January 2018

GeneRead™ QIAact RNA Custom Panel Handbook

REF Catalog number is defined for each custom panel

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

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

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

Sample to Insight

QIAGEN

Contents

Kit Contents

GeneRead QIAact RNA Custom Panel	
Number of reactions	**24**
GeneRead QIAact RNA Library Preparation and Target Enrichment Reagents (Box1)*	
QIAact RNA Target Enrichment Panel and QIAact Adapters (Box 2)*	

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

GeneRead QIAact RNA Library Preparation and Target Enrichment Reagents *	
Number of reactions	**24**
RP Primer	26 µl
EZ Reverse Transcriptase	26 µl
BC3 Buffer, 5x	52 µl
RNase Inhibitor	26 µl
RH RNase	26 µl
dNTP	26 µl
XC Buffer, 10x	52 µl
BX Enzyme	26 µl
ERA Enzyme	260 µl
ERA Buffer, 10x	130 µl
DNA Ligase	260 µl
Ligation Buffer, 5x	520 µl
UPCR Buffer, 5x	312 µl
Nuclease-free Water	2 x 2 ml
HotStarTaq® DNA Polymerase	2 x 50 µl
QIAseq Beads (provided in a bottle 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 RNA Target Enrichment Panel and QIAact Adapters *	
Number of reactions	**24**
GeneRead QIAact RNA Custom Panel Forward Primers	130 µl
GeneRead QIAact RNA 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 2–8°C.

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

Intended Use

The GeneRead QIAact RNA Custom Panel and GeneRead QIAact RNA 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 RNA Custom Panel is tested against the specification as indicated in the panel-specific certificate.

Introduction

NGS is a useful tool to detect genetic alterations in RNA, including translocations, fusions and exon-skipping events. Targeted-enrichment technology enables NGS-platform users to sequence specific regions of interest from RNA, effectively increasing sequencing depth and throughput with lower cost.

The GeneRead QIAact RNA Custom Panel integrates unique molecular index (UMI) technology into a primer-based target-enrichment process, enabling sensitive fusion detection by NGS on the GeneReader system.

The GeneRead QIAact RNA Custom Panel has been designed according to customer specifications to achieve highly efficient enrichment.

Principle and procedure

The GeneRead QIAact RNA Custom Panel relies on specific target enrichment in combination with UMIs for uniform coverage and sensitive fusion detection.

Unique molecular index

Unique molecular indexing is used to attach a unique sequence index to each original molecule prior to any amplification. This attachment is accomplished by the ligation of a QIAact adapter containing a UMI of 8 random bases to the double-stranded cDNA.

The double-stranded cDNA molecules are then amplified by PCR for target enrichment and library amplification. Due to intrinsic noise and sequence-dependent bias, double-stranded cDNA 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. For fusion detection, UMIs allow the retracing of tagged transcript fragments from the original purified RNA, allowing accurate quantification of the fusion event.

Process

The GeneRead QIAact RNA Custom Panel is provided as two primer mix tubes. The GeneRead QIAact RNA Custom Panel is designed to enrich selected fusion targets from total RNA.

Total RNA is first reverse transcribed to first-strand cDNA. A separate, second-strand synthesis is used to generate double stranded cDNA, which is then end-repaired and A-tailed in a single-tube protocol. The prepared double-stranded cDNAs are then ligated at their 5′ ends to a sequencing-platform-specific adapter containing a UMI and a sample-specific bar code.

Ligated double-stranded cDNA molecules are subjected to limited cycles of target-enrichment PCR using fusion-specific primers that target defined sequences for which the breakpoint and fusion partners are known (Figure 1). This reaction ensures that intended targets 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 RNA Custom Panel workflow, which automatically performs all steps necessary to generate a fusion report from the raw NGS data. All detected fusions can be further interpreted by QIAGEN Clinical Insight (QCI™) analysis.

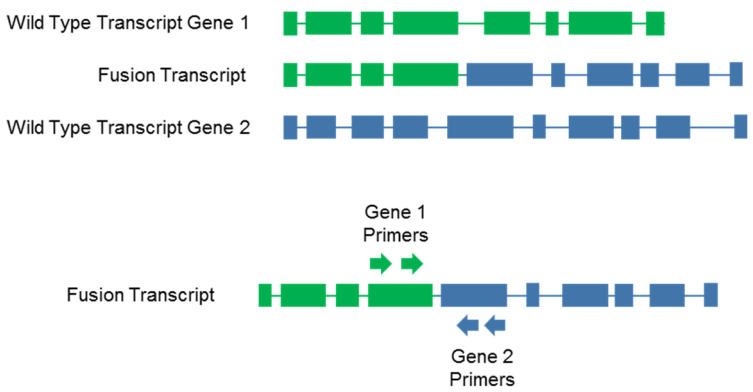

Figure 1. Principle of fusion detection. Fusion-specific primers are designed to target-defined sequences on both sides of the translocation breakpoint in the two fusion partners.

Recommendation for multiplexing and clonal amplification input

More than one sample can be sequenced in one flow cell due to the addition of a sample-specific bar code that is added during library preparation (see "Protocol: Adapter Ligation", page 16). 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).

RNA libraries can be multiplexed with DNA libraries, according to the corresponding panel configurations.

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 RNA Custom Panel, the following supplies are required:

For RNA isolation

- See "Recommended RNA preparation methods", page 10.

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
- Nuclease-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 RNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q32852)
- Qubit assay tubes (e.g., Thermo Fisher Scientific, cat. no. Q32856)
- DynaMag™-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)

Optional

- QIAxpert® System (for information, visit **www.qiagen.com**)
- Agilent® 2200 TapeStation®
- Agilent High Sensitivity D1000 Screentape (Agilent cat. no. 5067-5584) or equivalent
- Agilent High Sensitivity D1000 Reagents (Agilent cat. no. 5067-5585) or equivalent

Important Notes

RNA preparation

Maximizing RNA quality is essential for obtaining good sequencing results

The most important prerequisite for sequence analysis is maximizing the RNA quality of every experimental sample. Therefore, sample handling and RNA isolation procedures are critical to the success of the experiment.

Residual traces of proteins, salts or other contaminants may degrade the RNA or decrease efficiency (if not block completely) of the enzyme activities necessary for optimal target enrichment.

Recommended RNA preparation methods

The QIAGEN RNeasy® FFPE Kit (cat. no. 73504) is highly recommended for the preparation of total RNA samples from FFPE tissue. **Do not** omit the recommended DNase treatment step to remove DNA.

For best results, all RNA samples should be resuspended in RNase-free water. **Do not use DEPC-treated water**.

RNA quantification

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

RNA purity determined by UV spectrophotometry

The purity of RNA 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.

Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in 10 mM Tris•HCl, pH 7.5.

* 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.

RNA concentration

The concentration of RNA should be determined by fluorometric quantitation using equipment such as the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, cat. no. Q33216). RNA input of 100 ng is recommended for the GeneRead QIAact RNA Custom Panel.

Protocol: First-Strand cDNA Synthesis

Procedure

1. Dilute RNA to 20 ng/µl with nuclease-free water in a LoBind tube. For each sample, 100 ng (5 µl, 20 ng/µl) is required for first-strand cDNA synthesis (i.e., a total of 100 ng per sample).
2. Preheat a thermal cycler to 65°C. Set the heated lid to 103°C.
3. Place 0.2 ml PCR tubes on ice.
4. Add 100 ng total RNA to each tube, and then add 1 µl of RP Primer to each tube, according to Table 1.

Table 1. Primer priming reaction components

	Volume (µl)
Component	**1 sample**
RNA sample (20 ng/µl)	5.0
RP Primer	1.0
Total volume	**6.0**

5. Mix by pipetting up and down 7 times with a pipette set to 4 µl, and then centrifuge briefly.
6. Transfer the tube(s) from ice to the cycler and incubate at 65°C for 5 min, according to Table 2.

Table 2. Cycler setting for priming

Step	Incubation temperature	Incubation time
1	65°C	5 min

7. Remove the tube(s) from the cycler and place on ice for at least 2 min.

Briefly centrifuge before proceeding to “Protocol: Reverse Transcription”, page 13.

Protocol: Reverse Transcription

Procedure

1. Prepare a reverse transcription mix according to Table 3.

Table 3. Reverse-transcription mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
BC3 Buffer, 5x	2.0	13.0	25.0
RNase Inhibitor	1.0	6.5	12.5
EZ Reverse Transcriptase	1.0	6.5	12.5
Total volume	**4.0**	**26.0**	**50.0**

2. Transfer 4 µl of the reverse-transcription mix to the 0.2 ml PCR tube(s) containing the first-strand cDNA prepared in "Protocol: First-Strand cDNA Synthesis", page 12. Mix by pipetting up and down 7 times with a pipette set to 4 µl, and then centrifuge briefly.
3. Place the 0.2 ml PCR tube(s) into a cycler with a heated lid (e.g., set to 103°C) and incubate according to Table 4.

Table 4. Cycler settings for reverse transcription

Step	Incubation temperature	Incubation time
1	25°C	10 min
2	42°C	30 min
3	70°C	15 min
4	4°C	Hold

4. Remove the 0.2 ml PCR tube(s) from the thermal cycler, briefly centrifuge and place on ice.
5. Proceed to "Protocol: Second-Strand Synthesis", page 14. 0.

   Note: Reverse-transcription reactions can be stored at –20°C overnight.

Protocol: Second-Strand Synthesis

Procedure

1. Prepare the second-strand synthesis mix described in Table 5.

Table 5. Second-strand synthesis mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
Nuclease-free water	5.0	32.5	62.5
XC Buffer	2.0	13.0	25.0
RH RNase	1.0	6.5	12.5
dNTP	1.0	6.5	12.5
BX Enzyme	1.0	6.5	12.5
Total volume	**10.0**	**65.0**	**125.0**

2. Transfer 10 µl of the mix to each of the 0.2ml PCR tubes containing the reverse-transcription reaction from “Protocol: Reverse Transcription”, page 13. Mix by pipetting up and down 7 times with a pipette set to 10 µl, and then centrifuge briefly.
3. Place the 0.2 ml PCR tube(s) into a cycler with a heated lid (e.g., set to 103°C) and incubate according to Table 6.

Table 6. Cycler settings for second-strand synthesis

Step	Incubation temperature	Incubation time
1	37°C	7 min
2	65°C	10 min
3	80°C	10 min
4	4°C	Hold

4. Remove the 0.2 ml PCR tube(s) from the thermal cycler, briefly centrifuge and place on ice. Proceed to “Protocol: End Repair/dA Tailing”, page 15. 0.

Protocol: End Repair/dA Tailing

Procedure

1. Set the thermal cycler to the program described in Table 7. Set the heated lid to 70°C if possible.

Table 7. Cycler settings for end repair/dA tailing

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	20°C	30 min
3	65°C	30 min
4	4°C	Hold

2. When the cycler block reaches 4°C (Step 1), pause the program.

   IMPORTANT: It is important to follow the procedure described below to achieve optimal results. The final total reaction volume is 50 µl.

3. Prepare an end repair/dA tailing mix in a new LoBind tube on ice according to Table 8.

Table 8. End repair/dA tailing mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
ERA Buffer, 10x	5.0	32.5	62.5
Nuclease-free water	15.0	97.5	187.5
Total volume	**20.0**	**130.0**	**250.0**

4. Add 20 µl end repair/dA tailing mix to each tube containing the second-strand synthesis reaction from “Protocol: Second-Strand Synthesis”, page 14. Gently mix by pipetting up and down 7 times.

5. Add 10 µl ERA Enzyme to each reaction. Gently mix by pipetting up and down 7 times with a pipette set to 25 µl, and then centrifuge briefly.

   Note: It is recommended to keep the PCR tubes on ice during the entire reaction setup.

6. Immediately transfer the tubes to the pre-chilled thermal cycler (4°C). Resume the cycling program.

   Note: If using a non-temperature-controlled lid, run with cycler lid open for r step 2 of the program . When the cycler reaches step 3, close the lid to avoid evaporation.

7. When the program is complete and the cycler has returned to 4°C, remove the 0.2 ml PCR tube(s) from block, spin to remove any condensation and place on ice.
8. Proceed immediately to "Protocol: Adapter Ligation", page 16.

Protocol: Adapter Ligation

Procedure

1. Prepare an adapter ligation master mix in a separate tube on ice according to Table 9, and mix well by pipetting up and down.

Table 9. Adapter ligation master mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
Ligation Buffer, 5x	20.0	130.0	250.0
DNA Ligase	10.0	65.0	125.0
Nuclease-free water	15.0	97.5	187.5
Total volume	**45.0**	**292.5**	**562.5**

2. For each adapter ligation reaction, transfer 5 µl of a QIAact Adapter to the 0.2 ml PCR tube containing the end repair/dA tailing reaction from "Protocol: End Repair/dA Tailing", page 15.

   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. .
4. To each tube containing the QIAact Adapter and end repair/dA tailing reaction mix, add 45 µl of adapter ligation master mix. Mix gently by pipetting up and down 7 times with a pipette set to 25 µl, centrifuge briefly and place on ice.

   Note: The final ligation reaction volume may be less than 100 µl due to evaporation. It is important to measure the ligation reaction volume. If the volume is less than 100 µl, bring the final volume up 100 µl to by adding the appropriate volume of nuclease-free water.
5. Set up the cycler using the parameters in Table 10. Start the program and pause it when the temperature is 4°C. Place the tubes in the cycler and restart the program after the tubes are in the cycler. Incubate the adapter ligation reaction at 20°C for exactly 15 min.

   IMPORTANT: Do not use a heated lid.

Table 10. Cycler settings for adapter ligation

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	20°C	15 min
3	4°C	Hold

6. Proceed immediately to “Protocol: Cleanup of Adapter-Ligated DNA with QIAseq Beads”, page 18.

Protocol: Cleanup of Adapter-Ligated DNA with QIAseq Beads

1. Equilibrate the QIAseq Beads at room temperature (15–25°C) for at least 30 min before use.
2. Transfer the 100 µl adapter-ligation reaction from "Protocol: Adapter Ligation", page 16, to a 1.5 ml LoBind tube.

   Note: The final adapter ligation reaction volume may be less than 100 µl due to evaporation. It is important to measure the ligation reaction volume from "Protocol: Adapter Ligation". If the volume is less than 100 µl, bring the final volume up to 100 µl by adding the appropriate volume of nuclease-free water.
3. Add 90 µl (0.9x volume) QIAseq Beads to the 100 µl adapter ligation reaction. Mix well by pipetting up and down 10 times using a pipette set to 100 µl. Use a new tip for each 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. Completely remove residual supernatant.

   It is recommended to use a 10 µl tip to aspirate the residual supernatant remaining after the first aspiration.
7. Add 260 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. Incubate the tube on magnetic rack for 1 min. Carefully remove and discard the supernatant.
8. Repeat the previous step twice.
9. Completely remove ethanol with a 200 µl pipette tip first, 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.

   IMPORTANT: 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.
10. 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 each sample. Place the tube on the magnetic rack until the solution is clear.
11. Transfer 50 µl supernatant to a clean 1.5 ml tube.

12. Add 65 µl (1.3x volume) QIAseq Beads to the 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 each sample.
13. Incubate for 5 min at room temperature.
14. 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.
15. 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. Incubate with the tube 1 min on the magnetic rack. Carefully remove and discard the supernatant.
16. Repeat the previous step once.
17. Completely remove the ethanol with a 200 µl pipette tip first, 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 are no longer shiny. Ethanol carryover can reduce the efficiency of the PCR in "Protocol: Target Enrichment PCR", page 20.
18. 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 the solution is clear (10 min).
19. Prepare two sets of 0.2 ml PCR tubes, one for the forward target enrichment PCR and one for the reverse target enrichment PCR.
20. 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 20.

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 mixes described in Table 11 and Table 12 in two 1.5 ml LoBind tubes.

Table 11. Forward target enrichment PCR mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
UPCR Buffer, 5x	4.0	26.0	50.0
GeneRead QIAact RNA 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 12. Reverse target enrichment PCR mix

	Volume (µl)		
Component	**1 sample**	**6 samples**	**12 samples**
UPCR Buffer, 5x	4.0	26.0	50.0
GeneRead QIAact RNA 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**

2. Add 10.6ul of 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 18. Mix gently by pipetting up and down 7 times with a pipette set to 10 µl, and centrifuge briefly.
3. Set the thermal cycler to the cycling conditions provided in Table 13. 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 13. Cycler settings for target enrichment PCR

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

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

4. When the program is complete, place the reactions on ice and proceed to "Protocol: Cleanup of Target Enrichment PCR with QIAseq Beads", page 22.

   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 for at least 30 minutes before use.
2. Briefly centrifuge the forward and reverse PCRs from "Protocol: Target Enrichment PCR", page 20, and combine them in a 1.5 ml LoBind tube for each sample. Add nuclease-free water to bring the total combined volume of the forward and reverse PCRs to 100 µl.

   Note: The combined PCR volume may be less than 40 µl due to evaporation. It is important to measure the combined volume from "Protocol: Target Enrichment PCR", page 20. Add the appropriate volume of nuclease-free water to bring the final volume up to 100 µl.
3. Add 130 µl (1.3x volume) QIAseq Beads to the 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 each 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 the 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. Incubate the tube for 1 min on the magnetic rack. Carefully remove and discard the supernatant.
7. Repeat previous step once.
8. Completely remove ethanol with a 200 µl pipette tip first, then use a 10 µl tip to remove any residual ethanol. Air dry the 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 are no longer shiny. Avoid over drying the beads. Ethanol carryover can affect PCR efficiency in "Protocol: Universal PCR Amplification", page 24.
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 the tube on the magnetic rack until the 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 24.

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 14.

Table 14. 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 the universal PCR mix from Table 14 to the 0.2 ml PCR tube(s) containing the purified PCR product from "Protocol: Cleanup of Target Enrichment PCR with QIAseq Beads", page 22. Mix gently by pipetting up and down 7 times with a pipette set to 10 µl, and centrifuge briefly.
3. Set the thermal cycler to the cycling conditions provided in Table 15. 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 15. Cycler settings for universal PCR

Time	Temperature	Number of cycles
15 min	95°C	1 (Initial denaturation)
15 s 2 min	95°C 60°C	Specified in the Certificate of Analysis for each custom panel
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.

4. When the reaction is complete, place the reactions on ice and proceed to "Protocol: Cleanup of Universal PCR with QIAseq Beads", page 25.

   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 24, to a 1.5 ml LoBind tube. Add nuclease-free water to bring the volume up to 50 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", page 24. Add the appropriate volume of nuclease-free water to bring the final volume up to 50 µl.
3. Add 65 µl (1.3x volume) QIAseq Beads to the 50 µl PCR solution. Mix well by pipetting up and down 10 times using a pipette set to 55 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. Incubate the tube for 1 min on magnetic rack, then carefully remove and discard the supernatant.
7. Repeat previous step once.
8. Completely remove ethanol with a 200 µl pipette tip first, 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 are no longer shiny. Avoid over drying the beads. Ethanol carryover may affect downstream processing and sample assessment.
9. To elute the 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 15 µl. Use a new tip for each sample. Place the tube on the magnetic rack until the 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 the 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 29). Typically, 3–20 ng/µl PCR product is obtained after purification.

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

    Optional: The DNA library assessment can also be performed on the Agilent 2100 Bioanalyzer® using the Agilent High Sensitivity DNA Kit.

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

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see 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 that you may have regarding the information and/or protocols in this handbook or for any sample and assay technologies. To contact QIAGEN Technical Services, visit **www.qiagen.com**.

	Comments and suggestions
Library preparation and target enrichment	
No or low PCR product yield (<2ng/µl) after Universal PCR	Ensure that 100 ng of RNA is used as input for "Protocol: First-Strand cDNA Synthesis", page 12. Concentration of RNA should be determined by fluorometric quantitation. Ensure that all reaction components are thoroughly mixed as described before use. Ensure that all reaction components are correctly added at each stage and thoroughly mixed as described. Increase RNA input used for library preparation. If 100 ng was used initially, increase input to 200 ng RNA.

Symbols

Symbol	Symbol definition
	Contains reagents sufficient for <N> tests
	Catalog number
	Material number (i.e., component labeling)
	Manufacturer

Appendix A: Analyze the Library Using QIAxcel Advanced

After the library is constructed and purified, analyze it using the QIAxcel Advanced, in combination with the QIAxcel DNA High Resolution Kit (cat. no. 929002), to check the fragment size and concentration. The library fragments to be quantified are between 200–400 bp in size (Figure 2). Amounts of DNA under the peak can be used to quantify libraries. Additional peaks are observed at 150 bp and 600 bp, however these do not impact quantification and sequencing results.

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

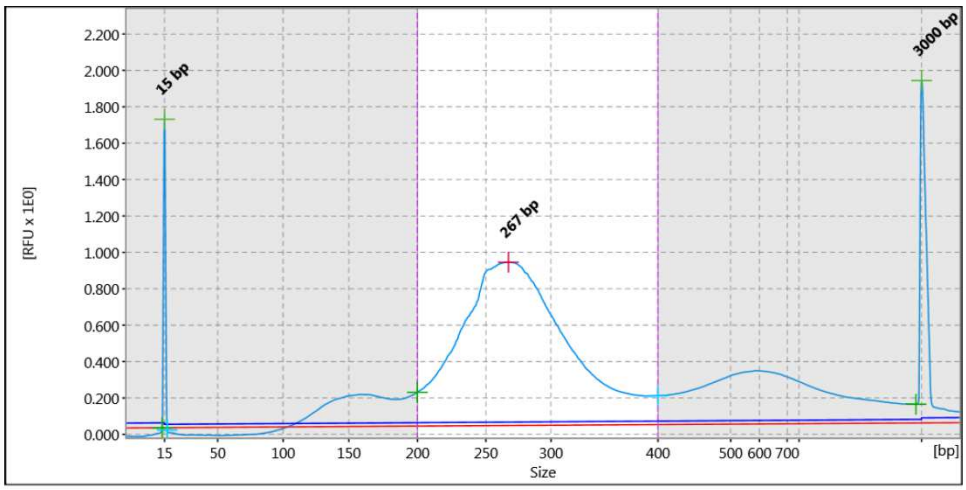

Figure 2. Sample QIAxcel Advanced image of a GeneRead QIAact Lung RNA library (cat. no. 181935)

Ordering Information

Product	Contents	Cat. no.
GeneRead QIAact RNA Custom Panel (24)	Two primer mixes, each containing customer-specific primers designed to enrich selected genomic regions. Library preparation and target enrichment reagents to process 500 samples. Includes QCI-Analyze custom workflow	Inquire
Related products		
GeneRead QIAact DNA Custom Panel	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
RNeasy FFPE Kit (50)	50 RNeasy MinElute® Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	73504
QIAxcel Advanced System	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel Software, installation, training, and 1-year warranty on parts and labor	9002123
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929002
QX DNA Size Marker 50–800 bp (50 µl) v2.0	DNA size marker with fragments of 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, and 800 bp; concentration 100 ng/µl	929561
QX Alignment Marker 15 bp/3 kb (1.5 ml)	Alignment marker with 15 bp and 3 kb fragments	929522
QX Nitrogen Cylinder (6)	6 QIAxcel Nitrogen Cylinders	929705

QIAxpert System	QIAxpert instrument with installation and startup training and 1 year warranty coverage including parts, labor, and shipping; repair by sending to a regional repair center	9002368

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

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Limited License Agreement for GeneRead QIAact RNA Custom Panel

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HB-2477-002 01/2018