July 2018

GeneRead™ QIAact Lung RNA Fusion UMI Panel Handbook

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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.

REF 181936

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Sample to Insight

QIAGEN

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Kit Contents

GeneRead QIAact Lung RNA Fusion UMI Panel	
Catalog no.	**181936**
Number of reactions	**24**
GeneRead QIAact RNA Library Prep and Target Enrichment Reagents (Kit Box1)*	
GeneRead QIAact RNA Fusion UMI Panel and Adapter Kit (Kit Box 2)*	

* Kit boxes 1 and 2 are components of the GeneRead QIAact Lung RNA Fusion UMI Panel.

GeneRead QIAact RNA Library Prep 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 2 ml
HotStarTaq® DNA Polymerase	2 x 50 µl
One bottle containing QIAact Beads	10 ml

* Not for individual sale; to order reagents, see cat. no. 181936.

QIAact RNA Target Enrichment Panel and QIAact Adapters *	
Number of reactions	**24**
GeneRead QIAact Lung RNA Fusion UMI Panel Forward Primers	130 µl
GeneRead QIAact Lung RNA Fusion UMI 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)	12µl
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. 181936.

Storage

The GeneRead QIAact Library Prep and Target Enrichment Reagents (except QIAact Beads) are shipped on dry ice and should be stored at –30°C to –15°C upon arrival. QIAact 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 Fusion UMI Panel is shipped on dry ice and should be stored at –30°C to –15°C upon arrival. When stored properly, components are stable for up to 3 months after delivery.

Intended Use

The GeneRead QIAact Lung RNA Fusion UMI Panel and GeneRead QIAact RNA Fusion UMI Library Prep 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

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

Introduction

NGS is a useful tool to detect genetic alterations in RNA, including translocation/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 Lung RNA Fusion UMI 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 Fusion UMI 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 Fusion UMI 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-stranded (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.

Fusion targets

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

Table 1. Fusion targets

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

Total RNA is first reverse-transcribed to first-strand cDNA. A separate, second-strand synthesis is used to generate 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 GeneReader-specific adapter containing an UMI and a 9 bp 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 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 Lung RNA Fusion UMI 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.

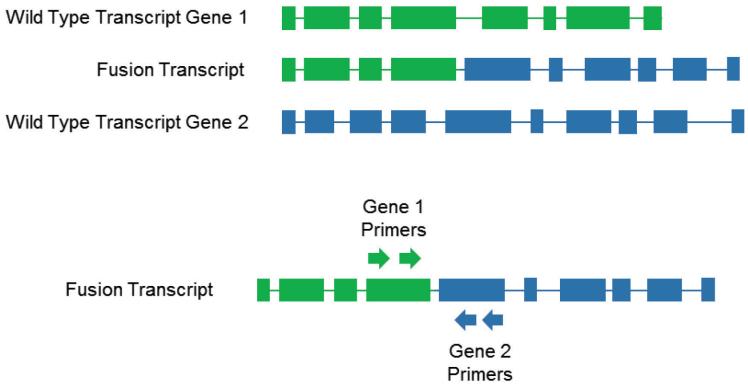

Figure 1. Principle of fusion detection. Fusion-specific primers are designed to target defined sequences on both sides of the translocation breakpoint in the two fusion partners.

Recommendation for multiplexing and clonal amplification input

More than one sample can be sequenced in one flow cell and this is made possible by the addition of a 9 bp sample-specific bar code that is added during library preparation (see "Protocol: Adapter Ligation", page 22). 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 *GeneRead Clonal Amp Q Handbook* for more information). Based on the total number of amplicons that are produced by the GeneRead QIAact Lung RNA Fusion UMI 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 625 pg pooled samples in the clonal amplification process (see "Preparing Libraries for Emulsion Making" in the *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 Prep and Target Enrichment Reagents and GeneRead QIAact Lung RNA Fusion UMI Panel, the following supplies are required:

For RNA isolation

- See "Recommended RNA preparation methods", page 13.

For library construction and targeted enrichment

- High-quality, nuclease-free water.

  IMPORTANT: 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 pipet tips and tubes
- QIAxcel® Advanced instrument* (for information, visit **www.qiagen.com**)

* Make sure that instruments have been checked, maintained, and calibrated regularly according to the manufacturer's instructions.

- 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® 2100 Bioanalyzer®
- Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)

* Make sure that instruments have been checked, maintained, and calibrated regularly according to the manufacturer's instructions.

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 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 the Qubit 3.0 Fluorometer (Thermo Fisher Scientific cat. no. Q33216). RNA input of 100 ng is recommended for the GeneRead QIAact Lung RNA Fusion UMI Panel.

> **Note**: Highly degraded RNA samples present challenges, such as providing insufficient intact mRNA transcripts for detecting fusions. Use of highly degraded RNA is not recommended.

* 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 0.2 ml PCR tubes on ice.
4. Prepare a reaction mixture for first-strand synthesis according to Table 2.

Table 2. Preparation of mixture for first-strand synthesis

Reagent	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 pipet set to 4 µl and then centrifuge briefly (10–15 seconds).
6. Transfer the tube(s) from ice to the cycler and incubate at 65°C for 5 minutes.

Table 3. Thermal cycler conditions for first-strand synthesis

Step	Temperature	Time
1	65°C	5 minutes
2	Ice	≥2 minutes

7. Remove the tube(s) from the cycler and place on ice for at least 2 minutes.
8. Briefly centrifuge (10–15 seconds) before next step.

Protocol: Reverse Transcription

Procedure

1. Prepare the reverse transcription reaction in Table 4 and mix the components well by pipetting up and down 10 times using a pipet set to 15 µl volume.
2. Add 4 µl the master mix (Table 4) to each 0.2 ml PCR tube(s) from "Protocol: First-Strand cDNA Synthesis", page 15, containing the random primed RNA.

Table 4. Preparation of mixture for reverse transcription

	Volume (µl) for the number of samples		
Reagent	1	6	12
Random primed RNA from previous section	6.0	–	–
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	**10.0**	**26.0**	**50.0**

3. Mix by pipetting up and down 7 times with a pipet set to 4 µl, and then centrifuge briefly (10–15 seconds).
4. Program a thermal cycler according to
5. Table 5. Be sure to use the instrument's heated lid (e.g., set to 103°C).

Table 5. Thermal cycler conditions for reverse transcription

Step	Temperature	Time
1	25°C	10 minutes
2	42°C	30 minutes
3	70°C	15 minutes
4	4°C	Hold

6. When the thermal cycler program is complete and the sample block has reached 4°C, remove the samples and place them 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. Prepare the second-strand synthesis reaction mixture in Table 6 and mix the components well by pipetting up and down 10 times using a pipet set to 30 µl volume.
2. Add 10 µl master mix (Table 6) to each 0.2 ml PCR tube(s) from "Protocol: Reverse Transcription", page 16, containing the cDNA.

Table 6. Preparation of mixture for second-strand synthesis

	Volume (µl) for the number of samples		
Component	1	6	12
cDNA from "Protocol: Reverse Transcription"	10.0	–	–
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	**20.0**	**65.0**	**125.0**

3. Mix by pipetting up and down 7 times with a pipet set to 10 µl and then centrifuge briefly (10–15 seconds).
4. Place the 0.2 ml PCR tube(s) into a cycler with a heated lid (e.g. set to 103°C) and incubate as described in Table 7:

Table 7. Thermal cycler conditions for second-strand synthesis

Step	Temperature	Time
1	37°C	7 minutes
2	65°C	10 minutes
3	80°C	10 minutes
4	4°C	Hold

5. Once incubation is completed, remove the 0.2 ml PCR tube(s) from the thermal cycler, centrifuge briefly (10–15 seconds) and place on ice.
6. Immediately proceed to "Protocol: End Repair and A-addition ", page 20.

Protocol: End Repair and A-addition

Procedure

1. Enter the program provided in Table 8 into a thermal cycler with a heated lit. Set the heated lid to 70°C.

Table 8. Thermal cycler conditions end repair and A-addition

Step	Temperature	Time
1	4°C	1 minute
2	20°C	30 minutes
3	65°C	30 minutes
4	4°C	Hold

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

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

3. Prepare a master mix in a new LoBind tube on ice by combining ERA Buffer and nuclease-free water as indicated in Table 9. Mix the components well by pipetting up and down 10 times using a pipet set to 50 µl volume.

Table 9. Preparation of mixture for end repair and A-addition

	Volume (µl) for the number of samples		
Reagent	1	6	12
Second-strand product from "Protocol: Second-Strand Synthesis"	20.0	–	–
ERA Buffer, 10x	5.0	32.5	62.5
Nuclease-free water	15.0	97.5	187.5
Total volume	**40.0**	**130.0**	**250.0**

4. Add 20 µl of End repair and A-addition mix (Table 9) to each tube from “Protocol: Second-Strand Synthesis”, page 18.
5. Add 10 µl ERA Enzyme to each 0.2 ml PCR and gently mix by pipetting up and down 7 times with a pipet set to 25 µl. Centrifuge briefly (10–15 seconds).

   Note: It is recommended to keep the PCR tube on ice for the whole time during reaction setup.
6. Immediately transfer the tubes to the pre-chilled thermal cycler (4°C). Resume the cycling program.

   Note: If using a thermal cycler that does not have a temperature-controlled lid, run with cycler lid open for step 2. When the cycler reaches step 3, close the lid to avoid evaporation.
7. When the program is complete and sample block has returned to 4°C, remove 0.2 ml PCR tube(s) from block. Centrifuge briefly (10–15 seconds) and place on ice.
8. Immediately proceed to “Protocol: Adapter Ligation”, page 22.

Protocol: Adapter Ligation

Procedure

1. Prepare on ice a ligation reaction master mix according to Table 10. Mix well by pipetting up and down 10 times using a pipet set to 50 µl volume.

Table 10. Preparation of mixture for ligation

	Volume (µl) for the number of samples		
Reagent	1	6	12
Ligation Buffer, 5x	20	130	250
DNA Ligase	10	65	125
Nuclease-free Water	15	97.5	187.5
Total volume	**45**	**292.5**	**562.5**

2. Each QIAact Adapter has a different 9 bp sample-specific bar code. Transfer 5 µl of one QIAact Adapter for each sample being prepared into a separate 0.2 ml PCR tube(s).

   Note: Only one single QIAact 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 and A-addition sample from "Protocol: End Repair and A-addition ", page 20, into each 0.2 ml PCR tube which contains an adapter.
4. Add 45 µl ligation master mix to each 0.2 ml PCR tube (using a fresh tip for each addition) and mix gently by pipetting up and down 7 times with a pipet set to 25 µl, centrifuge briefly (10–15 seconds) and then place on ice.
5. Program a thermal cycler to 20°C.
6. Transfer the tube(s) to the thermal cycler and incubate reaction for 15 minutes at 20°C.

IMPORTANT: Do not use a heated lid. If it is not possible to disable the lid on the thermocycler, leave the lid open.

7. Proceed immediately to “Protocol: Cleanup of Adapter-Ligated DNA with QIAact Beads”, page 24.

Protocol: Cleanup of Adapter-Ligated DNA with QIAact Beads

1. Let the QIAact Beads come to room temperature (15–25°C) for at least 30 minutes and vortex thoroughly before use

   Note: we recommend vortexing for 1 minute at maximum speed.

2. Transfer the 100 µl adaptor ligation from "Protocol: Adaptor Ligation", page 22, 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) QIAact Beads to 100 µl ligation product. Mix well by pipetting up and down 10 times using a pipet 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 to use a 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. Once complete wait until solution is clear (2–3 minutes). Completely remove ethanol with a 200 µl pipet tip.
8. Repeat previous step twice.
9. After completely removing the ethanol close the tube lid.

10. Remove tube from the magnetic rack and centrifuge briefly (10–15 seconds).
11. Replace tube on the magnetic rack and wait until solution is clear (2–3 minutes). Open lid and then use a 10 µl tip to remove any residual ethanol. Keeping the tube lid open, air dry the beads for up to 10 minutes 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 but do not appear cracked.

    Note: The beads in different tubes may dry at different rates. Once beads are dry, close tube lid to avoid over drying.
12. Elute DNA from beads into 52 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipet set to 25 µl. Use a fresh tip for each sample. Place tube on the rack until solution is clear (5–10 minutes).
13. Transfer 50 µl supernatant to a clean 1.5 ml tube.
14. Add 65 µl (1.3x volume) QIAact Beads to 50 µl DNA solution from previous step. Mix well by pipetting up and down 10 times using a pipet set to 50 µl. Use a fresh tip for each sample.
15. Incubate for 5 minutes at room temperature.
16. 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.
17. 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. Once complete wait until solution is clear (2–3 minutes). Completely remove ethanol with a 200 µl pipet tip.
18. Repeat previous step once.
19. After completely removing the ethanol close the tube lid.
20. Remove tube from the magnetic rack and centrifuge briefly (10–15 seconds).

21. Replace tube on the magnetic rack and wait until solution is clear (2–3 minutes). Open lid and then use a 10 µl tip to remove any residual ethanol. Keeping the tube lid open, air dry the beads for up to 10 minutes 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 but do not appear cracked. Ethanol carryover can affect enrichment PCR efficiency in the target enrichment PCR.

    Note: The beads in different tubes may dry at different rates. Once beads are dry, close tube lid to avoid over drying.

22. Elute DNA from beads in 22 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipet set to 15 µl. Use a fresh tip for each sample. Place tube on the magnetic rack until the solution is clear (10 minutes).

23. Prepare two sets of 0.2 ml PCR tubes, one for the forward target enrichment PCR and one for the reverse target enrichment PCR. 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 "Protocol: Target Enrichment PCR", page 27.

    Note: If reactions are to be stored after the QIAact Beads Cleanup, transfer them to a –20°C freezer. Samples are stable for up to 3 days.

Protocol: Target Enrichment PCR

Procedure

1. Prepare the following master mixes as indicated in Table 11 and Table 12 in separate 1.5 ml LoBind tubes. Mix well by pipetting up and down 10 times using a pipet set to 30 µl volume.

Table 11. Preparation of mixture for target enrichment PCR forward reaction

	Volume (µl) for the number of samples		
Reagent	1	6	12
Purified adaptor-ligated sample	9.4	–	–
UPCR Buffer, 5x	4.0	26	50
GeneRead QIAact Lung RNA Fusion UMI Panel Forward Primers	5.0	32.5	62.5
GeneReader TE-PCR Primer	0.8	5.2	10
HotStarTaq DNA Polymerase	0.8	5.2	10
Total volume	**20.0**	**68.9**	**132.5**

Table 12. Preparation of mixture for target enrichment PCR reverse reaction

	Volume (µl) for the number of samples		
Reagent	**1**	**6**	**12**
Purified adaptor-ligated sample	9.4	–	–
UPCR Buffer, 5x	4.0	26.0	50.0
GeneRead QIAact Lung RNA Fusion UMI 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	**20.0**	**68.9**	**132.5**

2. Add 10.6 µl of master mix from either Table 11 or Table 12 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 pipet 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. Thermal cycler 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. Transfer the tubes containing the target enrichment PCR into the thermal cycler and start the program.
5. When the reaction is complete, place the reactions on ice and proceed to "Protocol: Cleanup of Target Enrichment PCR with QIAact Beads", page 29.

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

Protocol: Cleanup of Target Enrichment PCR with QIAact Beads

Procedure

1. Let the QIAact Beads come to room temperature (15–25°C) for at least 30 minutes and vortex thoroughly before use.

   Note: We recommend vortexing for 1 minute at maximum speed.

2. Pulse-centrifuge the forward and reverse PCR from "Protocol: Target Enrichment PCR", page 27, 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 PCR to 100 µl.

   Note: The combined PCR volume may be less than 40 µl due to evaporation. It is important to measure the combined PCR 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) QIAact Beads to 100 µl PCR solution. Mix well by pipetting up and down 10 times using a pipet 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. Completely remove residual supernatant.

   Note: It is recommended to use a 10 µl tip to aspirate the trace amount of residual supernatant after the first aspiration.

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. Once complete wait until solution is clear (2–3 minutes). Completely remove ethanol with a 200 µl pipet tip.
7. Repeat previous step once.
8. After completely removing the ethanol close the tube lid.
9. Remove tube from the magnetic rack and centrifuge briefly (10–15 seconds).
10. Replace tube on the magnetic rack and wait until solution is clear (2–3 minutes). Open lid and then use a 10 µl tip to remove any residual ethanol. Keeping the tube lid open, air dry the beads for up to 10 minutes 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 but do not appearcracked. Ethanol carryover can affect PCR efficiency in the universal PCR.

    Note: The beads in different tubes may dry at different rate. Once beads are dry close tube lid to avoid over drying.
11. Elute DNA from beads in 16 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipet set to 8 µl. Use a fresh tip for each sample. Place on the magnetic rack until the solution is clear (5–10 minutes).
12. Transfer 13.4 µl supernatant to a clean 0.2 ml PCR tube. Proceed to “Protocol: Universal PCR Amplification”, page 31.

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

Protocol: Universal PCR Amplification

Procedure

1. Prepare a universal PCR master mix in a 1.5 ml LoBind tube according to Table 14. Mix well by pipetting up and down 10 times using a pipet set to 20 µl volume.

Table 14. Preparation of mixture for universal PCR amplification

	Volume (µl) for the number of samples		
Reagent	**1**	**6**	**12**
Purified target enrichment sample	13.4	–	–
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	**20.0**	**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 "Protocol: Cleanup of Target Enrichment PCR with QIAact Beads", page 29. Mix gently by pipetting up and down 7 times with a pipet set to 10 µl, and 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. Thermal cycler 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 "Protocol: Cleanup of Universal PCR with QIAact Beads", page 33.

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

Protocol: Cleanup of Universal PCR with QIAact Beads

Procedure

1. Let the QIAact Beads come to room temperature (15–25°C) for at least 30 minutes and vortex thoroughly before use.

   Note: We recommend vortexing for 1 minute at maximum speed.

2. Transfer 20 µl PCR from "Protocol: Universal PCR Amplification", page 31, to a 1.5 ml LoBind tube. Add nuclease-free water to bring the volume to 50 µl.

   Note: The PCR volume may be less than 20 µl due to evaporation. It is important to measure the PCR volume from the universal PCR. 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) QIAact Beads to the PCR solution. Mix well by pipetting up and down 10 times using a pipet 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. Completely remove residual supernatant (it is recommended to use a 10 µl tip to aspirate the trace amount of residual supernatant after the first aspiration).

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. Once complete wait until solution is clear (2–3 minutes). Completely remove ethanol with a 200 µl pipet tip.

7. Repeat previous step once.
8. After completely removing the ethanol close the tube lid.
9. Remove tube from the magnetic rack and centrifuge briefly (10–15 seconds).
10. Replace tube on the magnetic rack and wait until solution is clear (2–3 minutes). Open lid and then use a 10 µl tip to remove any residual ethanol. Keeping the tube lid open, air dry the beads for up to 10 minutes 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 but do not appear cracked. Ethanol carryover may affect downstream processing and sample assessment.

    Note: The beads in different tubes may dry at different rates. Once beads are dry, close tube lid to avoid over drying.
11. Elute DNA from beads in 30 µl nuclease-free water. Mix well by pipetting up and down 10 times using a pipet set to 15 µl. Use a fresh tip for each sample.
12. Place tube on the magnetic rack until the solution is clear (5–10 minutes). 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.
13. Assess the yield (ng) of PCR-enriched DNA library using a Qubit Fluorometer and Qubit dsDNA HS Assay Kit. Assess the product size (bp) using the QIAxcel Advanced instrument and the QIAxcel DNA High Resolution Kit 1200 (see "Appendix A: Analyze the Library using the QIAxcel Advanced", page 37).

    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	
No or low PCR product yield (<2 ng/µl) after Universal PCR	Ensure that 100 ng of RNA is used as input for "Protocol: First-Strand cDNA Synthesis", page 15. Concentration of RNA 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 RNA input used for library preparation. If 100 ng was used initially increase input to 200 ng of RNA.

Symbols

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

Appendix A: Analyze the Library using the 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. The library fragments to be quantified are between 200–400 bp* in size (Figure 2). Additional peaks are observed at approximately 150 bp and 600 bp; however, these do not impact quantification and sequencing results.

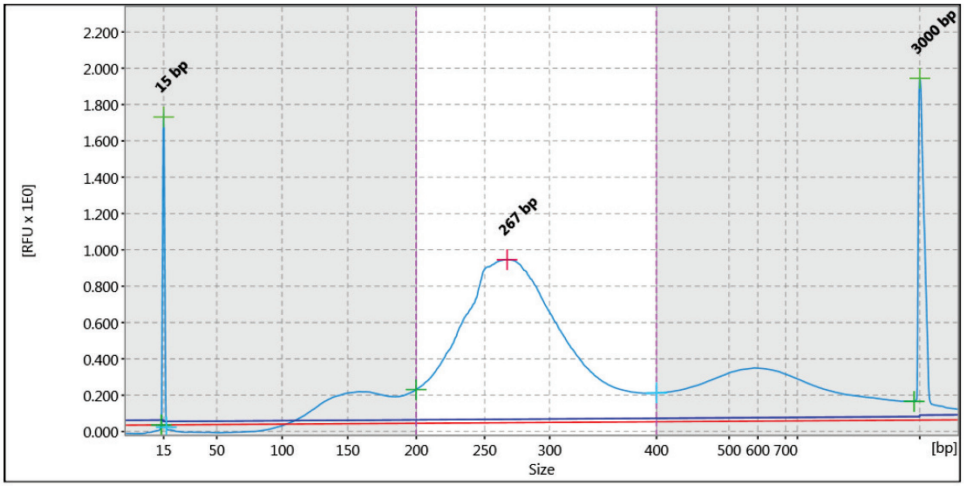

Figure 2. Example of a QIAxcel Advanced image from a GeneRead QIAact Lung RNA Fusion UMI library.

* Data generated from RNA reference standards.

Ordering Information

Product	Contents	Cat. no.
GeneRead QIAact Lung RNA Fusion UMI Panel (24)	GeneRead QIAact Library Prepand Target Enrichment Reagents (24) and QIAact Target Enrichment Panel and QIAact Adapters (24)	181936
Related products		
QIAGEN RNeasy FFPE Kit (50)		73504

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.

Revision History

Revision Date	Contents	Rev. no.
04/2018	Updated kit contents for cleanup beads. Added Revision History.	R2
06/2018	Updated to include change from QIAseq to QIAact beads. QuantiMIZE was removed and Qubit was added as a library quantification method.	R3

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