June 2014

GeneRead™ DNAseq Targeted Panels V2 Handbook

For targeted enrichment prior to next-generation sequencing

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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Kit Contents

GeneRead DNAseq Targeted Panel V2	
Catalog no.	181900*
Pools with enough primers for 12 or 96 samples, depending on pack size	1†/4
Handbook	1

* Gene panel pools are labeled A1, A2, A3, and A4.

† E.g., the Tumor Actionable Mutations GeneRead DNAseq Targeted Panel V2.

GeneRead DNAseq Targeted HC Panel V2	181901†‡
Pools with enough primers for 12 or 96 samples, depending on pack size	4
Handbook	1

‡ Gene panel pools are labeled A1, A2, A3 and A4.

§ E.g., the Human Comprehensive Cancer, Carrier Testing, and Cancer Predisposition GeneRead DNAseq Targeted HC Panel V2.

GeneRead DNAseq Custom Panel V2	181902
Pools with primers for any gene or region in the human genome, up to 9600 primer sets, for 480 samples	1/2/3/4¶
Handbook	1

¶ Number of pools determined by covered region.

GeneRead DNAseq Mix-n-Match Panel V2	181905
Pools with primers for a mix of any gene in catalog panels, up to 9600 primer sets, for 96 samples	4
Handbook	1

Catalog no.	Product name	No. of primer pairs	No. of pools
NGHS-001X	Human Breast Cancer Panel	2915	4
NGHS-002X	Human Colorectal Cancer Panel	1954	4
NGHS-003X	Human Myeloid Neoplasms Panel	2536	4
NGHS-004X	Human Liver Cancer Panel	2052	4
NGHS-005X	Human Lung Cancer Panel	3586	4
NGHS-006X	Human Ovarian Cancer Panel	2021	4
NGHS-007X	Human Prostate Cancer Panel	1837	4
NGHS-008X	Human Gastric Cancer Panel	2377	4
NGHS-009X	Human Cardiomyopathy Panel	2657	4
NGHS-011X	Human Carrier Testing Panel	6943	4
NGHS-013X	Human Cancer Predisposition Panel	6582	4
NGHS-501X	Human Comprehensive Cancer Panel	7951	4
NGHS-101X	Clinically Relevant Tumor Panel	602	4
NGHS-201X	Tumor Actionable Mutations Panel	118	1

GeneRead DNAseq Panel PCR Kit V2	181940 (12) (24/24/16/12* samples)	181942 (96) (192/192/128/96* samples)
GeneRead DNAseq Panel 5x PCR Buffer	230 µl	1800 µl
GeneRead HotStarTaq® DNA Polymerase 6 U/µl	80 µl	600 µl
DNase-free water	1000 µl	1000 µl

* Number of samples that can be processed for 1, 2, 3, or 4 pool panels, respectively.

Storage

GeneRead DNAseq Panel Kits are shipped on dry ice and should be stored at –15°C to –30°C upon arrival. When stored properly at –15°C to –30°C, all reagents are stable for up to 6 months after delivery.

GeneRead DNAseq Panel PCR Kits are shipped on cold packs. For long-term storage, keep tubes at –15°C to –30°C. If the entire volume will not be used at once, we recommend dividing into aliquots and storing at –15°C to –30°C. Avoid repeated freezing and thawing. If stored under these conditions, GeneRead DNAseq Panel PCR Kits are stable for 6 months after receipt.

Intended Use

GeneRead DNAseq Targeted Panels and GeneRead DNAseq Panel PCR Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient

and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

GeneRead DNAseq Panel Kits are tested, and each assay in the GeneRead DNAseq Targeted Panels is tested when designed, against predetermined specifications to ensure consistent product quality.

Introduction

DNA sequencing is a useful tool to detect genetic variations, including somatic mutations, SNPs, and small insertions and deletions. Targeted enrichment technology enables next-generation sequencing (NGS) platform users to sequence specific regions of interest instead of the entire genome, effectively increasing sequencing depth and throughput with lower cost. GeneRead DNAseq Targeted Panels V2 use multiplex PCR-based targeted enrichment technology, in combination with a sophisticated primer design algorithm, to enable amplification and enrichment of any gene or targeted region in the human genome in order to detect genetic variation using NGS (Figure 1). Adjacent and potentially interacting primer pairs are separated into different pools for optimal performance. GeneRead DNAseq Targeted Panels V2 are designed to analyze a panel of genes and/or regions related to a disease state and can be used with any major NGS platform. The targeted enrichment process is essential for the efficient utilization of medium- and high-throughput sequencers such as Life Technologies®' Ion Personal Genome Machine® (PGM™) Sequencer and Ion Proton™, as well as Illumina®'s MiSeq® Personal Sequencer, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500, and GAIIx.

GeneRead DNAseq Targeted Panels V2 have been optimized in combination with GeneRead DNAseq Panel PCR Kits V2 to provide superior sensitivity and linear multiplex amplification. The simplicity of the PCR method makes these panels accessible for routine use in every research laboratory.

Principle and procedure

GeneRead DNAseq Targeted Panels V2 are provided as sets of 1, 2, 3, or 4 pools, each containing primer mixes, with up to 9600 primer pairs per 4-pool set. The number of pools included is determined by the region covered by amplicons. Most panels cover full coding regions of genes, so one 4-pool set is provided. For special panels, like the Tumor Actionable Mutations Panel covering tumor mutation hotspots, one pool is provided. GeneRead DNAseq Targeted Panels V2 can enrich selected genes and/or regions using as little as 40 or 20 ng genomic DNA in 3 hours for a 4- or 1-pool panel, respectively (Figure 2). Briefly, genomic DNA samples are combined with primer mix and PCR reagent and PCR is performed in a standard thermocycler. After the reaction is complete, the reactions for each sample are pooled and the enriched DNA is purified. The purified DNA then is ready for NGS library construction and sequencing using the NGS platform of your choice. The sequencing results can be analyzed using the GeneRead DNAseq Analysis

Software at http://ngsdataanalysis.sabiosciences.com, which will automatically perform all steps necessary to generate a DNA sequence variant report from your NGS data (Figure 3).

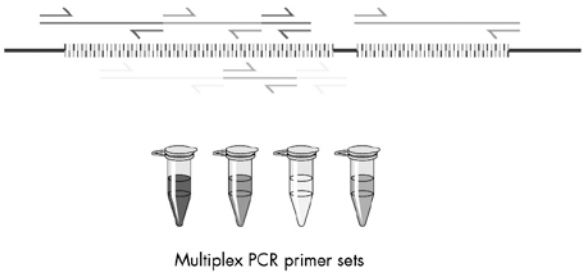


Figure 1. Multiplex PCR-based targeted enrichment scheme. GeneRead DNAseq Targeted Panels V2 use multiplex PCR-based targeted enrichment technology in combination with a sophisticated primer design algorithm to maximize design coverage and minimize nonspecific amplification. The adjacent primer sets are distributed across an appropriate number of pools to minimize nonspecific amplification products.

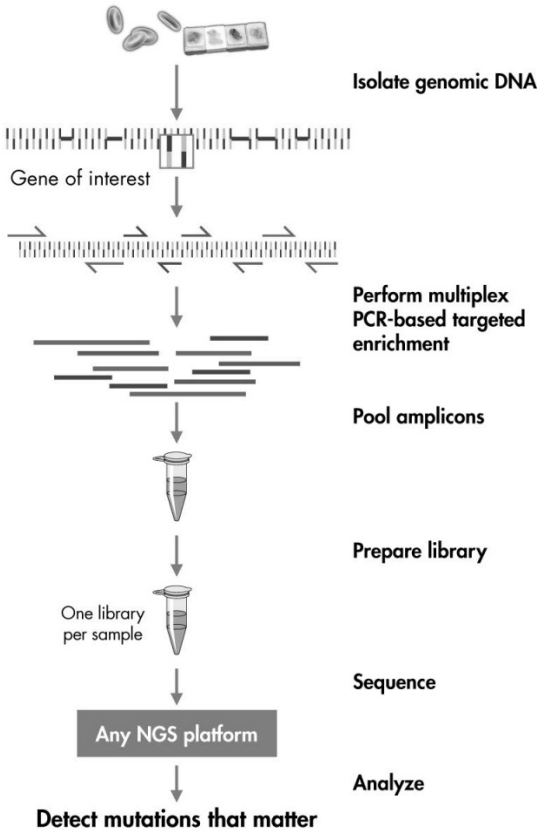


Figure 2. GeneRead DNAseq Targeted Panel V2 procedure.

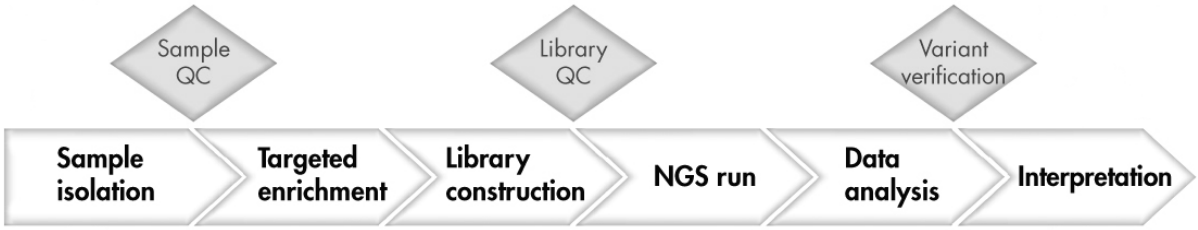


Figure 3. Overview of the complete NGS workflow with GeneRead DNAseq Targeted Panels V2. The complete sample-to-interpretation procedure begins with DNA extraction, followed by targeted enrichment with GeneRead DNAseq Targeted Panels V2, NGS library construction, sequencing, and data analysis using the QIAGEN NGS Data Analysis Web Portal. Follow-up experiments or sample verification against specific targets can be performed with qBiomarker Somatic Mutation PCR Assays.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

In addition to the GeneRead DNAseq Targeted Panel V2 and GeneRead DNAseq Panel PCR Kit V2, the following supplies are required:

For genomic DNA isolation:

- See page 6 for specific recommendations.

For targeted enrichment:

- High-quality, nuclease-free water. Do not use DEPC-treated water.
- GeneRead DNA QuantiMIZE Array or Assay Kit if using FFPE samples (QIAGEN cat. nos. 180642/180654)
- Agencourt® AMPure® XP Kit (Beckman Coulter cat. no. A63880)
- Microcentrifuge
- 1.5 ml LoBind tubes
- 0.2 ml PCR tubes, 96-well reaction plates, or PCR strips and caps
- Thermal cycler
- Multichannel pipettor
- Single-channel pipettor
- DNase-free pipet tips and tubes
- Agilent® 2100 Bioanalyzer®
- Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)

For NGS library construction for Ion PGM and Proton Sequencers:

- GeneRead DNA Library L Core Kit (QIAGEN cat. no. 180462)
- GeneRead DNA L Amp Kit (QIAGEN cat. no. 180485)
- GeneRead Adapter L Set 1-plex (QIAGEN cat. no. 180922) or GeneRead Adapter L Set 12-plex (QIAGEN cat. no. 180994)
- GeneRead Size Selection Kit (QIAGEN cat. no. 180514)
- Agencourt AMPure® XP Kit (Beckman Coulter cat. no. A63880)

- QIAquick® PCR Purification Kit (QIAGEN cat. no. 28104 or 28106)
- GeneRead Library Quant Kit for Ion PGM Sequencer (QIAGEN cat. no. 180601)
- 80% ethanol
- Thermal cycler
- A real-time PCR machine compatible with 96-well/100-well/384-well plates

For NGS library construction for Illumina MiSeq/HiSeq:

- GeneRead DNA Library I Core Kit (QIAGEN cat. no. 180432)
- GeneRead DNA I Amp Kit (QIAGEN cat. no. 180455)
- GeneRead Adapter I Set 1-plex (QIAGEN cat. no. 180912) or GeneRead Adapter I Set 12-plex (QIAGEN cat. no. 180984)
- GeneRead Size Selection Kit (QIAGEN cat. no. 180514)
- QIAquick PCR Purification Kit (QIAGEN cat. no. 28104 or 28106)
- GeneRead Library Quant Kit for Illumina (QIAGEN cat. no. 180601)
- Agencourt AMPure® XP Kit (Beckman Coulter cat. no. A63880)
- 80% ethanol
- Thermal cycler
- A real-time PCR machine compatible with 96-well/100-well/384-well plates

Important Notes

DNA preparation and quality control

High-quality DNA is essential for obtaining good sequencing results

The most important prerequisite for DNA sequence analysis is consistent, high-quality DNA from every experimental sample. Therefore, sample handling and DNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts, or other contaminants may either degrade the DNA or decrease the efficiency of, if not block completely, the enzyme activities necessary for optimal targeted genome amplification and real-time PCR performance.

Recommended genomic DNA preparation method

The QIAGEN QIAamp® DNA Mini Kit (cat. no. 51304), QIAamp DNA FFPE Tissue Kit (cat. no. 56404), and GeneRead DNA FFPE Kit (cat. no. 180134) are highly recommended for the preparation of genomic DNA samples from fresh tissues and FFPE tissue samples. Ensure that samples have been treated for the removal of RNA, as RNA contamination will cause inaccuracies in DNA concentration measurements. Do not omit the recommended RNase treatment step to remove RNA. If genomic DNA samples need to be harvested from biological samples for which kits are not available, please contact Technical Support representatives for suggestions.

For best results, all DNA samples should be resuspended in DNase-free water or alternatively in DNase-free 10 mM Tris buffer pH 8.0. Do not use DEPC-treated water.

DNA quantification and quality control

For best results, all DNA samples should also demonstrate consistent quality according to the following criteria:

Concentration and purity determined by UV spectrophotometry

The concentration and purity of DNA should be determined by measuring the absorbance in a spectrophotometer. Prepare dilutions and measure absorbance

in 10 mM Tris·Cl,* pH 8.0. The spectral properties of nucleic acids are highly dependent on pH.

A_{260}:A_{280} ratio should be greater than 1.8

Concentration determined by A_{260} should be >2.5 µg/ml DNA.

DNA integrity

For best results, the genomic DNA should be greater than 2 kb in length with some fragments greater than 10 kb. This can be checked by running a fraction of each DNA sample on a 1% agarose gel.

FFPE DNA

If FFPE DNA will be used for GeneRead DNAseq Targeted Panels, the QIAGEN GeneRead DNA QuantiMIZE Array or Assay Kit is recommended for determining optimal DNA amount and PCR cycling conditions for each FFPE DNA sample.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs), available from the product supplier.

Protocol: PCR Setup

Procedure

1. Dilute DNA to 2.5 ng/µl with DNase-free water in a LoBind tube. For each sample, 20 ng (8 µl, 2.5 ng/µl) DNA is required for the 1-pool panel, or 40 ng (16 µl, 2.5 ng/µl) for the 4-pool panel.

   Note: Dilution of FFPE DNA samples should be determined by QIAGEN GeneRead DNA QuantiMIZE Array or Assay Kit for optimal results.
2. Determine the number of reactions needed. For a 1-pool panel, one 40 µl reaction for each sample is required. For 2, 3, or 4-pool panels, 2, 3, or 4 x 20 µl reactions for each sample are required. Prepare PCR strips or a PCR plate according to the number of reactions. Label with sample names and pool numbers.
3. Aliquot 8 µl (1-pool panel) or 4 µl (2, 3, or 4-pool panels) of each DNA sample into each PCR reaction.

   Note: If the GeneRead DNA QuantiMIZE Array or Assay Kit is used, the same volume of FFPE DNA will be used after diluting DNA samples according to the GeneRead DNA QuantiMIZE Array or Assay Kit.
4. Prepare the PCR mix on ice according to Table 1 (1-pool panel) or Table 2 (2, 3, or 4-pool panel). For each sample, 1, 2, 3, or 4 PCR mixes will be needed. Mix gently by pipetting up and down.

Table 1. Preparation of PCR mix for each primer mix pool (1-pool panel)

Component	Per 1 sample (µl)	Per *n* samples (µl)
GeneRead DNAseq Panel PCR Buffer (5x)	8.8	8.8 x *n*
Primer mix pool (2x)	22	22 x *n*
GeneRead HotStarTaq DNA Polymerase (6U/µl)	2.9	2.9 x *n*
DNase-free water	1.5	1.5 x *n*
Total volume	35.2	35.2 x *n*

Table 2. Preparation of PCR mix for each primer mix pool (2, 3, or 4-pool panels)

Component	Per 1 sample (µl)	Per *n* samples (µl)
GeneRead DNAseq Panel PCR Buffer (5x)	4.4	4.4 x *n*
Primer mix pool x* (2x)	11	11 x *n*
GeneRead HotStarTaq DNA Polymerase (6 U/µl)	1.5	1.5 x *n*
DNase-free water	0.7	0.7 x *n*
Total volume	17.6	17.6 x *n*

* The number of primer mix pools is determined by the panel size.

5. Aliquot 32 µl (1-pool panel) or 16 µl (2, 3, or 4-pool panel) of each PCR mix, and add it to the well with DNA samples accordingly. Mix gently by pipetting up and down.
6. Seal the wells with PCR tube caps. Place strips or plate in thermocycler and set up reaction parameters according to Tables 3 and 4.

Table 3. PCR program

Cycle	Temperature	Time
1	95°C	15 min
Number of cycles according to table 4	95°C	15 s
	60°C	4/8 min*
1	72°C	10 min
1	4°C	∞

* 4 min if number of primer pairs is ≤1200 in each pool; 8 min if number of primer pairs is 1201–2500 per pool. To determine number of primer pairs per pool, divide the total number of primer pairs (page 6) by 4 (except for the Tumor Actionable Mutations panel)

Table 4. PCR cycles

Primer pairs per pool	No. of cycles for standard DNA*
1–11	25
12–23	24
24–47	23
48–95	22
96–191	21
192–287	20
288–399	19
400–1200	18
1201–2500	16

*Number of cycles for FFPE samples should be determined by QIAGEN GeneRead DNA QuantiMIZE Array or Assay Kit.

7. After the reaction is complete, place on ice and proceed with sample pooling and purification using AMPure XP beads.

   Note: If the samples are to be stored prior to purification, transfer them to a –20°C freezer.

Protocol: Sample Pooling and Purification

1. For a 2, 3, or 4-pool panel, combine all 2, 3, or 4 reactions from the same sample into one well of a PCR plate/strip. Mix thoroughly. The volume of each sample should be approximately 40 µl for a 1- or 2-pool panel and 60 or 80 µl for a 3- or 4-pool panel.
2. Transfer 40 µl (1- or 2-pool panel), 60 µl (3-pool panel), or 80 µl (4-pool panel) from each sample to a 1.5 ml LoBind tube for purification. Add the appropriate volume of DNase-free water to 1-, 2-, or 3-pool panel to bring total volume to 80 µl.
3. Add 72 µl (0.9x volume) AMPure XP beads to 80 µl PCR product. Mix well on a vortex mixer or by pipetting up and down at least 10 times.
4. Incubate for 5 min at room temperature.
5. Pulse-spin the tube. Place the tube on magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), carefully transfer 140 µl the supernatant to a new tube without disturbing the beads. Discard the beads, which contain unwanted large DNA fragments.

   Note: Do not discard the supernatant.

   IMPORTANT: Transferring 140 µl supernatant will leave behind about 12 µl supernatant. This is to ensure that no beads are carried over into the supernatant. Any bead carryover will result in a significant amount of larger fragments present in the library, which will affect sequencing specificity.
6. Add 128 µl (1.6x the original volume of PCR product, which was 80 µl) AMPure XP beads to the supernatant, mix well, and incubate for 5 min at room temperature.
7. Pulse-spin the tube. Place the tube on a magnetic rack and wait until solution is clear (about 5 min). Carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

   Note: Do not discard the beads.
8. Add 400 µl fresh 80% ethanol to the tube while it is on the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.
9. Repeat previous step once.
10. Briefly spin the tube, and place on the magnetic rack. Completely remove residual ethanol and dry beads for 15 min while the tube is on the rack with the lid open.

11. Elute DNA target beads into 28 µl sterile water. Mix well by vortexing. Spin down briefly and place tube on the rack until solution is clear. Transfer 25 µl supernatant to a clean LoBind tube.
12. Determine the amount of your sample with an Agilent 2100 Bioanalyzer, using the High Sensitivity DNA Kit. Normally 25–125 ng of PCR product will be obtained after purification.
13. Proceed to library construction according to the sequencing platform of your choice. Refer to Appendix A for the recommended library construction protocol for sequencing with Ion PGM Sequencer. Refer to Appendix B for the recommended library construction protocol for sequencing with Illumina MiSeq/HiSeq.

    Note: If reactions are to be stored prior to library construction, transfer them to a –20°C freezer.

Troubleshooting Guide

For technical support, please call us at 1-888-503-3187 or 1-301-682-9200. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Appendix A: Library Construction Using GeneRead Library Prep Kits for Ion PGM Sequencer/Proton

The following kits are required for NGS library construction for the Ion PGM Sequencer or Ion Proton:

- GeneRead DNA Library L Core Kit (QIAGEN cat. no. 180462)
- GeneRead DNA L Amp Kit (QIAGEN cat. no. 180485)
- GeneRead Adapter L Set 1-plex (QIAGEN cat. no. 180922) or GeneRead Adapter L Set 12-plex (QIAGEN cat. no. 180994)
- GeneRead Size Selection Kit (QIAGEN cat. no. 180514)
- QIAquick PCR Purification Kit (QIAGEN cat. no. 28104 or 28106)
- Agencourt AMPure® XP Kit (Beckman Coulter cat. no. A63880)
- GeneRead Library Quant Kit for Ion PGM Sequencer (QIAGEN cat. no. 180601)

End repair of DNA

Note: 10–200 ng PCR-enriched DNA should be used for library construction. Starting amounts <10 ng or >200 ng will decrease the efficiency of library construction.

A1. Prepare a reaction mix for end-repair according to Table 5, dispensing the reagents into a PCR tube or the wells of a PCR plate on ice.

Table 5. DNA end-repair reaction components

Component	Volume (µl)
PCR-enriched DNA from previous step	20.5
End-Repair Buffer, 10x	2.5
End-Repair Enzyme Mix	2.0
Total	25

A2. Mix the components by pipetting up and down several times.

A3. Incubate in a thermal cycler for 20 min at 25°C, followed by 10 min at 70°C.

A4. Pulse-spin the microfuge tube and return to ice.

Adapter ligation

A5. Prepare a reaction mix for adapter ligation according to Table 6, adding the components to the PCR tube containing the end-repaired DNA from previous step. Mix thoroughly.

Note: If analyzing 1 sample only, use the GeneRead Adapter L Set 1-plex. If analyzing up to 12 samples in a single run, use the GeneRead Adapter L Set 12-plex.

Note: When using barcode adapters, open one adapter tube at a time and change gloves between pipetting the different barcode adapters to avoid cross-contamination.

IMPORTANT: Only one of the 12 adapters (Adapter Bc1–Bc12) should be used per ligation reaction, in combination with the universal adapter BcGen.

Table 6. Reaction setup for adapter ligation

Component	Singleplex Volume/reaction (µl)	Multiplex Volume/reaction (µl)
End-repaired DNA (from step A4)	25	25
Ligation Buffer, 2x	40	40
Adapter (singleplex) (50 µM)	0.8*	—
Universal Adapter BcGen (25 µM)	—	1.6*
Barcode Adapter 1–12 (25 µM)	—	1.6*
Ligation and Nick Repair Mix	4	4
dNTP Mix (10 mM)	1	1
DNase-free water	9.2	6.8
Total	80.0	80.0

* Use 0.5 µM final concentration of GeneRead Adapter L Set 1-plex or GeneRead Adapter L Set 12-plex. Alternatively, add the correct amount of adapter according to supplier's directions. If more than 12 samples will be multiplexed, Ion Xpress™ Barcode Adapters from Life Technologies can be used. For each reaction, use 1.6 µl Ion Xpress P1 Adapter, and 1.6

µl of one of the Ion Xpress Barcodes. Only one of the Ion Xpress Barcodes is used for each sample.

A6. Mix the contents by pipetting up and down several times.

A7. Program a thermocycler to incubate for 10 min at 25°C, followed by 5 min at 72°C.

IMPORTANT: Do not use a thermocycler with a heated lid.

A8. After the reaction is complete, place the reactions on ice and proceed with purification using GeneRead Size Selection Kit (QIAGEN cat. no. 180514).

Purification of adaptor-ligated DNA using GeneRead Size Selection Kit

A9. Transfer the DNA library sample (~80 µl) from step A7 into a 1.5 ml LoBind tube. Add 320 µl Buffer SB1 (4 x volume) to the DNA library sample and mix.

A10. To bind DNA, apply the sample to the MinElute® spin column and centrifuge for 1 min. Discard the flow-through. Place the MinElute spin column back into the same tube.

Note: All centrifugation steps in this section are carried out at full speed (maximum 20,000 x *g*) in a conventional tabletop microcentrifuge at room temperature (15–25°C).

A11. To wash, add 700 µl freshly made 80% ethanol to the MinElute spin column and centrifuge for 1 min. Discard the flow-through. Place the MinElute spin column back into the same tube.

A12. Repeat step A11.

A13. Centrifuge the MinElute spin column for an additional 1 min at maximum speed.

A14. Place the MinElute spin column into a clean 1.5 ml microcentrifuge tube (provided).

A15. Add 90 µl Buffer TE to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min. Keep the MinElute spin column.

A16. Add 4 volumes of Buffer SB1 (~360 µl) to 1 volume of the flow-through and mix. Place the MinElute spin column from step A15 into a new 2 ml collection tube (provided).

A17. Re-apply the mixture to the same MinElute spin column and centrifuge for 1 min. Discard the flow-through.

A18. To wash, add 700 µl 80% ethanol to the MinElute spin column and centrifuge for 1 min. Discard the flow-through. Place the MinElute spin column back into the same tube.

A19. Repeat step A18.

A20. Centrifuge the MinElute spin column for an additional 1 min at maximum speed.

A21. Place the MinElute spin column in a clean 1.5 ml microcentrifuge tube (provided).

A22. For elution, add 19 µl Buffer EB to the center of the membrane, let the MinElute spin column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the buffer is dispensed directly onto the center of the membrane for complete elution of the bound DNA.

Note: The median size of library will be 220 bp. Cleanup step should remove adapter monomers and dimers from ligated library.

PCR amplification of the purified library

Note: This step is required to ensure that only properly-made libraries proceed to the next-generation sequencing step.

A23. Mix the components in Table 7 in a 0.2 ml PCR tube.

Table 7. Reaction components for PCR amplification

Component	Volume (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix (10 µM each)	1.5
Library DNA (from step A22)	17
RNase-free water	6.5
Total	50

A24. Set up the cycler using the cycling conditions in Table 8.

Table 8. Cycling conditions for amplification of adaptor-ligated DNA

Step	Temperature	Time
Initial denaturation	98°C	2 min
5 cycles	98°C	20 sec
	60°C	30 sec
	72°C	30 sec
1 cycle	72°C	1 min
Hold	4°C	∞

A25. After the reaction is complete, place the reactions on ice and proceed with cleanup using the QIAquick PCR Purification Kit in Appendix C.

Appendix B: Library Construction Using GeneRead Library Prep Kits for Illumina

The following kits are required for NGS library construction for Illumina MiSeq/HiSeq:

- GeneRead DNA Library I Core Kit (QIAGEN cat. no. 180432)
- GeneRead DNA I Amp Kit (QIAGEN cat. no. 180455)
- GeneRead Adapter I Set 1-plex (QIAGEN cat. no. 180912) or GeneRead Adapter I Set 12-plex (QIAGEN cat. no. 180984)
- GeneRead Size Selection Kit (QIAGEN cat. no. 180514)
- QIAquick PCR Purification Kit (QIAGEN cat. no. 28104 or 28106)
- Agencourt AMPure® XP Kit (Beckman Coulter cat. no. A63880)
- GeneRead Library Quant Kit for Illumina (QIAGEN cat. no. 180601)

End repair of DNA

Note: 10–200 ng PCR-enriched DNA should be used for library construction. Starting amounts <10 ng or >200 ng will decrease the efficiency of library construction.

B1. Prepare a reaction mix for end-repair according to Table 9, dispensing the reagents into a PCR tube or the well of a PCR plate on ice.

Table 9. Reaction mix for end-repair

Component	Volume (µl)
PCR-enriched DNA from previous step	20.5
End-Repair Buffer, 10x	2.5
End-Repair Enzyme Mix	2.0
Total	25

B2. Mix the components by pipetting up and down several times.

B3. Incubate in a thermal cycler for 30 min at 25°C, followed by 20 min at 75°C.

B4. Pulse-spin the microfuge tube and return to ice.

Note: For panels with <100 primer pairs, add 25 µl of DNase-free water to finished end-repair reaction and clean up with QIAquick PCR Purification Kit according to Appendix C before A-addition. For panels with >100 primer pairs, proceed directly to B5 for A-addition.

A-addition

B5. Prepare a reaction mix for A-addition according to Table 10, adding the components to the PCR tube containing the end-repaired DNA from step B4.

Table 10. Reaction mix for A-addition

Component	Volume
End-repaired DNA (from step B4 or C10)	25 µl
A-addition Buffer, 10x	3 µl
Klenow Fragment (3'→5' exo-)	3 µl
Total	31 µl

B6. Mix the components by pipetting up and down several times.

B7. Incubate in a thermal cycler for 30 min at 37°C, followed by 10 min at 75°C.

Adapter ligation

Note: If preparing 1 sample, use the GeneRead Adapter I Set 1-plex. If preparing up to 12 samples in a single run, use the GeneRead Adapter I Set 12-plex.

B8. Prepare a reaction mix for adapter ligation according to Table 11, adding the components to the PCR tube containing DNA that has undergone end-repair and A-addition (step B7).
Note: When using barcode adapters, open one adapter tube at a time and change gloves between pipetting the different barcode adapters to avoid cross-contamination.

IMPORTANT: Only one of the 12 adapters (Adapter Bc1–Bc12) should be used per ligation reaction.

Table 11. Reagents for adapter ligation of PCR product

Component	Volume/reaction (µl)
DNA from step B7	31
Ligation Buffer, 2x	45
Adaptor (50 µM)	2.5*
T4 DNA Ligase	4
DNase-free water	7.5
Total	90

* Use 1.4 µM final concentration of GeneRead Adapter I Set 1-plex or GeneRead Adapter I Set 12-plex. Alternatively, add the correct amount of adapter according to supplier's directions. If more than 12 samples will be multiplexed, use GeneRead Adapter I Set 1-plex in ligation and add index during library amplification (step B36) with NEBNext® Multiplex Oligos for Illumina.

B9. Mix the components by pipetting up and down several times.

B10. Program a thermocycler to incubate at 25°C for 10 min.

IMPORTANT: Do not use a thermocycler with a heated lid.

B11. After the reaction is complete, place the reactions on ice and proceed with purification using GeneRead Size Selection Kit (QIAGEN cat. no. 180514).

Cleanup of adaptor-ligated DNA with GeneRead Size Selection Kit

B12. Transfer the DNA library sample (~90 µl) from step B11 into a 1.5ml LoBind tube. Add 360 µl Buffer SB1 (4x volume) to the DNA library sample and mix.

B13. To bind DNA, apply the sample to the MinElute spin column and centrifuge for 1 min. Discard the flow-through. Place the MinElute spin column back into the same tube.

Note: All centrifugation steps in this section are carried out at full speed (maximum 20,000 x *g*) in a conventional tabletop microcentrifuge at room temperature (15–25°C).

B14. To wash, add 700 µl freshly made 80% ethanol to the MinElute spin column and centrifuge for 1 min. Discard the flow-through. Place the MinElute spin column back into the same tube.

B15. Repeat step B14.

B16. Centrifuge the MinElute spin column for an additional 1 min at maximum speed.

B17. Place the MinElute spin column into a clean 1.5 ml microcentrifuge tube (provided).

B18. Add 90 µl Buffer TE to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min. Keep the MinElute spin column.

B19. Add 4 volumes of Buffer SB1 (~360 µl) to 1 volume of the flow-through and mix. Place the MinElute spin column from step B18 into a new 2 ml collection tube (provided).

B20. Re-apply the mixture to the same MinElute spin column and centrifuge for 1 min. Discard the flow-through.

B21. To wash, add 700 µl 80% ethanol to the MinElute spin column and centrifuge for 1 min. Discard the flow-through. Place the MinElute spin column back into the same tube.

B22. Repeat step B21.

B23. Centrifuge the MinElute spin column for an additional 1 min at maximum speed.

B24. Place the MinElute spin column in a clean 1.5 ml microcentrifuge tube (provided).

B25. For elution, add 90 µl Buffer EB to the center of the membrane, let the MinElute spin column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the buffer is dispensed directly onto the center of the membrane for complete elution of the bound DNA.

Fine size selection with AMPure XP beads

B26. Add 72 µl (0.8x volume) AMPure XP beads to 90 µl DNA solution from B25. Mix well on a vortex mixer or by pipetting up and down at least 10 times.

B27. Incubate for 5 min at room temperature.

B28. Pulse-spin the tube. Place the tube on magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), carefully transfer 155 µl of the supernatant to a new tube without disturbing the beads. Discard the beads, which contain unwanted large DNA fragments.

Note: Do not discard the supernatant.

IMPORTANT: Transferring 155 µl supernatant will leave behind about 7 µl supernatant. This is to ensure that no beads are carried over into the supernatant. Any bead carryover will result in a significant amount of larger fragments after library amplification, which will affect sequencing specificity.

B29. Add 36 µl (0.4x the original volume of DNA solution, which was 90 µl) AMPure XP beads to the supernatant, mix well, and incubate for 5 min at room temperature.

B30. Pulse-spin the tube. Place the tube on magnetic rack and wait until solution is clear (about 5 min). Carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

Note: Do not discard the beads.

B31. Add 400 µl freshly made 80% ethanol to the tube while it is on the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant.

B32. Repeat previous step once.

B33. Briefly spin the tube, and place on the magnetic rack. Completely remove residual ethanol and dry beads for 10 min while the tube is on the rack with the lid open.

B34. Elute DNA target beads into 19 µl sterile water. Mix well by vortexing. Spin down briefly and place tube on the rack until solution is clear.

B35. Transfer 17 µl supernatant to a clean PCR tube and proceed to PCR amplification.

Note: The median size of the library will be 280 bp. Cleanup and size selection steps should remove adapter monomer and dimers as well as most of DNA fragments longer than the library.

PCR amplification of purified library

B36. Mix the components in Table 12 in a 0.2 ml PCR tube.

Table 12. Reaction components for PCR amplification

Component	Volume/reaction (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix (10 µM each)*	1.5
Library DNA (from step B35)	17
RNase-free water	6.5
Total	50

* Use 0.3 µM final concentration of the PCR primer mix. Alternatively, add the correct amount of primer according to supplier's directions. If NEBNext Multiplex Oligos for Illumina from NEB are used, for each reaction, use 0.3 µM (0.6 µl) of NEBNext Universal PCR Primer for Illumina (25 µM stock), and 0.3 µM (0.6 µl) of one of the NEBNext Index Primers for Illumina (25 µM stock). Only one of the NEBNext Index Primers for Illumina is used for each sample.

B37. Set up the cycler using the cycling conditions in Table 13.

Table 13. Cycling conditions for amplification of the DNA library

Step	Temperature	Time
Initial denaturation	98°C	2 min
10 cycles	98°C	20 sec
	60°C	30 sec
	72°C	30 sec
1 cycle	72°C	1 min
Hold	4°C	∞

B38. After the reaction is complete, place the reactions on ice and proceed with cleanup using the QIAquick PCR Purification Kit in Appendix C.

Appendix C: Cleanup Using QIAquick PCR Purification Kit

C1. For each sample (Ion PGM library, step A25; Illumina library, step B38; or end-repaired reaction from a <100 primer pairs panel, step B4), add 250 µl Buffer PB (5x volume) to the sample and mix by pipetting up and down.

C2. Place a QIAquick spin column in a provided 2 ml collection tube.

C3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.

Note: All centrifugation steps in this section are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature (15–25°C).

C4. Discard flow-through. Place the QIAquick column back into the same tube.

C5. To wash, add 0.75 ml prepared Buffer PE to the QIAquick column and centrifuge for 30–60 s.

Note: If this is the first time using Buffer PE from a new kit, add ethanol (96–100%) to Buffer PE before use (see bottle label for volume; see the *QIAquick Spin Handbook* for more details).

C6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

C7. Place QIAquick column in a clean 1.5 ml LoBind tube.

C8. To elute DNA, add 30 µl buffer EB (for Ion PGM or Illumina final library) or 27 µl DNase-free water (for end-repaired reaction) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

C9. The library can be stored in a –20°C freezer prior to quantification using the GeneRead DNAseq Library Quant Array.

C10. For cleaned end-repaired reaction, go to B5 to finish the rest of Illumina library construction steps.

Appendix D: FFPE DNA Quality and Quantity

Genomic DNA present in FFPE archives is usually damaged and fragmented to an uncertain extent. Commonly used DNA quantification methods including spectrometers or fluorometers do not differentiate between amplifiable and non-amplifiable DNA. Therefore, they cannot reliably measure the amplifiable amounts of DNA that are able to participate in the multiplex PCR-based targeted enrichment step in the NGS workflow.

The QIAGEN GeneRead DNA QuantiMIZE System is a qPCR-based approach that determines the quantity and quality of DNA that is amenable to PCR-based targeted enrichment prior to NGS. The system provides the most cost-effective approach to qualify and quantify DNA isolated from biological samples, mainly for FFPE samples. Please refer to the corresponding user manual for determining FFPE DNA quantity and quality with GeneRead DNA QuantiMIZE System.

Appendix E: Library Quantification and Quality Control

Quality control for the targeted enrichment and library construction process can be performed using QIAGEN's GeneRead DNAseq Library Quant Array. With this array, the correct dilution of the library can also be determined for sequencing. Please refer to the corresponding user manual for library quantification and quality control.

Appendix F: Data Analysis using QIAGEN's GeneRead DNAseq Analysis Software

After sequencing, results can be analyzed using QIAGEN's GeneRead DNAseq Analysis Software. Our data analysis software will perform mapping to the reference genome, read trimming (removing primer sequences), and variant identification. Please refer to the corresponding document for data analysis.

Appendix G: Combine Libraries for Multiplex Sequencing

Libraries can be combined into one sequencing run, as long as each library uses a different barcode.

Ion PGM Sequencer libraries

Barcoded libraries can be constructed using the GeneRead Adapter L Set 12-plex. If more than 12 samples will be multiplexed, the Ion Xpress Barcode Adapters from Life Technologies (cat. nos. 4471250, 4474009, 4474518, 4474519, 4474520, 4474521, or 4474517) can be used to replace the "GeneRead Adapter L Set 12-plex" in Table 6, page 21.

After the library is constructed, follow Appendix E to determine the library dilution factor and dilute each individual library according to this factor. Combine equal amounts of all the libraries you need, and mix well. At least 25 µl of the mixture is required. Proceed to template preparation using the mixture.

Illumina libraries

Barcoded libraries can be constructed using GeneRead Adapter I Set 12-plex as described in Appendix B, Table 11. If more than 12 samples will be multiplexed, use the GeneRead Adapter I Set 1-plex during ligation in Appendix B, Table 11, and an index will be added with NEBNext Multiplex Oligos for Illumina (NEB cat. no. E7335 or E7500) during the library amplification step, as described in Appendix B, Table 12.

After the library is constructed, follow Appendix E to determine the library concentration. Dilute individual libraries to 4 nM, then combine equal amounts of each, and mix well. At least 5 µl of the mixture is needed. Proceed to generate clusters using this mixture.

Appendix H: Analyze the PCR amplicons and Library Using the Agilent 2100 Bioanalyzer

The Bioanalyzer can be used at multiple steps of the targeted enrichment workflow as a quality control checkpoint.

After the multiplex PCR run, sample pooling, and purification, the PCR product can be analyzed using the Bioanalyzer. A sample Bioanalyzer image is shown in Figure 4. The amplicons should be in the correct size range (usually around 160 bp), and the measured amount should be greater than 10 ng total. The OD reading (NanoDrop®) method is not recommended for determination of DNA concentration because the product may be below the detection limitation of the instrument.

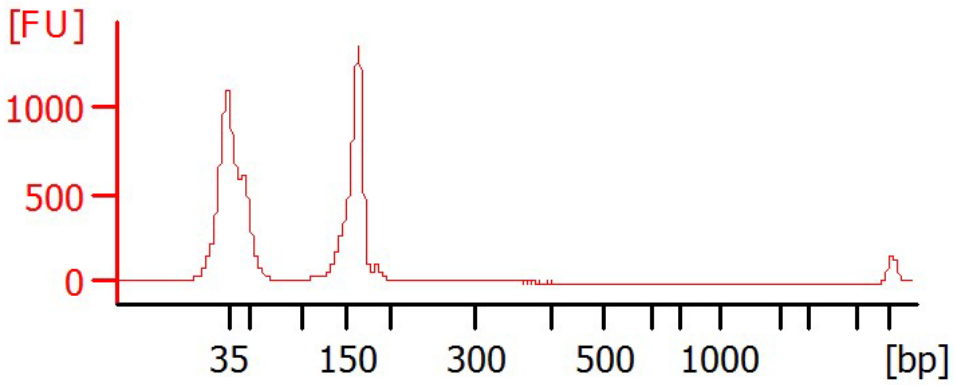


Figure 4. Bioanalyzer trace of pooled and purified multiplex PCR product. A peak of around 160 bp is observed, which represents the amplicons. The primer dimers will be removed during the size selection step after adapter ligation and will have no effect on the final library (see Figure 5).

After the library is constructed and purified, the Bioanalyzer can be used to check the fragment size and concentration. For Ion PGM Sequencer libraries, a peak at 220 bp is expected. There should be no significant peak at around 100 bp, which represents adaptor dimers. For Illumina libraries, a peak at 280 bp is expected (Figure 5) and no significant peak should be observed around 150 bp.

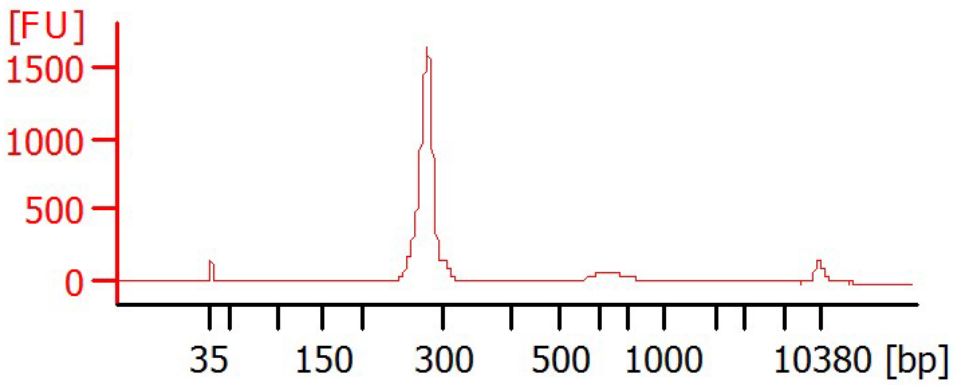


Figure 5. Sample Bioanalyzer image of a MiSeq Sequencer library. A peak of around 280 bp is observed.

Appendix I: Splitting a Single Run File with a Multiplex Sample on Ion PGM Sequencer

I1. When the run is finished, navigate to the report page on the Torrent Browser. Locate the "Output Files" section near the end of the report.

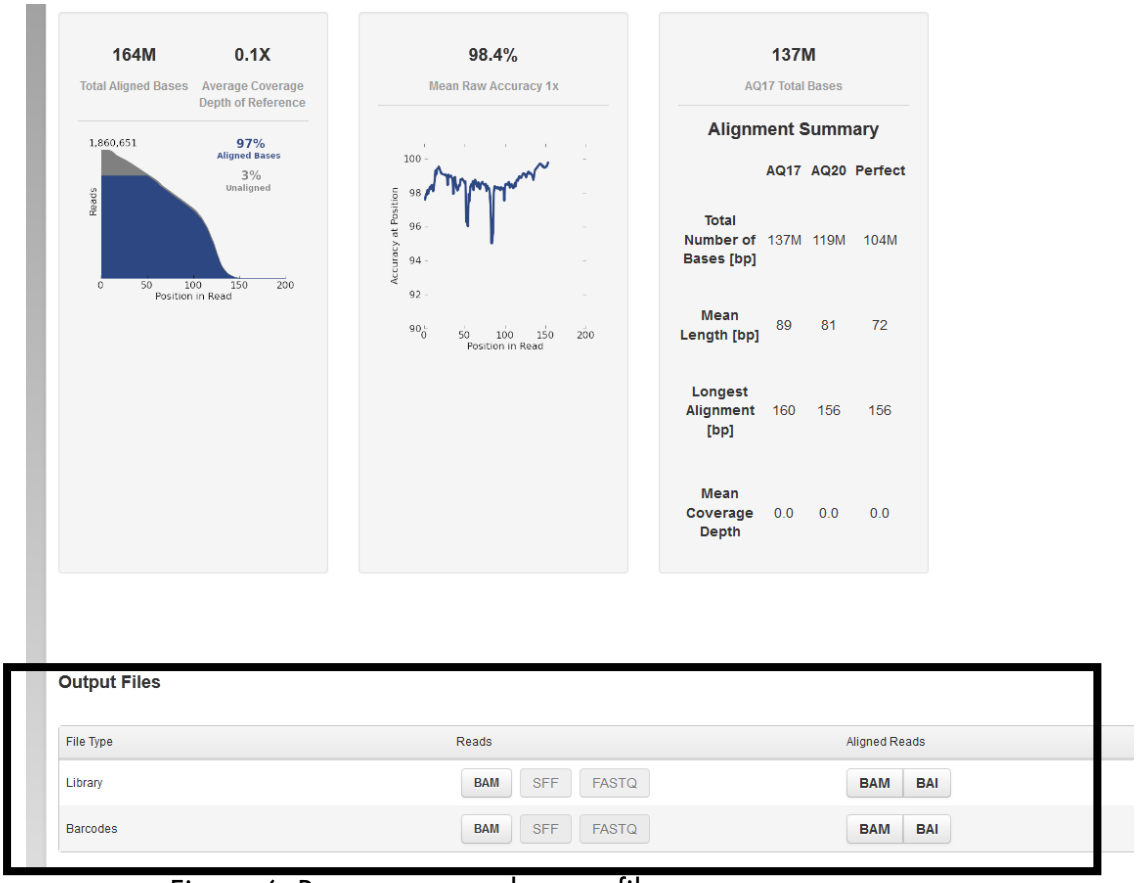


Figure 6. Report page and output files.

I2. Click the "BAM" button in the row labeled "Barcodes" and column labeled "Reads" in the table. These are the unaligned reads in BAM format, with the barcode separated for each sample. Save the .zip file to your local disk. The file is usually several hundred megabytes to several gigabytes, depending on the size of the sequencing chip being used.

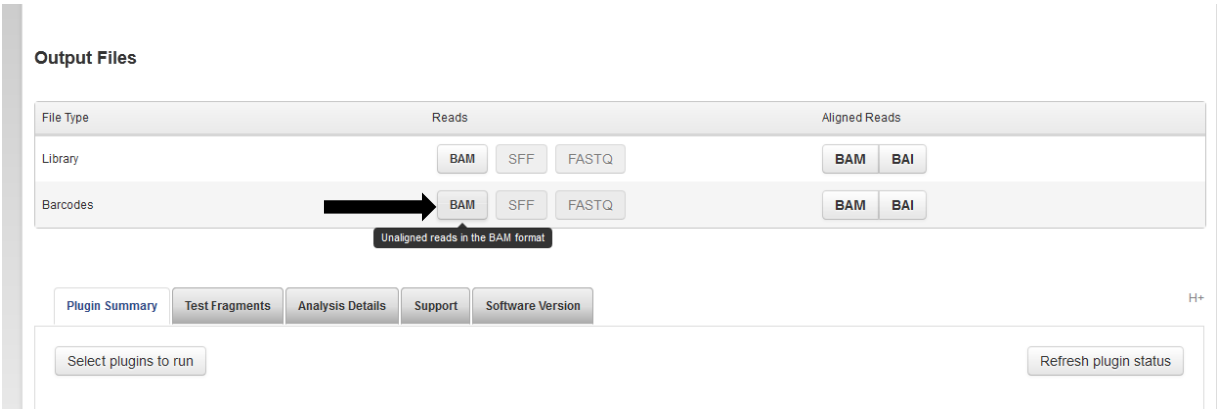


Figure 7. Unaligned reads in BAM format.

I3. Locate the file that was just downloaded to the local disk, and extract the .zip file using a file decompression software such as 7-Zip, Winzip®, or WinRAR.

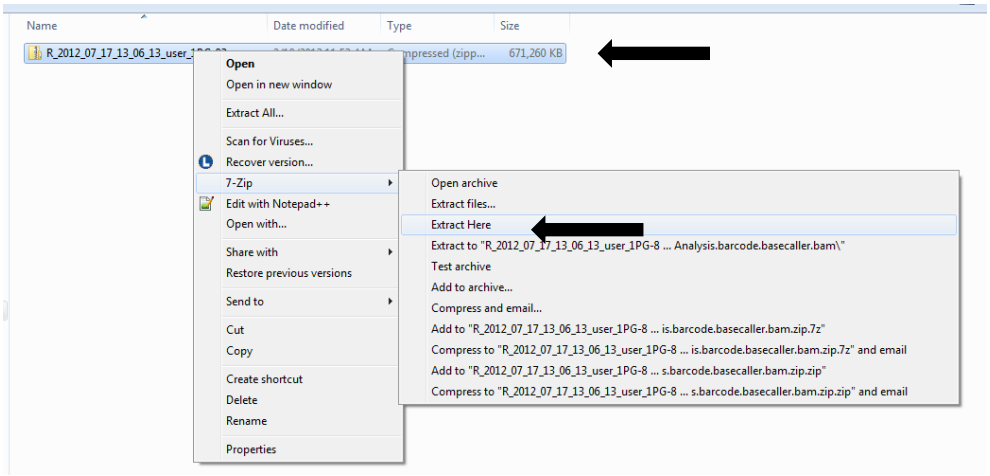
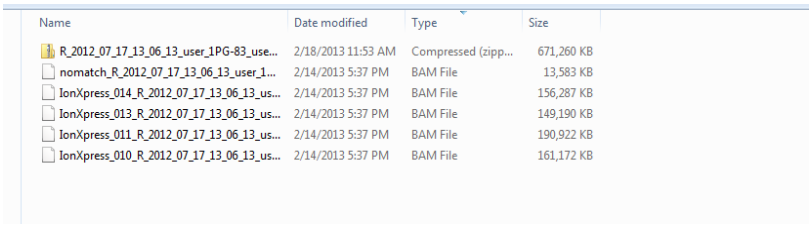


Figure 8. Extracting the .zip files.

I4. Extracting the .zip file will yield several .BAM files, each one corresponding to one barcode sample. The file name starts with the barcode name being used. The one starting with "no match" contains

reads whose barcodes cannot be clearly identified. In most cases, this file can be ignored.



15. Upload the individual .BAM file to QIAGEN's NGS Data Analysis Web Portal for analysis.

Ordering Information

Product	Contents	Cat. no.
GeneRead DNAseq Targeted Panels V2	Sets of 1 or 4 pools containing wet-bench verified primer sets for targeted enrichment of a focused panel of <100 genes	181900
GeneRead DNAseq Targeted HC Panel V2	Sets of 4 pools containing wet-bench verified primer sets for targeted enrichment of a focused panel of >100 genes	181901
GeneRead DNAseq Custom Panel V2	Pools containing primer sets for targeted enrichment of a customized panel of genes or genomic regions	181902
GeneRead DNAseq Mix-n-Match Panel V2	Pools containing wet-bench verified primer sets for targeted enrichment of a custom panel of genes	181905
GeneRead DNAseq Panel PCR Kit V2	PCR chemistry for use with the GeneRead DNAseq Panel V2 System	Varies
Related products		
GeneRead DNAseq Library Quant Array	Reagents for NGS sample library quantification following targeted exon enrichment with the GeneRead DNAseq Gene Panel System	180601
GeneRead qPCR SYBR® Green Mastermix	Master mix for use with the GeneRead Library Quant Arrays and Kit	Varies
GeneRead DNA QuantiMIZE Array Kit	qPCR arrays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA	180642

Product	Contents	Cat. no.
GeneRead DNA QuantiMIZE Assay Kit	qPCR assays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA	180654
GeneRead Size Selection Kit (50)	For 50 reactions: Spin columns and buffers	180514
QIAamp DNA Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Collection Tubes (2 ml), reagents and buffers	51304
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Collection Tubes (2 ml), buffers	56404
GeneRead DNA FFPE Kit (50)	QIAamp MinElute columns, Proteinase K, UNG, Collection Tubes (2 ml), Buffers, Deparaffinization Solution, RNaseA	180134
Ion PGM Sequencer/Proton Library Prep		
GeneRead DNA Library L Core Kit (12)	For 12 reactions: Buffers and reagents for end-repair, ligation, and nick repair, for use with Ion PGM Sequencer/Proton Instruments from Life Technologies	180462
GeneRead DNA L Amp Kit (100)	For 100 reactions: Buffers and reagents for library amplification, for use with Ion PGM Sequencer/Proton Instruments from Life Technologies	180485
GeneRead Adapter L Set 1-plex (12)	For 12 reactions: Adapters for ligation to DNA library, for use with Ion PGM Sequencer/Proton Instruments from Life Technologies	180922

Product	Contents	Cat. no.
GeneRead Adapter L Set 12-plex (72)	For 72 reactions: 12 barcoded adapters for ligation to DNA library, for use with instruments from Life Technologies	180994
Illumina Library Prep		
GeneRead DNA Library I Core Kit (12)	For 12 reactions: Buffers and reagents for end-repair, A-Addition, and ligation, for use with Illumina instruments	180432
GeneRead DNA I Amp Kit (100)	For 100 reactions: Buffers and reagents for library amplification, for use with Illumina instruments	180455
GeneRead Adapter I Set 1-plex (12)	For 12 reactions: Adapters for ligation to DNA library, for use with Illumina instruments	180912
GeneRead Adapter I Set 12-plex (72)	For 72 reactions: 12 barcoded adapters for ligation to DNA library, for use with Illumina instruments	180984

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Australia ■ techservice-au@qiagen.com

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Brazil ■ suportetecnico.brasil@qiagen.com

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China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

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Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

Korea (South) ■ techservice-kr@qiagen.com

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

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USA ■ techservice-us@qiagen.com

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