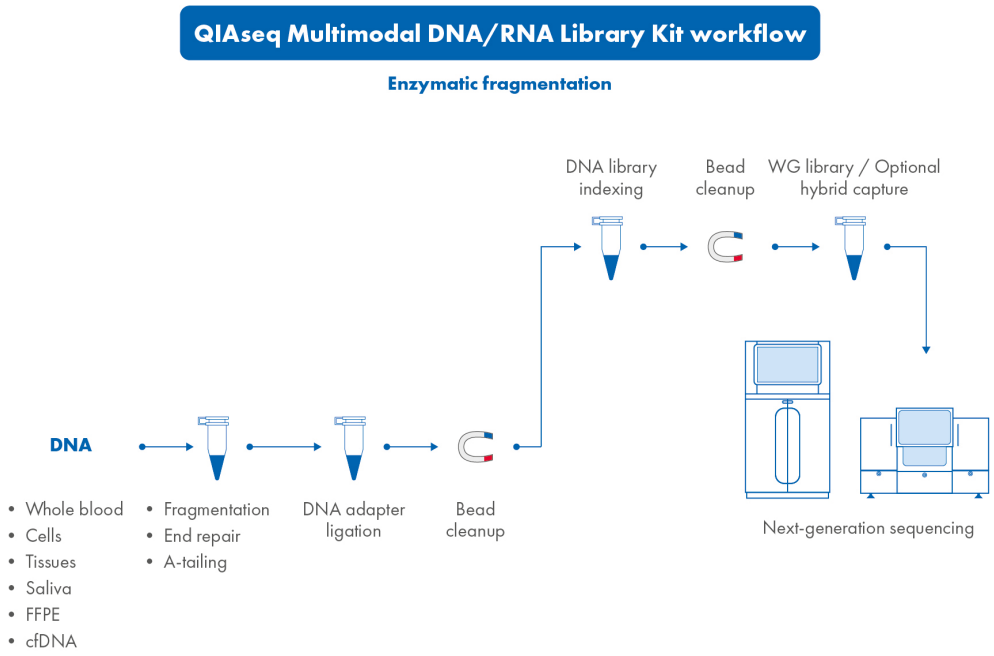
June 2025

Supplementary Protocol

QIAseq® Multimodal DNA Kit-EnzFrag

For the construction of WGS libraries with an enzymatic fragmentation workflow using the QIAseq Multimodal DNA Kit-EnzFrag (cat. nos. 335022 and 335025) and QIAseq Multimodal DNA UDI (cat. nos. 334972 and 334975).



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.

The following are required:

- 80% ethanol (made fresh daily)*
- Nuclease-free pipette tips and tubes
- 1.5 mL LoBind® tubes (Eppendorf®, cat. no. 022431021)
- PCR tubes (0.2 mL individual tubes [VWR®, cat. no. 20170-012] or tube strips [VWR, cat. no. 93001 118]) or plates
- Ice
- Microcentrifuge
- Thermal cycler
- Magnet for bead cleanups:
  - **Tubes**: MagneSphere® Technology Magnetic Separation Stand (Promega, cat. no. Z5342)
  - **Plates**: DynaMag™-96 Side Magnet (Thermo Fisher Scientific Inc., cat. no. 12331D)
- QIAxcel® Connect System: QIAxcel DNA High Resolution Kit

*Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone.

Protocol: Nucleic Acid Fragmentation

Important points before starting

- This protocol describes fragmentation of nucleic acids samples.
- Recommended starting amounts of nucleic acid:
  - The recommended starting amount of DNA is 10–100 ng. For samples where hybrid-capture will be performed, the minimal recommendation is 50 ng.
  - For FFPE samples, the recommended amount of FFPE DNA is 50–250 ng.
  - For cfDNA samples, the recommended starting amount is 20 ng.
- Set up reactions on ice.
- Unless specifically indicated, do not vortex any reagents or reactions.

Procedure

1. Thaw nucleic acid sample(s) on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Prepare the reagents required for the fragmentation.

   5x WGS FX Mix and Side Reaction Reducer should be removed from the freezer and placed on ice just before use. After use, immediately return the enzymes to the freezer.
3. On ice, prepare the fragmentation mix according to Table 1 (for Standard or FFPE samples) or Table 2 (for cfDNA samples). Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 1. Reaction mix for Standard or FFPE samples

Component	Volume/reaction
DNA (see input recommendation in the "Important points before starting" section)*	Variable
10x FX Buffer	2 µL
5x WGS FX Mix	4 µL
Side Reaction Reducer	1.6 µL
Nuclease-free Water	12.4 µL – Variable
Total	**20 µL**

Table 2. Reaction mix for nucleic acid fragmentation of cfDNA samples

Component	Volume/reaction
DNA (see input recommendation in the "Important points before starting" section)	Variable
10x FX Buffer	2 µL
5x WGS FX Mix	4 µL
FG Solution	1.25 µL
Nuclease-free Water	12.75 µL – Variable
Total	**20 µL**

4. Program the thermal cycler according to Table 3. Use the instrument's heated lid.

   Important: The thermal cycler must be prechilled and paused at 4°C.

Table 3. Incubation conditions for nucleic acid fragmentation

Step	Incubation temperature (°C)	Incubation time for standard sample (min)	Incubation time for cfDNA or FFPE (min)
1	4	1	1
2	32	16	12
3	65	30	30
4	4	Hold	Hold

5. Transfer the tubes/plate wells prepared in step 3 to the prechilled thermal cycler and resume the program.
6. Upon completion, allow the thermal cycler to return to 4°C.
7. Place the samples on ice and immediately proceed to "Protocol: DNA Ligation".

Protocol: DNA Ligation

Important points before starting

- In this protocol, there is the option to use the non-UMI Adapter (that does not contain a UMI) or the UMI Adapter (that contains a UMI). Depending on the experiment of choice, choose one adapter or the other.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for the DNA ligation.
   a. Thaw non-UMI Adapter or UMI Adapter and UPH Ligation Buffer, 2.5x, at room temperature.

      Note: Non-UMI Adapter does not contain a UMI, while the UMI Adapter contains a UMI.

   b. Mix by flicking the tube, and then centrifuge briefly.

      Note: DNA Ligase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzyme to the freezer.
2. Prepare the DNA ligation mix according to Table 4. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

   Note: Refer to Table 5 for the recommended proportion of dilution.

Table 4. Reaction mix for DNA ligation

Component	Volume/reaction (µL)
DNA sample from previous step	20
UPH Ligation Buffer, 2.5x	40
Non-UMI Adapter or UMI Adapter*	2.5
DNA Ligase	10
Nuclease-free Water	27.5
Total	**100**

Table 5. Adapter dilution factors for low input

Standard DNA amount	FFPE samples	Adapter dilution
100–999 pg		1:100
1–10 ng		1:10
	50 ng < input < 100 ng	1:10

3. Incubate the reactions in a thermal cycler according to Table 6.

   Important: Do not use the heated lid.

Table 6. Incubation conditions for DNA ligation

Step	Temperature (°C)	Time (min)
1	4	1
2	20	15
3	4	Hold

4. Add 90 µL QIAseq Beads, and then mix by vortexing.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates).

   Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads because they contain the DNA of interest.

   Note: For plates, the following may improve performance. After 8 min, remove 90 µL supernatant. Leave it on the magnetic stand for 2 min and remove 90 µL supernatant. Then, briefly centrifuge the plate, return it to the magnetic stand for 1 min, and use a 10 µL pipette to remove the remaining supernatant.
7. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash.

   Important: Completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
9. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.
10. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 52 µL Nuclease-free Water. Mix well by pipetting.
11. Return the tubes/plate wells to the magnetic rack until the solution has cleared.
12. Transfer 50 µL of the supernatant to clean tubes/plate wells.
13. Add 55 µL of QIAseq Beads and mix by vortexing.
14. Incubate for 5 min at room temperature.
15. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates).

    Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads because they contain the DNA of interest.
16. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.

17. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.

18. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.

19. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 17 µL Nuclease-free Water. Mix well by pipetting.

20. Return the tubes/plate wells to the magnetic rack until the solution has cleared.

    Aliquot 14 µL of the eluate into a separate clean tubes/plate wells and proceed to "Protocol: DNA Library Indexing".

    Alternatively, the samples can be stored at –30°C to –15°C in a constant-temperature freezer.

Protocol: DNA Library Indexing

Important points before starting

- The starting material is a 14 µL sample aliquot from "Protocol: DNA Ligation".
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Use MDNA index plates, either MDNA-24X or MDNA-96AX.
- Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for DNA Library indexing.
   a. Thaw HiFi Ultra Buffer, 5x; MM F-R Primer Mix, and MDNA-24X or MDNA-96AX index plate at room temperature.
   b. Mix by either flicking the tube or vortexing the index plate, and then centrifuge briefly. Plate should be centrifuged at 1000 x *g* for 1 min.

      Note: HiFi Ultra Polymerase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzyme to the freezer.
2. Prepare the reactions according to Table 7. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 7. Reaction mix for DNA indexing

Component	Volume/reaction (µL)
Sample aliquot (from "Protocol: DNA Ligation")	14
HiFi Ultra Buffer, 5x	8
MM F-R Primer Mix	1.6
Nuclease-free Water	9.9
Well from MDNA-24X or MDNA-96AX index plate*	2.5
HiFi Ultra Polymerase	4
Total	**40**

*Ensure proper technique to prevent cross-contamination. Additionally, ensure that every sample has a unique index and that no well is used twice.

3. Program a thermal cycler as described in Table 8, using cycle numbers described in Table 9.

   Important: Set up the ramp rate of the thermal cycler at ≤ 2°C/s.

Table 8. Cycling conditions for DNA indexing

Step	Time	Temperature (°C)
Hold	2 min	98
2-step cycling		
Denaturation	20 s	98
Annealing/Extension	1 min	60
Cycle number	See Table 9	
Hold	3 min	72
Hold	∞	4

Table 9. Cycle number recommendations for DNA indexing, based on original sample input

DNA Input	Standard Sample (cycles)	FFPE sample (cycles)
100 pg	18	–
1 ng	15	–
10 ng	12	–
100 ng	9	12
250 ng	–	11

4. After the reaction is complete, add 36 µL QIAseq Beads, and then mix by vortexing or pipetting up and down several times.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant.

   After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads because they contain the DNA of interest.
7. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash twice, for a total of 3 washes.

   Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
9. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.
10. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 24 µL Nuclease-free Water. Mix well by pipetting.
11. Return the tubes/plate wells to the magnetic rack until the solution has cleared.
12. Transfer 22 µL of the supernatant to clean tubes/plate wells.
13. Proceed to "Recommendations: Library QC & Quantification" in the *QIAseq Multimodal DNA/RNA Library Kit Handbook*. Alternatively, the samples can be stored at –30°C to –15°C in a constant-temperature freezer.

Document Revision History

Date	Description
06/2025	Initial release

Limited License Agreement for QIAseq Multimodal DNA-EnzFrag Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this Instructions for Use and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this Instructions for Use, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.
3. This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the panel and/or its components.

For updated license terms, see **www.qiagen.com**.

This page intentionally left blank.