July 2017

GeneRead™ QIAact Lung RNA Panel Handbook

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.

REF 181935

QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden

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Sample to Insight

QIAGEN

Contents

Kit Contents 4

Storage 5

Intended Use 6

Safety Information 6

Quality Control 6

Introduction 7

Principle and procedure 7

Equipment and Reagents to Be Supplied by User 11

Important Notes 12

RNA preparation 12

RNA quantification 13

Protocol: First strand cDNA synthesis 14

Protocol: Reverse transcription 15

Protocol: Second strand synthesis 16

Protocol: End repair / dA tailing 18

Protocol: Adaptor ligation 20

Protocol: Cleanup of Adapter-ligated DNA with QIAseq Beads 22

Protocol: Target Enrichment PCR 25

Protocol: Cleanup of Target Enrichment PCR with QIAseq Beads 27

Protocol: Universal PCR Amplification 29

Protocol: Cleanup of Universal PCR with QIAseq Beads 31

Troubleshooting Guide 33

Symbols 34
Appendix A: Analyze the Library Using QIAxcel Advanced 35
Ordering Information 36

Kit Contents

GeneRead QIAact Lung RNA Panel	
Catalog no.	**181935**
Number of reactions	**24**
GeneRead QIAact RNA Library Preparation and Target Enrichment Reagents (Kit Box1)*	
QIAact RNA Target Enrichment Panel and QIAact Adapters (Kit Box 2)*	

* Kit boxes 1 and 2 are components of the GeneRead QIAact Lung RNA 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	2 x 130 µl
Ligation buffer, 5x	2 x 260 µl
UPCR buffer, 5x	2 x 220 µl
Nuclease-free water	2 x 2ml
HotStarTaq® DNA Polymerase	2 x 50 µl
One bottle containing QIAseq Beads	34 ml

* Not for individual sale; to order reagents, see cat. no. 181935.

QIAact RNA Target Enrichment Panel and QIAact Adapters *	
Number of reactions	**24**
GeneRead™ QIAact Lung RNA Panel Forward Primers	130 µl
GeneRead QIAact Lung RNA 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, see cat. no. 181935.

Storage

The GeneRead QIAact Library Preparation and Target Enrichment Reagents (except QIAseq Beads) are shipped on dry ice and should be stored at –15°C to –30°C upon arrival. QIAseq Beads are shipped on cold packs and should be stored at 4°C. When stored properly, all reagents are stable for up to 3 months after delivery.

GeneRead QIAact Lung RNA Panel is shipped on dry ice and should be stored at –15°C to –30°C upon arrival. When stored properly, components are stable for up to 3 months after delivery.

Intended Use

The GeneRead QIAact Lung RNA 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.

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

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the GeneRead QIAact Lung RNA Panel is tested against predetermined specifications, to ensure consistent product quality.

Introduction

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

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

The GeneRead QIAact Lung RNA 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 Lung RNA Panel relies on fusion-specific targeting in combination with UMIs for uniform coverage and sensitive fusion detection.

Unique molecular index

The concept of unique molecular indexing is that prior to any amplification, each original molecule is attached to a unique sequence index. This attachment is accomplished by the ligation of double strand complimentary (ds)-cDNA with a QIAact adapter containing a UMI with 8 random bases.

The (ds)-cDNA molecules are then amplified by PCR for target enrichment and library amplification. Due to intrinsic noise and sequence-dependent bias, (ds)-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, while 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.

Procedure

The GeneRead QIAact Lung RNA Panel is provided as two primer mix tubes. The GeneRead QIAact Lung RNA Panel is designed to enrich selected fusion targets (Table 1) starting from 100 ng of total RNA.

Table 1. Fusion Targets

AGTRAP-BRAF	DCTN1-ALK	FGFR1-ZNF703	HOOK3-RET	NCOA4-RET	RNF130-BRAF	TPM3-ALK
AKAP9-BRAF	EML4-ALK	FGFR3-TACC3	KIF5B-ALK	NPM1-ALK	SDC4-ROS1	TPM3-NTRK1
ATIC-ALK	ERC1-RET	FN1-ALK	KIF5B-RET	NTRK1-TPM3	SEC31A-ALK	TPM3-ROS1
CCDC6-RET	ERC1-ROS1	GATM-BRAF	KLC1-ALK	PCM1-RET	SLC34A2-ROS1	TPM4-ALK
CD74-NRG1	ESRP1-RAF1	GNAI1-BRAF	LMNA-NTRK1	PPFIBP1-ALK	SLC45A3-BRAF	TRIM24-RET
CD74-NTRK1	EZR-ROS1	GOLGA5-RET	LRIG3-ROS1	PPFIBP1-ROS1	SQSTM1-ALK	TRIM27-RET
CD74-ROS1	FAM131B-BRAF	GOPC-ROS1	LSM14A-BRAF	PRKAR1A-RET	STRN-ALK	TRIM33-RET
CEP89-BRAF	FCHSD1-BRAF	HACL1-RAF1	MET exon 14 skipping	PWWP2A-ROS1	TFG-ALK	UBE2L3-KRAS
CLCN6-BRAF	FGFR1-PLAG1	HERPUD1-BRAF	MKRN1-BRAF	RAF1-DAZL	TFG-NTRK1	VCL-ALK
CLTC-ALK	FGFR1-TACC1	HIP1-ALK	MYO5A-ROS1	RANBP2-ALK	TP53-NTRK1	ZSCAN30-BRAF

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

Ligated (ds)-cDNA molecules are subject to limited cycles of target enrichment PCR using fusion-specific primers that target defined sequences where 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 Lung RNA Panel workflow, which will automatically perform 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.

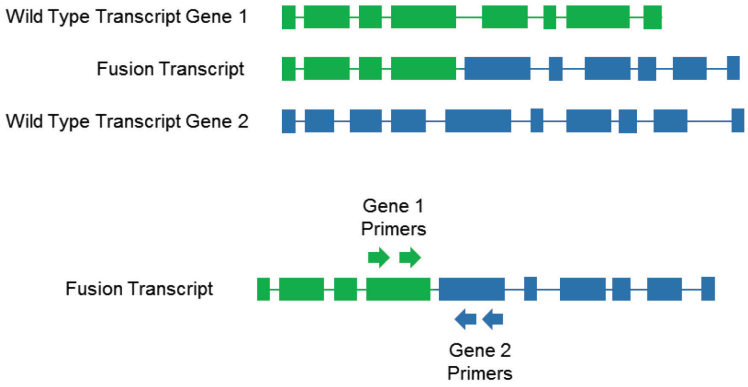

Figure 1. Principle of fusion detection. Fusion-specific primers are designed to target defined sequences on both sides of the translocation breakpoint in the two fusion partners.

Recommendation for multiplexing and clonal amplification input

More than one sample can be sequenced in one flow cell and this is made possible by the addition of a sample-specific bar code that is added during library preparation (see "Protocol: Adapter Ligation", page 20). 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). Based on the total number of amplicons that are produced by the GeneRead QIAact Lung RNA Panel, we recommend a maximum multiplex of 12 samples for FFPE samples.

When using the RNA libraries as part of the GeneRead QIAact Lung All-in-One Assay, we recommend a maximum multiplex of 6 samples for FFPE samples (see GeneRead QIAact Lung All-in-One Assay Handbook for more information).

After target enrichment and library preparation, use 500 pg 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 Lung RNA Panel, the following supplies are required:

For RNA isolation:

- See "Recommended RNA preparation methods", page 12

For library construction and targeted enrichment:

- High-quality, nuclease-free water. **Do not use DEPC-treated water**
- 80% ethanol, made fresh
- Microcentrifuge
- 1.5 ml LoBind tubes (Eppendorf®)
- 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 (QIAGEN cat. no. 929002)
- QX DNA Size Marker 50–800 bp (50 µl) (QIAGEN cat. no. 929561)
- QX Alignment Marker 15 bp/3 kb (1.5 ml) (QIAGEN cat. no. 929522)

- QX Nitrogen Cylinder (x 6) (QIAGEN 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 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 either degrade the RNA or decrease the efficiency of (if not block completely) 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 also 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·Cl* 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.

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 Lung RNA Panel.

* 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: 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. Pre-heat PCR cycler to 65°C with a heated lid (at 103°C).
3. Place 0.2ml PCR tubes on ice.
4. Add 100 ng total RNA to each tube then add 1 µl of RP primer to each tube.

Table 2. Primer priming

Component	1x Volume (µl)
RNA sample (20 ng/µl)	5
RP Primer	1
Total volume	**6**

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

Table 3. Cycler setting for priming

Step	Incubation Temperature	Incubation Time
1	65°C	5 min
2	Ice	≥ 2 min

7. Remove the tube(s) from the cycler and place on ice for at least 2 min.
8. Briefly centrifuge before next step.

Protocol: Reverse transcription

Procedure

1. Add 4 µl of the following to the same 0.2ml PCR tube(s) from previous reaction (Table 3).

Table 4. Reverse transcription mix

Component	1x Volume (µl)	6x Volume (µl)	12x Volume (µl)
Random primed RNA from previous section	6	-	-
BC3 buffer, 5x	2	13	25
RNase inhibitor	1	6.5	12.5
EZ Reverse Transcriptase	1	6.5	12.5
Total volume	**10**	**26**	**50**

2. Mix by pipetting up and down 7 times with a pipette set to 4 µl and then spin down briefly.
3. Place the 0.2ml PCR tube(s) into a cycler with a heated lid (e.g. 103°C) and incubate as follows:

Table 5. 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 0.2ml PCR tube(s) from the thermal cycler, briefly spin down and place on ice.

Note: If reactions are to be stored after reverse transcription, transfer them to a –20°C freezer. Samples are stable overnight.

Protocol: Second strand synthesis

Procedure

1. Add 10 µl of the following to the same 0.2ml PCR tube(s) of the previous reaction (Table 5).

Table 6. Second strand synthesis mix

Component	1x Volume (µl)	6x Volume (µl)	12x Volume (µl)
cDNA from previous section	10	-	-
Nuclease-free water	5	32.5	62.5
XC buffer	2	13	25
RH Rnase	1	6.5	12.5
dNTP	1	6.5	12.5
BX enzyme	1	6.5	12.5
Total volume	**20**	**65**	**125**

2. Mix by pipetting up and down 7 times with a pipette set to 10 µl and then spin down briefly.
3. Place the 0.2ml PCR tube(s) into a cycler with a heated lid (e.g. 103°C) and incubate as follows:

Table 7. 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 0.2ml PCR tube(s) from the thermal cycler, briefly spin down and place on ice.

Protocol: End repair / dA tailing

Procedure

1. Enter the following program into a thermal cycler (Table 8).

   Note: Be certain to use the instrument's heated lid with the lid temperature setting being ~70°C if possible.

Table 8. Cycler settings for reverse transcription

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.
3. It is important to follow the procedure described below in order to achieve optimal results. The final total reaction volume is 50 µl.
4. Prepare a reaction mix in a new LoBind tube on ice by combining ERA Buffer and nuclease-free water as indicated in Table 9.

Table 9. End repair / dA tailing mix

Component	1x Volume (µl)	6x Volume (µl)	12x Volume (µl)
Second strand product from previous section	20	-	-
ERA buffer, 10x	5	32.5	62.5
Nuclease-free water	15	97.5	187.5
Total volume	**40**	**130**	**250**

5. Add 10 µl ERA enzyme to each separate 0.2ml PCR tube(s) and gently mix by pipetting up and down 7 times with a pipette set to 25 µl and then spin down briefly.

Note: It is recommended to keep the PCR tube on ice for the whole time during reaction setup.

6. Immediately transfer 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 step 2. When the cycler reaches step 3, close the lid to avoid evaporation. Spin 0.2ml PCR tube(s) after step 4 to remove any condensation.

7. When the program is complete and sample block has returned to 4°C, remove 0.2ml PCR tube(s) from block and place on ice.

8. Immediately proceed to the next step.

Protocol: Adaptor ligation

Procedure

1. Prepare the following ligation reaction master mix in a separate tube on ice and mix well by pipetting (Table 10).

Table 10. Ligation mix

Component	1x Volume (µl)	6x Volume (µl)	12x Volume (µl)
5x ligation buffer	20	130	250
DNA ligase	10	65	125
Nuclease-free water	15	97.5	187.5
Total volume	**45**	**292.5**	**562.5**

2. Transfer 5 µl of a separate adapter with molecular tags, for each sample, into a separate 0.2ml PCR tube(s).

   Note: Only one single sample-specific bar code adapter should be used per ligation reaction. Open one adapter tube at a time to avoid cross-contamination. It is also recommended to change gloves between each adapter addition to avoid cross-contamination.

3. Transfer 50 µl of each End repair / dA tailing sample generated in the ERA (End Repair / dA Tailing Protocol above), into each 0.2ml PCR tube(s) which contains an adapter.

4. Add 45 µl of ligation master mix to each 0.2ml PCR tube(s) and mix gently by pipetting up and down 7 times with a pipette set to 25 µl, spin down briefly, and then cool 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 from "Protocol: Adapter Ligation". If the volume is less than 100 µl, add the appropriate volume of nuclease-free water to bring the final volume to 100 µl.

5. Incubate the ligation reaction at 20°C for exactly 15 min. Pause the cycler during the first step before adding the samples, then restart the program.

   Important: Do not use a heated lid.

Table 11. Cycler settings for reverse transcription

Step	Incubation Temperature	Incubation Time
1	4°C	1 min
2	20°C	15 min
3	4°C	hold

6. Proceed immediately to adapter ligation cleanup.

Protocol: Cleanup of Adapter-ligated DNA with QIAseq Beads

1. Let the QIAseq Beads come to room temperature for at least 30 minutes before use.
2. Transfer the 100 µl adaptor ligation product into a 1.5 ml LoBind tube for sample cleanup.

   Note: The final 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, add the appropriate volume of nuclease-free water to bring the final volume to 100 µl.
3. Add 90 µl (0.9x volume) QIAseq Beads to 100 µl ligation product. Mix well by pipetting up and down 10 times using a pipette set to 100 µl. Use a fresh tip for each sample.
4. Incubate for 5 minutes at room temperature.
5. Place the tube on the magnetic rack for 10 minutes 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. Completely remove residual supernatant (it is recommended using 10 µl tip to aspirate the trace amount of residual supernatant 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. Wait 1 min with the tube on magnetic rack. Carefully remove and discard the supernatant.
8. Repeat 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 minutes 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 no longer appear shiny.

10. Elute DNA target beads into 52 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipette set to 25 µl. Use a fresh tip for each sample. Place tube on the rack until 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 50 µl DNA solution from previous step. Mix well by pipetting up and down 10 times using a pipette set to 50 µl. Use a fresh tip for each sample.
13. Incubate for 5 minutes at room temperature.
14. Place the tube on the magnetic rack for 10 minutes 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. Wait 1 min with the tube on magnetic rack. Carefully remove and discard the supernatant.
16. Repeat previous step once.
17. Completely remove ethanol with a 200 µl pipette tip first, then use a 10 µl tip to remove any residual ethanol. Dry beads for up to10 minutes 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 no longer appear shiny. Ethanol carryover can affect enrichment PCR efficiency in the next protocol, "Target Enrichment PCR".
18. Elute DNA target beads in 22 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipette set to 15 µl. Use a fresh tip for each sample. Place tube on the magnetic rack until the solution is clear (10 minutes).

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 transfer 9.4 µl supernatant to each of two PCR tubes, one for the forward target enrichment PCR and one for the reverse target enrichment PCR. Proceed to the next protocol, "Target Enrichment PCR", page 25.

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

Protocol: Target Enrichment PCR

Procedure

1. Prepare the following master mixes as indicated in Tables 12a and 12b in separate 1.5 ml LoBind tubes.

Table 12a. Reaction components for forward target enrichment PCR

Component	1x Volume (µl)	6 x Volume (µl)	12x Volume (µl)
Purified Adaptor-ligated sample from previous section	9.4	-	-
UPCR Buffer, 5x	4	26	50
GeneRead QIAact Lung RNA Panel Forward Primers	5	32.5	62.5
GeneReader TE-PCR Primer	0.8	5.2	10
HotStarTaq DNA Polymerase	0.8	5.2	10
Total volume	**20**	**68.9**	**132.5**

Table 12b. Reaction components for reverse target enrichment PCR

Component	1x Volume (µl)	6 x Volume (µl)	12x Volume (µl)
Purified Adaptor-ligated sample from previous section	9.4	-	-
UPCR Buffer, 5x	4	26	50
GeneRead QIAact Lung RNA Panel Reverse Primers	5	32.5	62.5
GeneReader TE-PCR Primer	0.8	5.2	10
HotStarTaq DNA Polymerase	0.8	5.2	10
Total volume	**20**	**68.9**	**132.5**

2. Add 10.6 µl of master mix from either Table 12a or 12b to the corresponding 0.2 ml PCR tube(s) containing the purified adaptor-ligated sample from the previous section. Mix gently by pipetting up and down 7 times with a pipette set to 10 µl, spin down briefly.

3. Set up the thermal cycler using the cycling conditions provided in Table 13.

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

Table 13. Cycling conditions for target enrichment PCR

Time	Temperature	Number of cycles
15 minutes	95°C	1 (Initial denaturation)
15 seconds 10 minutes	95°C 68°C	8
5 minutes	72°C	1
5 minutes*	4°C	1
∞	4°C	Hold

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

4. When the reaction is complete, place the reactions on ice and proceed to the next protocol, "Cleanup of Target Enrichment PCR with QIAseq Beads", page 27.

   If reactions are to be stored after target enrichment PCR, transfer them to a –20°C freezer. Samples are stable for 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. Pulse-centrifuge the Forward and Reverse PCR reactions from "Protocol: Target Enrichment PCR", page 25, and combine them in a 1.5 ml LoBind tube. Add nuclease-free water to bring the total combined volume of the Forward + Reverse PCR reactions 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 PCR reaction volume from "Protocol: Target Enrichment PCR". Add the appropriate volume of nuclease-free water to bring the final volume to 100 µl.
3. Add 130 µl (1.3x 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 fresh tip for each sample. Incubate for 5 minutes at room temperature.
4. Place the tube on the magnetic rack for 10 minutes to separate beads from supernatant. Carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

   IMPORTANT: Do not discard the beads.
5. 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. Wait 1 min with the tube on magnetic rack, then carefully remove and discard the supernatant.
6. Repeat previous step once.
7. Completely remove ethanol with a 200 µl pipette tip first, then use a 10 µl tip to remove any residual ethanol. Dry beads for up to10 minutes 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 no longer appear shiny. Ethanol carryover can affect PCR efficiency in the next protocol, "Universal PCR Amplification".

8. Elute DNA beads in 16 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipette set to 8 µl. Use a fresh tip for each sample. Place on the magnetic rack until the solution is clear (5–10 minutes).
9. Transfer 13.4 µl supernatant to a clean 0.2ml PCR tube. Proceed to the next protocol, "Universal PCR Amplification", page 29.

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

Protocol: Universal PCR Amplification

Procedure

1. Prepare the following master mix as indicated in Table 14 in a 1.5 ml LoBind tube.

Table 14. Reaction components for universal PCR amplification

Component	1x Volume (µl)	6 x Volume (µl)	12x Volume (µl)
Purified PCR product sample from previous section	13.4	-	-
UPCR Buffer, 5x	4	26	50
GeneReader Universal PCR Primer A	0.8	5.2	10
GeneReader Universal PCR Primer B	0.8	5.2	10
HotStarTaq DNA Polymerase	1	6.5	12.5
Total volume	**20**	**42.9**	**82.5**

2. Add 6.6 µl of master mix from Table 14 to the 0.2 ml PCR tube(s) containing the purified PCR product from the previous section. Mix gently by pipetting up and down 7 times with a pipette set to 10 µl, spin down briefly.
3. Set up the thermal cycler using the cycling conditions provided in Table 15.

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

Table 15. Cycling conditions for universal PCR amplification

Time	Temperature	Number of cycles
15 minutes	95°C	1 (Initial denaturation)
15 seconds 2 minutes	95°C 60°C	25
5 minutes	72°C	1
5 minutes*	4°C	1
∞	4°C	Hold

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

4. When the reaction is complete, place the reactions on ice and proceed to the next protocol, "Cleanup of Universal PCR with QIAseq Beads", page 31.

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

Protocol: Cleanup of Universal PCR with QIAseq Beads

Procedure

1. Let the QIAseq Beads come to room temperature for at least 30 minutes before use.
2. Transfer 20 µl PCR reaction from "Protocol: Universal PCR Amplification", page 29, to a 1.5 ml LoBind tube. Add nuclease-free water to bring the volume to 50 µl.

   Note: The PCR reaction volume may be less than 20 µl due to evaporation. It is important to measure the PCR reaction volume from "Protocol: Universal PCR Amplification ". If the volume is less than 50 µl, add the appropriate volume of nuclease-free water to bring the final volume to 50 µl.
3. Add 65 µl (1.3x volume) QIAseq Beads to the PCR solution. Mix well by pipetting up and down 10 times using a pipette set to 55 µl. Use a fresh tip for every sample. Incubate for 5 minutes at room temperature.
4. Place the tube on the magnetic rack for 10 minutes to separate beads from supernatant. Carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

   IMPORTANT: Do not discard the beads.
5. 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. Wait 1 min with the tube on magnetic rack, then carefully remove and discard the supernatant.
6. Repeat previous step once.
7. Completely remove ethanol with a 200 µl pipette tip first, then use a 10 µl tip to remove any residual ethanol. Dry beads for up to10 minutes 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 no longer appear shiny. Ethanol carryover may affect downstream processing and sample assessment.

8. Elute DNA library beads in 30 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipette set to 15 µl. Use a fresh tip for each sample.
   Place 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.

9. 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). 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 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 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 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		
a)	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". Concentration of RNA should be determined by fluorometric quantitation.
b)	No or low PCR product yield (<2ng/µl) after Universal PCR	Ensure that all reaction components are thoroughly mixed as described in this handbook before use. Ensure that all reaction components are correctly added at each stage and thoroughly mixed as described in this handbook.
d)	No or low PCR product yield (<2ng/µl) after Universal PCR	Increase RNA input used for library preparation. If 100ng was used initially increase input to 200ng of 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 analyse 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 approximately 150bp and 600bp, however, these do not impact quantification and sequencing results.

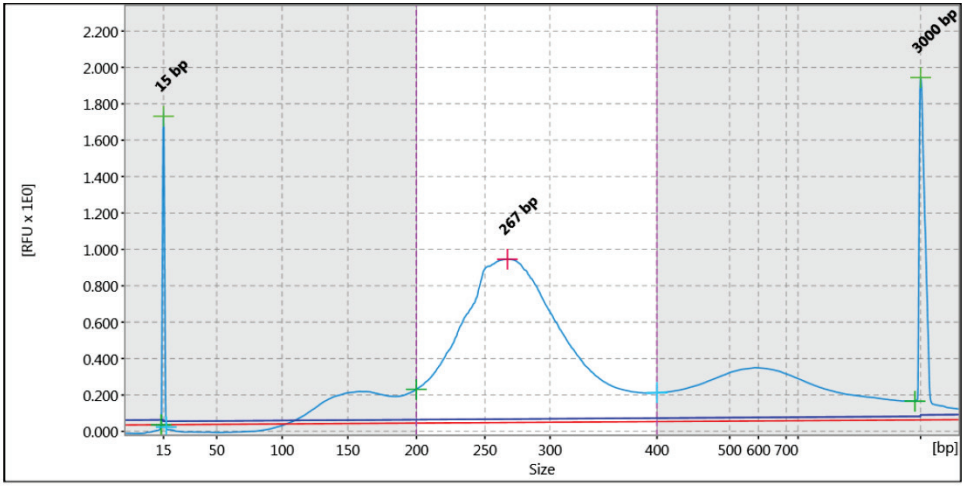

Figure 2. Sample QIAxcel Advanced image of a GeneRead QIAact Lung RNA library

Ordering Information

Product	Contents	Cat. no.
GeneRead QIAact Lung RNA Panel (24)	GeneRead QIAact Library Preparation and Target Enrichment Reagents (24) and QIAact Target Enrichment Panel and QIAact Adapters (24)	181935
Related Products		
QIAGEN RNeasy® FFPE Kit (50)		73504

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Limited License Agreement for GeneRead QIAact Lung RNA Panel

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