January 2018

GeneRead™ QIAact DNA Custom Panel Handbook

For constructing targeted, molecularly bar-coded libraries from DNA for digital sequencing with next-generation sequencing (NGS)

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

REF Catalog number is defined for each custom panel

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

Sample to Insight

QIAGEN

Contents

Kit Contents

GeneRead QIAact DNA Custom Panel	
Number of reactions	**24**
GeneRead QIAact DNA Library Preparation and Target Enrichment Reagents (Box 1)*	
QIAact DNA Target Enrichment Panel and QIAact Adapters (Box 2)*	

* Boxes 1 and 2 are components of the GeneRead QIAact DNA Custom Panel.

GeneRead QIAact DNA Library Preparation and Target Enrichment Reagents*	
Number of reactions	**24**
Fragmentation Buffer, 10x	65 µl
Fragmentation Enzyme Mix	130 µl
FERA Solution	20 µl
Ligation Buffer, 5x	260 µl
Ligation Solution	200 µl
DNA Ligase	130 µl
Nuclease-Free Water	2 x 2 ml
TEPCR Buffer, 5x	220 µl
UPCR Buffer, 5x	220 µl
HotStarTaq® DNA Polymerase	2 x 50 µl
QIAseq Beads (provided in a separate cold-packed shipment)	34 ml

* Not for individual sale; to order reagents, contact your sales representative or QIAGEN Technical Service (see back cover).

QIAact DNA Target Enrichment Panel and QIAact Adapters*	
Number of reactions	**24**
GeneRead QIAact DNA Custom Panel Forward Primers	130 µl
GeneRead QIAact DNA Custom Panel Reverse Primers	130 µl
QIAact Adapters (contains 12 tubes with each tube corresponding to one sample-specific bar code; each tube can process up to 2 samples)	10 µl
GeneReader™ TE-PCR Primer	40 µl
GeneReader Universal PCR Primer A	40 µl
GeneReader Universal PCR Primer B	40 µl

* Not for individual sale; to order products, contact your sales representative or QIAGEN Technical Service (see back cover).

Storage

The GeneRead QIAact Library Preparation and Target Enrichment Reagents (except QIAseq Beads) are shipped on dry ice and should be stored at –30°C to –15°C upon arrival. QIAseq Beads are shipped on cold packs and should be stored at 4°C.

GeneRead QIAact DNA Custom Panel is shipped on dry ice and should be stored at –30°C to –15°C upon arrival.

Intended Use

The GeneRead QIAact DNA Custom Panel and GeneRead QIAact Library Preparation and Target Enrichment Reagents are intended for Research Use Only and 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

Each lot of the GeneRead QIAact DNA Custom Panel is tested against the specification as indicated in the panel-specific certificate.

Introduction

DNA sequencing is a useful tool to detect genetic variations, including somatic mutations, single nucleotide variants (SNVs), copy number variation (CNVs) and small insertions and deletions (InDels). 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.

The GeneRead QIAact DNA Custom Panel integrates unique molecular index (UMI) technology into a gene-specific, primer-based target enrichment process, enabling sensitive variant detection of targeted genomic regions by NGS on the GeneReader system.

The GeneRead QIAact DNA Custom Panel has been optimized in combination with a specially formulated enrichment chemistry to achieve highly efficient enrichment on both regular and GC-rich regions at high multiplex levels.

Principle and procedure

The GeneRead QIAact DNA Custom Panel relies on gene-specific primer enrichment and UMIs for uniform coverage and sensitive variant detection.

Unique molecular index

Unique molecular indexing is used to attach a unique sequence index to each molecule prior to amplification. This attachment is accomplished by the ligation of a QIAact Adapter containing a UMI of 8 random bases to each original DNA molecule.

The DNA molecules are then amplified by PCR for target enrichment and library amplification. Due to intrinsic noise and sequence-dependent bias, DNA molecules with UMIs may be amplified unevenly across the target regions. Even target region coverage can be achieved by counting the number of UMIs in the reads rather than counting the number of total reads

for each region. Sequence reads with varying UMIs represent different original molecules, whereas sequence reads with the same UMI are the result of PCR duplication from one original molecule.

PCR amplification and the sequencing process generate biases and artefacts which lead to the recording of false-positive DNA variants in the sequencing reads. These false-positive variants may mask the detection of true low-frequency variants in heterogeneous samples, such as tumors. To reduce the number of false-positive variants, variants are called across all reads within a UMI , in contrast to calling only variants from the original sequencing read (Figure 1).

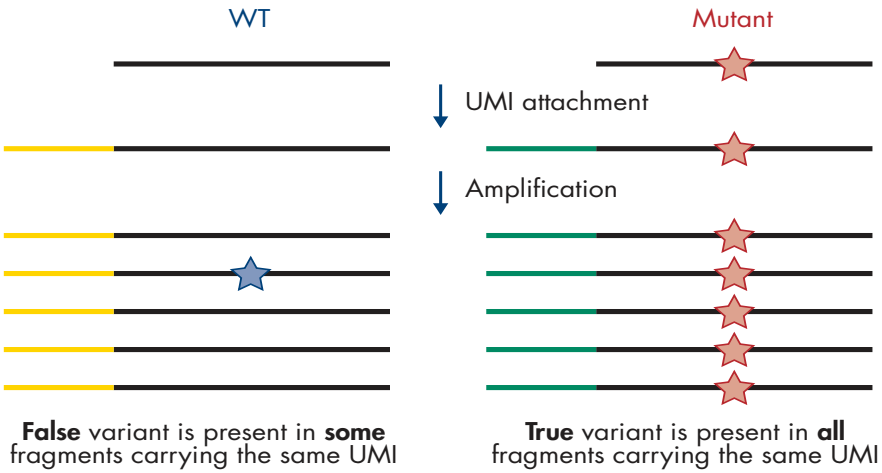

Figure 1. Principle of variant detection with UMI. Each original molecule is tagged by a UMI. True variants are those mutations present in the majority of reads within a UMI, while errors are mutations present in only one or a few reads within a UMI. Description of the variant calling algorithm can be found at **www.qiagen.com**.

Process

The GeneRead QIAact DNA Custom Panel is provided as two tubes containing the primer mixes. The GeneRead QIAact DNA Custom Panel is designed to enrich selected genes and regions using 40–100 ng DNA.

Genomic DNA samples are first fragmented, end-repaired and A-tailed within a single, controlled multiple enzyme reaction. The prepared DNA fragments are then ligated at their 5' ends with a specific adapter containing a UMI and a sample-specific bar code.

Ligated DNA molecules are subjected to limited cycles of target enrichment PCR, with one gene-specific primer targeting a region and one universal forward primer complimentary to an adapter sequence. This reaction ensures that intended targets and UMIs are enriched sufficiently to be represented in the final library. A universal PCR with GeneReader-specific sequences is then carried out to amplify the targets and complete the library.

Once the library is sequenced, results can be analyzed using the GeneRead QIAact DNA Custom Panel workflow, which automatically performs all steps necessary to call variants and generate a secondary analysis report from your raw NGS data summarizing all of the DNA mutation information. All detected variants can be further interpreted by QIAGEN Clinical Insight (QCI™) analysis.

Recommendation for multiplexing and clonal amplification input

More than one DNA sample can be sequenced in one flow cell due to a sample-specific bar code that is added during library preparation (see "Protocol: Adapter Ligation", page 18). The sequencing libraries prepared from multiple samples must be pooled prior to clonal amplification to allow them to be sequenced together in one flow cell (see "Protocol: Library Concentration Normalization and Pooling" in the *QIAGEN GeneRead Clonal Amp Q Handbook* for more information).

After target enrichment and library preparation, use the pooled samples in the clonal amplification process (see "Preparing Libraries for Emulsion Making" in the *QIAGEN GeneRead Clonal Amp Q Handbook* for more information).

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 QIAact Library Preparation and Target Enrichment Reagents and GeneRead QIAact DNA Custom Panel, the following supplies are required:

For DNA isolation:

- See "Recommended genomic DNA preparation methods", page 13

For library construction and targeted enrichment:

- High-quality, nuclease-free water. Do not use DEPC-treated water
- 80% ethanol, freshly made
- Microcentrifuge
- 1.5 ml LoBind tubes (Eppendorf® AG)
- 0.2 ml PCR tubes, 96-well PCR plates or PCR strips and caps
- Thermal cycler (e.g., Bio-Rad® C1000™)
- Multichannel pipettor
- Single-channel pipettor
- DNase-free pipette tips and tubes
- QIAxcel® Advanced instrument (for information, visit **www.qiagen.com**)
- QIAxcel DNA High Resolution Kit (cat. no. 929002)

- QX DNA Size Marker 50–800 bp (50 µl) (cat. no. 929561)
- QX Alignment Marker 15 bp/3 kb (1.5 ml) (cat. no. 929522)
- QX Nitrogen Cylinder (x 6) (cat. no. 929705)
- Qubit® 3.0 Fluorometer (Thermo Fisher Scientific cat. no. Q33216) or equivalent
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific cat. no. Q32851)
- Qubit assay tubes (e.g., Thermo Fisher Scientific cat. no. Q32856)
- DynaMag™-2 Magnet (Thermo Fisher Scientific cat. no. 12321D)

Optional

- QIAseq DNA QuantiMIZE Array or Assay Kit (configured for specific thermal cycler) if using FFPE samples (QIAGEN cat. nos. 333404 and 333414)
- QIAxpert® System (for information, visit **www.qiagen.com**)
- Agilent® 2100 Bioanalyzer®
- Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)

Important Notes

DNA preparation

High-quality DNA from every experimental sample is essential for obtaining good sequencing results. Therefore, sample handling and DNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants may degrade the DNA or decrease the efficiency (if not block completely) of the enzyme activities necessary for optimal targeted genome amplification.

Recommended genomic DNA preparation methods

The QIAGEN QIAamp® DNA Mini Kit (cat. no. 51304), GeneRead DNA FFPE Kit (cat. no. 180134) and QIAamp Circulating Nucleic Acid Kit (cat no. 55114) are highly recommended for the preparation of genomic DNA samples from fresh tissues, FFPE tissue and plasma or serum samples, respectively. 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 QIAGEN Technical Services for suggestions.

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

Recommended circulating-free DNA preparation method for liquid biopsy samples

Optimal extraction of circulating-free DNA (cfDNA) from plasma samples can be safeguarded by drawing blood into sample collection tubes that provide efficient stabilization of plasma

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samples (e.g., PAXgene® Blood ccfDNA Tube, cat.no. 768115). To maximize DNA yields, we recommend extraction from 4–5 ml plasma and elution in the lowest volume stated in the *QIAamp Circulating Nucleic Acid Kit Handbook*. Low DNA yields can present challenges, such as providing insufficient input material for detecting low frequency variants.

DNA quantification

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

DNA purity determined by UV spectrophotometry

The purity of DNA should be determined by measuring absorbance in a spectrophotometer, such as the QIAxpert System. Prepare dilutions and measure absorbance in 10 mM Tris·HCl* buffer, 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.

DNA concentration

The concentration of DNA should be determined by fluorometric quantitation using equipment such as the Qubit 3.0 Fluorometer (Thermo Fisher Scientific cat. no. Q33216). A minimum input of 40 ng DNA is recommended for the GeneRead QIAact Panels†.

If DNA purified from FFPE samples will be used with the GeneRead QIAact DNA Custom Panel, the QIAGEN GeneRead DNA QuantiMIZE Array Kit or QIAGEN GeneRead DNA QuantiMIZE Assay Kit can be used to check the integrity of the obtained DNA (for QC score

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† For unknown or low quality FFPE DNA, an input of 40—100 ng DNA should be used for optimal performance.

≥0.04, proceed, as the genomic DNA is of good quality; for QC score >0.04, proceed with caution, as the DNA is highly fragmented or damaged).

Automation

The manual cleanup steps described in this handbook can be automated on the QIAcube instrument. Three protocols for automating cleanup are available from the GeneRead QIAact Panel Cleanup Kit (cat. no. 185446).

- "Protocol: GR QIAact Panel Cleanup 1" describes the cleanup procedure after fragmentation, end repair and adapter ligation.
- "Protocol: GR QIAact Panel Cleanup 2" describes the cleanup procedure after target enrichment PCR.
- "Protocol: GR QIAact Panel Cleanup 3" describes the cleanup procedure after universal library amplification.

Protocol: Fragmentation, End-repair and A-addition

Important points before starting

- Ensure input DNA is in 10 mM Tris, QIAGEN Buffer EB, QIAGEN's Buffer ATE or low concentration TE (0.1x TE, 0.1 mM EDTA).*
- Pre-chill a thermal cycler to 4°C.

Procedure

1. Adjust the concentration of the input DNA with nuclease-free water in a LoBind tube. Use 4 µl of the diluted DNA for fragmentation.

   Note: If using 40 ng DNA as input, dilute to 10 ng/µl. If using 100 ng DNA as input, dilute to 25 ng/µl.

2. Prepare a reaction mixture for fragmentation, end-repair and A-addition according to Table 1, dispensing the reagents into a PCR tube on ice. It is important to keep the mixture on ice and mix the components well by pipetting up and down 10 times.

Table 1. Fragmentation, end-repair and A-addition mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
Fragmentation Buffer, 10x	2.5	16.2	31.2
FERA Solution	0.75	4.9	9.4
Nuclease-Free Water	12.75	82.9	159.4
Total volume	**16.0**	**104**	**200**

* 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.

3. Transfer 5 µl fragmentation, end-repair and A-addition mix to the LoBind tube containing the cDNA prepared in step 1. Mix by pipetting up and down 10 times using a pipette set to 15 µl volume (do not vortex).

   IMPORTANT: It is critical to keep the PCR tube or plate on ice for the entire time during reaction setup.

4. Set the thermal cycler to the program described in Table 2. Use the heated lid.

Table 2. Cycler settings for fragmentation, end-repair and A-addition

Time	Temperature	Number of cycles
1 min	4°C	1
24 min	32°C	1
30 min	72°C	1
∞*	4°C	Hold

* Samples should not remain at 4°C for prolonged period. See steps 6 and 7 below.

5. Start the program. When the thermal cycler block reaches 4°C (cycler step 1), pause the program.
6. Transfer the PCR tube to the pre-chilled thermal cycler and resume the cycling program.
7. When the thermal cycler program is complete and the sample block has returned to 4°C (cycler step 4), remove the samples and place them on ice.
8. Immediately proceed to “Protocol: Adapter Ligation”, page 18.

Protocol: Adapter Ligation

Procedure

1. Prepare the adapter ligation mix in a separate tube on ice according to Table 3, and mix well by pipetting up and down 10 times using a pipette set to 25 µl.

Table 3. Adapter ligation mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
Ligation Buffer, 5x	10.0	65.0	125
DNA Ligase	5.0	32.5	62.5
Ligation Solution (PEG6000, 50%)	7.2	46.8	90.0
Total volume	**25.0**	**162.5**	**312.5**

2. For each adapter ligation reaction, add 2.8ul of adapter to the PCR tube prepared in "Protocol: Fragmentation, End-repair and A-addition", page 16.

   Note: Use only one QIAact Adapter for each ligation reaction. Open one adapter tube at a time to avoid cross-contamination. It is recommended to change gloves between each adapter addition to avoid cross-contamination.

3. Transfer 25 µl of the adapter ligation mix to the PCR tube.Mix the components thoroughly by pipetting up and down 10 times using a pipette set to 25 µl.

4. Set a thermal cycler to 20°C. Incubate the reaction (DNA and adapter ligation mix) for 15 min.

   IMPORTANT: Do not use heated lid.

5. After the reaction is complete, place the reactions on ice and proceed to "Protocol: Cleanup of Adapter-ligated DNA with QIAseq Beads ", page 20.

   If reactions are to be stored after ligation, transfer them to a –20°C freezer. Samples are stable for 3 days.

Protocol: Cleanup of Adapter-ligated DNA with QIAseq Beads

Procedure

1. Equilibrate the QIAseq Beads at room temperature (15–25°C) for at least 30 min before use.
2. Transfer the 50 µl adapter-ligation reaction from "Protocol: Adapter Ligation", page 18, to a 1.5 ml LoBind tube. Add nuclease-free water to bring the volume to 100 µl.

   Note: The final adapter ligation reaction volume may be less than 50 µl due to evaporation. It is important to measure the ligation reaction volume from "Protocol: Adapter Ligation" and then add the appropriate volume of nuclease-free water to bring the final volume to 100 µl.
3. Add 100 µl (1.0x volume) QIAseq Beads to 100 µl diluted adapter ligation reaction. Mix well by pipetting up and down 10 times using a pipette set to 100 µl. Use a new tip for every sample.
4. Incubate for 5 min at room temperature.
5. Place the tube on the magnetic rack for 10 min to separate beads from the supernatant. After the solution is clear, carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

   IMPORTANT: Do not discard the beads.
6. Add 200 µl freshly made 80% ethanol to the tube while it is on the magnetic rack. Rotate the tube 180° on the magnetic rack four times, shifting the position of the pellet, to wash the beads. Carefully remove and discard the supernatant.
7. Repeat previous step once.

8. First remove ethanol completely with a 200 µl pipette tip, then use a 10 µl tip, to remove any residual ethanol. Air dry the beads for up to 10 min while the open tube is on the rack.

   Note: Avoid over drying the beads. As drying depends on temperature and air flow, the drying time may vary. Adapt the drying time until the beads are no longer shiny.

9. To elute the DNA, add 52 µl nuclease-free water to the beads. Mix well by pipetting up and down 10 times using a pipette set to 25 µl. Use a new tip for every sample. Place tube on the rack until solution is clear.
10. Transfer 50 µl supernatant to a clean 1.5 ml tube.
11. Add 50 µl (1.0x volume) QIAseq Beads to 50 µl eluted DNA solution from the previous step. Mix well by pipetting up and down 10 times using a pipette set to 50 µl. Use a new tip for every sample.
12. Incubate for 5 min at room temperature.
13. Place the tube on the magnetic rack for 10 min to separate beads from supernatant. After the solution is clear, carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

    IMPORTANT: Do not discard the beads.

14. Add 200 µl freshly made 80% ethanol to the tube while it is on the magnetic rack. Rotate the tube 180° on the magnetic rack four times, shifting the position of the pellet, to wash the beads. Carefully remove and discard the supernatant.
15. Repeat previous step once.
16. First remove completely the ethanol with a 200 µl pipette tip, then use a 10 µl tip to remove any residual ethanol. Air dry beads for up to10 min while the tube is on the rack.

    IMPORTANT: It is critical to dry beads completely before elution. As drying depends on temperature and air flow, the drying time may vary. Adapt the drying time until the beads they are no longer shiny. Ethanol carryover can affect enrichment PCR efficiency in "Protocol: Target Enrichment PCR", page 23.

17. To elute the DNA, add 22 µl nuclease-free water to the beads. Mix well by pipetting up and down 10 times using a pipette set to 15 µl. Use a new tip for each sample. Place the tube on the magnetic rack until solution is clear.
18. Prepare two sets of 0.2 ml PCR tubes, one for the forward target enrichment PCR and one for the reverse target enrichment PCR.
19. From each 1.5 ml LoBind tube containing beads and eluate while still placed on the magnetic rack, transfer 9.4 µl supernatant to each of the two prepared PCR tubes (i.e., forward target enrichment PCR and reverse target enrichment PCR). Proceed to "Protocol: Target Enrichment PCR", page 23.

Note: After QIAseq Beads cleanup, samples can be stored at –20°C for up to 3 days.

Protocol: Target Enrichment PCR

Procedure

1. Prepare the target enrichment PCR mixes described in Table 4 and Table 5 in two 1.5 ml LoBind tubes.

Table 4. Forward target enrichment PCR mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
TEPCR Buffer, 5x	4.0	26.0	50.0
GeneRead QIAact DNA Custom Panel Forward Primers	5.0	32.5	62.5
GeneReader TE-PCR Primer	0.8	5.2	10.0
HotStarTaq DNA Polymerase	0.8	5.2	10.0
Total volume	**10.6**	**68.9**	**132.5**

Table 5. Reverse target enrichment PCR mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
TEPCR Buffer, 5x	4.0	26.0	50.0
GeneRead QIAact DNA Custom Panel Reverse Primers	5.0	32.5	62.5
GeneReader TE-PCR Primer	0.8	5.2	10.0
HotStarTaq DNA Polymerase	0.8	5.2	10.0
Total volume	**10.6**	**68.9**	**132.5**

Add 10.6 µl of either the forward and reverse target enrichment PCR mixes to the corresponding prepared tubes containing the purified adapter-ligated samples from "Protocol: Cleanup of Adapter-ligated DNA with QIAseq Beads", page 20. Mix gently by pipetting up and down 7 times with a pipette set to 10 µl, and centrifuge briefly.

2. Set the thermal cycler to the program described in Table 6. Place the tubes in the cycler and start the program.

   IMPORTANT: Ensure the heated lid on the thermal cycler (is turned on for the PCR.

Table 6. Cycler settings for target enrichment PCR

Time	Temperature	Number of cycles
13 min 2 min	95°C 98°C	1 (Initial denaturation)
15 s 10 min	98°C 68°C	8
5 min	72°C	1
5 min*	4°C	1
∞	4°C	Hold

* Samples must be held at 4°C for at least 5 min.

3. When the reaction is complete, place the tubes on ice and proceed to "Protocol: Cleanup of Target Enrichment PCR with QIAseq Beads", page 25.

   Note: Target enrichment PCR amplification reactions can be stored at –20°C for up to 3 days.

Protocol: Cleanup of Target Enrichment PCR with QIAseq Beads

Procedure

1. Let the QIAseq Beads come to room temperature (15–25°C) for at least 30 min before use.
2. Briefly centrifuge the forward and reverse PCRs from "Protocol: Target Enrichment PCR", page 23, and combine them in a 1.5 ml LoBind tube. Add nuclease-free water to bring the total combined volume of the forward and reverse PCRs to 100 µl.

   Note: The combined PCR reaction volume may be less than 40 µl due to evaporation. It is important to measure the combined volume from "Protocol: Target Enrichment PCR". Add the appropriate volume of nuclease-free water to bring the final volume to 100 µl.
3. Add 100 µl (1.0x volume) QIAseq Beads to 100 µl diluted PCR solution. Mix well by pipetting up and down 10 times using a pipette set to 100 µl. Use a new tip for every sample.
4. Incubate for 5 min at room temperature.
5. Place the tube on the magnetic rack for 10 min to separate beads from supernatant. After the solution is clear, carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

   IMPORTANT: Do not discard the beads.
6. Add 200 µl fresh 80% ethanol to the tube while it is on the magnetic rack. Rotate the tube 180° on the magnetic rack four times, shifting the position of the pellet, to wash the beads, then carefully remove and discard the supernatant.
7. Repeat previous step once.

8. Fist remove completely the ethanol with a 200 µl pipette tip, then use a 10 µl tip to remove any residual ethanol. Air dry the beads for 10 min while the tube is on the rack.

   IMPORTANT: It is critical to dry beads completely before elution. As drying depends on temperature and air flow, the drying time may vary. Adapt the drying time until the beads are no longer shiny. Avoid over drying the beads. Ethanol carryover can affect enrichment PCR efficiency in "Protocol: Universal PCR Amplification".

9. To elute the DNA, add 16 µl nuclease-free water to the beads. Mix well by pipetting up and down 10 times using a pipette set to 8 µl. Use a new tip for each sample. Place on the magnetic rack until solution is clear (5–10 min).

10. Transfer 13.4 µl supernatant to a clean 0.2 ml PCR tube. Proceed to "Protocol: Universal PCR Amplification", page 27.

    Note: After QIAseq Beads cleanup, samples can be stored at –20°C for up to 3 days.

Protocol: Universal PCR Amplification

Procedure

1. Prepare the universal PCR mix in a 1.5 ml LoBind tube according to Table 7.

Table 7. Universal PCR mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
UPCR Buffer, 5x	4.0	26.0	50.0
GeneReader Universal PCR Primer A	0.8	5.2	10.0
GeneReader Universal PCR Primer B	0.8	5.2	10.0
HotStarTaq DNA Polymerase	1.0	6.5	12.5
Total volume	**6.6**	**42.9**	**82.5**

2. Add 6.6 µl of universal PCR master mix from Table 7 to the 0.2 ml PCR tube(s) containing the purified PCR product from "Protocol: Cleanup of Target Enrichment PCR with QIAseq Beads", page 25. Mix gently by pipetting up and down 7 times with a pipette set to 10 µl, spin down briefly.
3. Set the thermal cycler to the program described in Table 8. Place the tubes in the cycler and start the program.

   IMPORTANT: Ensure the heated lid on the thermal cycler is turned on for the PCR.

Table 8. Cycler settings for universal PCR

Time	Temperature	Number of cycles
13 min 2 min	95°C 98°C	1 (Initial denaturation)
15 s 2 min	98°C 60°C	Variable†
5 min	72°C	1
5 min*	4°C	1
∞	4°C	Hold

* Samples must be held at 4°C for at least 5 min.

† Number of cycles will be specified in the panel-specific Certificate of Analysis.

4. When the reaction is complete, place the tubes on ice and proceed to "Protocol: Cleanup of Universal PCR with QIAseq Beads", page 29.

   Note: Universal PCR amplification reactions can be stored at –20°C for up to 3 days.

Protocol: Cleanup of Universal PCR with QIAseq Beads

Procedure

1. Equilibrate the QIAseq Beads at room temperature (15–25°C) for at least 30 min before use.
2. Transfer the 20 µl reaction from "Protocol: Universal PCR Amplification", page 27, to a 1.5 ml LoBind tube. Add nuclease-free water to bring the volume to 100 µl.

   Note: The reaction volume may be less than 20 µl due to evaporation. It is important to measure the reaction volume from "Protocol: Universal PCR Amplification". Add the appropriate volume of nuclease-free water to bring the final volume to 100 µl.
3. Add 100 µl (1.0x volume) QIAseq Beads to 100 µl PCR solution. Mix well by pipetting up and down 10 times using a pipette set to 100 µl. Use a new tip for every sample.
4. Incubate for 5 min at room temperature.
5. Place the tube on the magnetic rack for 10 min to separate beads from supernatant. After the solution is clear, carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

   IMPORTANT: Do not discard the beads.
6. Add 200 µl freshly made 80% ethanol to the tube while it is on the magnetic rack. Rotate the tube 180° on the magnetic rack four times, shifting the position of the pellet, to wash the beads, then carefully remove and discard the supernatant.
7. Repeat previous step once.

8. First remove completely ethanol with a 200 µl pipette, then use a 10 µl tip to remove any residual ethanol. Air dry beads for up to 10 min while the tube is on the rack.

   IMPORTANT: It is critical to dry beads completely before elution. As drying depends on temperature and air flow, the drying time may vary. Adapt the drying time until the beads are no longer shiny. Ethanol carryover may affect downstream processing and sample assessment.

9. To elute DNA library, add 30 µl nuclease-free water to the beads. Mix well by pipetting up and down 10 times using a pipette set to 20 µl. Use a new tip for every sample. Place the tube on the magnetic rack until solution is clear. Transfer 28 µl supernatant to a clean LoBind 1.5 ml tube or PCR tube.

   Note: Reactions can be stored after universal PCR amplification cleanup at –20°C for up to 6 months.

10. Assess the product size (bp) and yield (ng) of PCR-enriched DNA library using the QIAxcel Advanced instrument and the QIAxcel DNA High Resolution Kit (1200) (see "Appendix A: Analyze the Library Using QIAxcel Advanced", page 33). Typically, 3–20 ng/µl of PCR product will be obtained after purification.

    Note: Dilute the sample 1:2 in QX DNA Dilution Buffer.

    Optional: The DNA library assessment could also be performed on the Agilent 2100 Bioanalyzer with the Agilent High Sensitivity DNA Kit (see "Appendix B: Analyze the Library Using the Agilent 2100 Bioanalyzer", page 34).

    Note: It is not recommended to proceed to sequencing when the yield of the Universal PCR is less than 2 ng/µl, as this may impact performance.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. 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/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
Library preparation and target enrichment	
a) No or low PCR product yield (<2ng/µl) after Universal PCR	Concentration of DNA to be used for library preparation and target enrichment should be determined by fluorometric quantitation. Ensure that all reaction components are thoroughly mixed as described in these instructions before use Ensure that all reaction components are correctly added at each stage and thoroughly mixed as described in these instructions. Increase DNA input used for library preparation. If 40 ng was used initially, increase input to 100 ng. If 100 ng was used initially, increase input to 250 ng.
b) No or low PCR product yield (<2ng/µl) after Universal PCR from FFPE sample-derived DNA	When using DNA derived from FFPE samples, ensure that the DNA is of suitable quality. It is recommended to first assess the extracted DNA using the QIAseq DNA QuantiMIZE Array or Assay Kit (QC score ≥0.04, proceed, as the genomic DNA is of good quality; QC Score >0.04, proceed with caution, as the DNA is highly fragmented or damaged).

Symbols

Symbol	Symbol definition
	Contains reagents sufficient for <N> tests
	Catalog number
	Manufacturer

Appendix A: Analyze the Library Using QIAxcel Advanced

After the library is constructed and purified, analyze using the QIAxcel Advanced, in combination with the QIAxcel DNA High Resolution Kit (cat. no. 929002), to check the fragment size and concentration. The majority of the library fragments to be quantified are between 200–800 bp in size (Figure 2). Amounts of DNA under the peak can be used to quantify the libraries.

The results for an example panel are shown. Peak size and limits may vary depending on the custom panel.

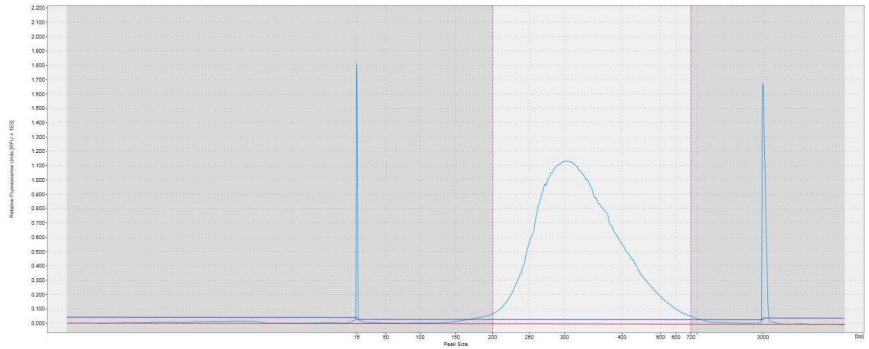

Figure 2. Sample QIAxcel Advanced image of a GeneRead QIAact Lung DNA library (cat. no. 181930). The majority of the library fragments are between 200–800 bp in size.

Appendix B: Analyze the Library Using the Agilent 2100 Bioanalyzer

After the library is constructed and purified, a Bioanalyzer can be used to check the fragment size and concentration with the High Sensitivity DNA Kit. The majority of the library fragments are between 200–800 bp in size (Figure 3). Amounts of DNA under the appropriate peaks can be used to quantify libraries.

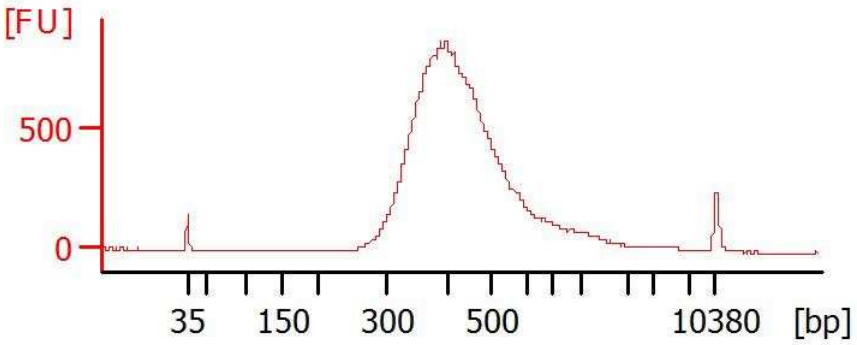

Figure 3. Sample Bioanalyzer image of a GeneRead QIAact Lung DNA library (cat. no. 181930). The majority of the library fragments are between 200–800 bp in size.

Ordering Information

Product	Contents	Cat. no.
GeneRead QIAact DNA Custom Panel (24)	Two primer mixes, each containing customer specific primers designed to enrich selected regions. Library preparation and target enrichment reagents to process 500 samples. Includes QCI-Analyze custom workflow	Inquire
Related Products		
GeneRead QIAact RNA Custom Panel (24)	Two primer mixes, each containing customer specific primers designed to enrich selected RNA fusions. Library preparation and target enrichment reagents to process 500 samples. Includes QCI-Analyze custom workflow	Inquire
QIAseq DNA QuantiMIZE Array Kit	2 arrays in Formats A, C, D, or F; or 2 discs in Format R; or 1 array in Format E or G; QuantiMIZE Control gDNA (50 µl; GeneRead qPCR SYBR Green Mastermix (2 x 1.35 ml)	333404
QIAseq DNA QuantiMIZE Assay Kit	Assay 100 and Assay 200 for 400 x 25 µl reactions (400 µl) each; QuantiMIZE Control gDNA (50 µl); RNase- and DNase-free water (1 ml x 4); GeneRead qPCR SYBR Green Mastermix (1.35 ml x 4)	333414
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
GeneRead DNA FFPE Kit (50)	For 50 preps: QIAamp MinElute® Columns, Collection Tubes, Deparaffinization Solution, Uracil-N-Glycosylase, RNase-Free Water, RNase A, and Buffers	180134

QIAamp Circulating Nucleic Acid Kit (50)	For 50 preps: QIAamp Mini Columns, Tube Extenders (20 ml), QIAGEN Proteinase K, Carrier RNA, Buffers, VacConnectors, and Collection Tubes (1.5 ml and 2 ml)	55114

Limited License Agreement for GeneRead Custom Panel

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HB-2393-002 01/2018