
6. Remove the supernatant with a pipette.
7. Add 200 µl of 80% Ethanol to each sample well.
8. Incubate at room temperature for 30 seconds.
9. Carefully remove the Ethanol with a pipette.
10. Wash a second time by adding 200 µl of 80% Ethanol to each sample well.
11. Incubate at room temperature for 30 seconds.
12. Carefully remove the Ethanol with a pipette. Remove all residual Ethanol without disturbing the beads.
13. Dry the beads by placing at room temperature for 3-5 minutes.
14. Remove the plate from the magnet.
15. Add 25 µl of 10mM Tris-HCl to each well.
16. Resuspend the beads by gentle vortexing and/or pipetting up and down at least 15-20 times.
17. Incubate at room temperature for 2 minutes.
18. Place the plate on a magnet to capture the beads, incubate for 2-5 minutes or until the liquid is clear.
19. Transfer 20 µl of supernatant to the wells of a new plate.

SAFE STOPPING POINT: If you are stopping, seal the plate with an adhesive seal and store at -25°C to -15°C.

6.7 Library Amplification

Estimated Time for 48 samples

- Hands on: 5 minutes
- Incubation: 10 minutes
- Total duration: 15 minutes

In this step the DNA fragments are amplified to enrich for products that have adapters ligated to both ends.

Consumables:

- Purified Adapter Ligated DNA from the previous step 6.6
- PCR ReadyMix
- Library Amplification Primer

Preparation:

- Remove Library Amplification Primer from the freezer and thaw.
- Transfer PCR ReadyMix from the freezer to ice.
- Mix Library Amplification Primer and PCR ReadyMix well then briefly centrifuge to collect contents at the bottom of the tube.

Procedure:

1. Prepare the Library Amplification master mix by adding the reagents according to the following table:

<table>
<tr><th>Component</th><th>Volume for 1x Library Amplification</th><th>Volume for 55x Library Amplification</th></tr>
<tr><td>Purified, adapter-ligated library</td><td>20 µL</td><td></td></tr>
<tr><td>PCR ReadyMix</td><td>25 µL</td><td>1375 µL</td></tr>
<tr><td>Library Amplification Primer</td><td>5 µL</td><td>275 µL</td></tr>
<tr><td>Total</td><td>50 µL</td><td></td></tr>
<tr><td colspan="3">It is recommended that a 55x master mix is made when processing 48 samples to allow for pipetting loss due to the viscous nature of the master mix.</td></tr>
</table>

2. Mix master mix well then dispense 30 µL into each sample well containing purified adapter ligated library.
3. Use a multichannel pipette set to 30 µL and pipette mix up and down at least 15-20 times.
4. Briefly spin down sample plate.
5. Incubate the sample plate in a thermocycler according to the following program.

Library Amplification:

<table>
<tr><th>Process</th><th>Temperature</th><th>Duration</th><th>Cycles</th><th>Hotlid</th></tr>
<tr><td>Initial denaturation</td><td>98°C</td><td>45 seconds</td><td>1</td><td rowspan="5">105°C</td></tr>
<tr><td>Denaturation</td><td>98°C</td><td>15 seconds</td><td rowspan="3">4</td></tr>
<tr><td>Annealing</td><td>60°C</td><td>30 seconds</td></tr>
<tr><td>Extension</td><td>72°C</td><td>30 seconds</td></tr>
<tr><td>Final extension</td><td>72°C</td><td>1 minute</td><td>1</td></tr>
<tr><td>Cooling</td><td>4°C</td><td>hold</td><td>1</td><td></td></tr>
</table>

SAFE STOPPING POINT: If you are stopping, seal the plate with an adhesive seal and store at -25°C to -15°C.

6.8 Purification of final indexed library DNA

Estimated Time: 48 samples

- Hands on: 30 minutes
- Total duration: 30 minutes

In this step the Amplified libraries are purified to remove residual primer and other components which may affect the performance of the sequencing.

Consumables:

- Amplified library from the previous step
- Purification beads
- 80% Ethanol (user supplied)
- 10mM Tris-HCl pH 8.0-8.5 (user supplied)
- 96 well plate (user supplied)
- Magnetic bead plate (user supplied)

Preparation:

- Prepare fresh 80% Ethanol.
- Thaw Purification Beads to room temperature.
- Vortex the Purification beads for 30 seconds to ensure even dispersion.

Procedure:

1. In a 96 well plate add 50 µl of library amplified sample to individual wells of a 96 well plate.
2. Add 50 µl of Purification beads to each sample.

Note: Mix the Purification Beads between each aliquot to ensure even dispersion.

3. Mix thoroughly by pipetting up and down 15 times.
4. Incubate the plate at room temperature for 5 minutes.
5. Place the plate on a magnet to capture the beads, incubate for 2-5 minutes or until the liquid is clear.
6. Remove the supernatant with a pipette.
7. Add 200 µl of 80% Ethanol to each sample well.
8. Incubate at room temperature for 30 seconds.
9. Carefully remove the Ethanol with a pipette.
10. Wash a second time by adding 200 µl of 80% Ethanol to each sample well.
11. Incubate at room temperature for 30 seconds.
12. Carefully remove the Ethanol with a pipette. Remove all residual Ethanol without disturbing the beads.
13. Dry the beads by placing at room temperature for 3-5 minutes.
14. Remove the plate from the magnet.
15. Add 25 µl of 10mM Tris-HCl to each well.
16. Resuspend the beads by pipetting up and down at least 15 times.
17. Incubate at room temperature for 2 minutes.
18. Place the plate on a magnet to capture the beads, incubate for 2-5 minutes or until the liquid is clear.
19. Transfer 20 µl of supernatant to the wells of a new plate.

SAFE STOPPING POINT: If you are stopping, seal the plate with an adhesive seal and store at -25°C to -15°C.

6.9 Sample Quantification

Estimated Time for 48 samples

- Hands on: 30 minutes
- Total duration: 30 minutes

In this step libraries are quantified and measured for size to ensure equal library representation in the pooled multiplexed sample. It is recommended that the Qubit dsDNA High Sensitivity Assay kit (tube format) or Quant-iT HS Assay Kit (96-well plate format) is used for quantification. Alternatively, samples can be quantified with qPCR library quantification kits such as the KAPA Library Quantification Kit for Illumina® Platforms.

Consumables (user supplied):

- Purified library from the previous step
- Qubit dsDNA HS Assay Kit
- Qubit Assay tubes
- Qubit Fluorometer

Preparation:

- Ensure all quantification reagents are at room temperature before use.
- If required, perform standard measurements according to the manufactures instructions.
- Prepare Qubit working solution for the required number of samples by diluting the dsDNA HS reagent 1:200 in the dsDNA HS Buffer.
- If required, dilute samples to within Qubit quantitation range.

Procedure:

1. Add 2 µl of purified library to 198 µl of Qubit working solution in a Qubit Assay tube.
2. Vortex to mix for 3-4 seconds then briefly spin down to collect contents in the bottom of the tube.
3. Incubate sample at room temperature for 2 minutes.
4. Quantify each sample according to manufacturers instructions and convert the units to ng/µl.

Quality Control

The purified sample DNA yield should be 20-100 ng/µl to ensure a high quality NGS result. If this yield is not achieved exclude the sample from the NGS run and contact support@rhsc.com.au for further assistance.

6.10 Sample Size Distribution Estimation

Estimated Time for 48 samples

- Hands on: 10 minutes
- Total duration: 30 minutes

In order to determine the size distribution of each sample library, it is recommended that an Agilent Bioanalyzer or Agilent Tapestation is used with DNA High Sensitivity reagents. In this guide, the protocol for the Agilent Tapestation is described. To determine the fragment size using other technologies refer to the manufacturers instructions.

Agilent Tapestation Consumables (user supplied):

- Purified library from the previous step 6.9
- Agilent Tapestation
- Agilent High Sensitivity D1000 Screentape
- Agilent High Sensitivity D1000 Reagents
- Tapestation Loading Tips
- Tapestation Optical Tubes

Agilent Tapestation Preparation:

- Allow reagents to equilibrate at room temperature for 30 minutes.
- Mix well before use.
- Dilute each sample to less than 10 ng/µl in 10mM Tris-HCl pH 8.0-8.5.

Agilent Tapestation Procedure:

1. Add 2 µl of High Sensitivity D1000 Buffer to 2 µl of DNA sample in the TapeStation Optical tubes.
2. Mix well then briefly centrifuge to collect contents in the bottom of the tube.
3. Load samples, tips and screentape into the TapeStation.
4. Select the required number of samples on the controller software then press start.
5. Follow the manufacturers guide for using the controller software.

Quality Control

The mode fragment size should be 300-500 bp in length. If your fragment size is not within this range contact support@rhsc.com.au for further assistance.

6.11 Sample pooling

Estimated Time for 48 samples

- Hands on: 20 minutes
- Total duration: 20 minutes

In this step the quantification and average size distribution of each sample is used to calculate what volume is required to create a 4nM pool with equal representation for all samples.

Preparation:

- Open the PKI(A) 4nM Pool Calculator spreadsheet.
- Enter the fragment size for each sample (or average fragment size if only a selection of samples were measured) into the "Size of Fragment" column.
- Enter the sample Qubit quantification result into the "Concentration from Qubit" column.
- Document the "Volume of sample library to add to final 4nM pool" value for each sample.
- Document the "Final volume of water to add to final pool".

Procedure:

1. Add the specified volume of PCR-grade water to a 1.5mL tube.
2. Add the specified volume of each sample to the same 1.5mL tube.
3. Mix well then briefly centrifuge to collect contents at the bottom of the tube.
4. Quantify the final pool according to the previously described Qubit quantification protocol.
5. Determine the concentration of the final pool by using the second tab "final pool concentration calc" of the 4nM Pool Calculator spreadsheet.

6.12 Library Denaturation and MiSeq Sample Loading

Estimated Time for 48 samples

- Hands on: 10 minutes
- Total duration: 10 minutes

In this step the 4nM pool of samples is denatured with sodium hydroxide then diluted in hybridization buffer in preparation for sequencing. PKI(A) has determined a final pool concentration of 15pM is required for optimal cluster density on a MiSeq for PG-Seq samples. The cluster density has a large impact on sequencing performance and the final pool concentration may need optimising depending on the sample type.

Consumables (user supplied):

- 4nM pool from the previous step 6.11
- Illumina HT1 (Hybridization Buffer)
- Illumina MiSeq v3 150bp reagent cartridge catalogue number: MS-102-3001
- Ice
- 1.0N NaOH
- PCR-grade H2O
- 1.5mL tube
- 20pM PhiX (if available, can be omitted)

Preparation:

- Dilute the NaOH to 0.2N by combining 80 µl of PCR-grade H_2O with 20 µl of 1.0N NaOH. This dilution should be made freshly before each use.
- Remove HT1 from the freezer and thaw, store in ice at 2-8°C until use.

Procedure:

Denature:

1. Add 5 µl of 4nM pooled library to a 1.5mL tube.
2. Add 5 µl of 0.2N NaOH to the same tube then vortex briefly.
3. Centrifuge at 280 x g for 1 minute.
4. Incubate for 5 minutes at room temperature.
5. Place on ice.

Dilute to 20pM

6. Add 990 µl of pre-chilled HT1.

Dilute to 15pM

7. Further dilute the pooled library by adding 450 µl of the 20pM pool to a 1.5mL tube.
8. Add 145 µl of pre-chilled HT1.
9. Add 5 µl of 20pM PhiX.

Sequencing

10. Ensure MiSeq Reporter is installed on the MiSeq instrument (Illumina Document # 15042295 v05)
11. Set up the run Sample Sheet according to the PKI(A) Sample Sheet Template. Enter the sample names and the corresponding adapter sequence into the required locations.
12. Sequence your library as indicated in the Illumina MiSeq System User Guide (Illumina Document # 15027617 v01) using 1x75bp read lengths.

7 Sequencing Quality Control

To determine whether a sequencing run was successful and each sample passes quality control, it is recommended that the criteria below are checked.

Metric	
Cluster Density A measure of the density of clusters detected during image analysis. It is influenced by the concentration of DNA loaded (i.e. too high or too low) and the size of the DNA fragments within the library. An optimal cluster density results in good image resolution for the sequencer, high quality data and high data output.	1000-1600 K/mm^2
Reads The total number of reads relates to how many distinct DNA molecules are located on the flow cell surface. The number is ideally between 25 and 30 million. Less than 25 million reads results in a lower output which may affect the accuracy of the analysis whereas more than 30 million reads may result in poor image resolution and inaccurate results.	25 million - 30 million
Reads PF The number of reads passing filter (PF). Some reads that form on the flow cell may be of poor quality and are excluded from further downstream processing. Ideally the number of reads PF is over 19 million. If a lower percentage of reads PF is seen, it could indicate that the concentration of denatured library loaded onto the flow cell was suboptimal.	>19 million
% ≥Q30 The percentage of bases with a quality score of Q30 or higher. A quality score of Q30 means one base in 1000 is predicted to be incorrect or 0.001 error probability.	>90%
Reads identified (PF) per sample (%) For a well balanced library the percentage of reads attributed to each sample should be approximately 2% for a 48 sample run. Higher or lower percentages could indicate that the sample quantification was inaccurate or there was a problem with the library preparation.	1.0% - 8.0%

Refer to the *PG-Seq Software N9 Module TDS V1.0 April 2018* for information on how to analyse the result.

For technical support please contact: support@rhsc.com.au

8 Ordering Information

Please contact your local distributor or PKI(A) for ordering information. For more information on the products use, limitations, and licenses visit www.rhsc.com.au

PG-Seq™ is a Research Use Only product and is not to be used for diagnostic procedures

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