xGen™ cfDNA & FFPE DNA Library Prep v2 MC Kit

REVISION HISTORY

Version	Release date	Description of changes
5	May 2024	Minor text changes for step naming consistency
4	March 2023	Added clarification for protocol and contamination concerns
3	August 2022	Updated the HiFi formulation to xGen HiFi PCR Mix
2	December 2021	Updated product name
1	February 2020	Initial release

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OVERVIEW

The xGen™ cfDNA & FFPE DNA Library Prep v2 MC Kit is designed specifically for 1–250 ng of degraded samples, such as cell-free DNA (cfDNA) or DNA extracted from formalin-fixed, paraffin-embedded (FFPE) samples. The technology utilized for this library construction workflow enables:

- High conversion of degraded or damaged samples into sequencer-ready libraries
- Adapter titration is no longer needed since the kit uses a unique chemistry to minimize adapter dimers
- Barcoding of samples is done via indexing PCR
- Independent tagging of the top and bottom strands of the library, using our proprietary ligation strategy with fixed UMI sequences

An overview of the workflow is depicted in the schematic below:

Fragmented input DNA

End repair
input DNA blunting

Ligation 1
Single-stranded ligation of
Ligation 1 Adapter to 3′ ends of insert

Ligation 2
Ligation 2 Adapter primes to the L1 adapter
to gap fill, followed by 5′ ligation

PCR
Amplification with xGen 2x HiFi PCR Mix and
xGen Unique Dual Index (UDI) Primer Pairs

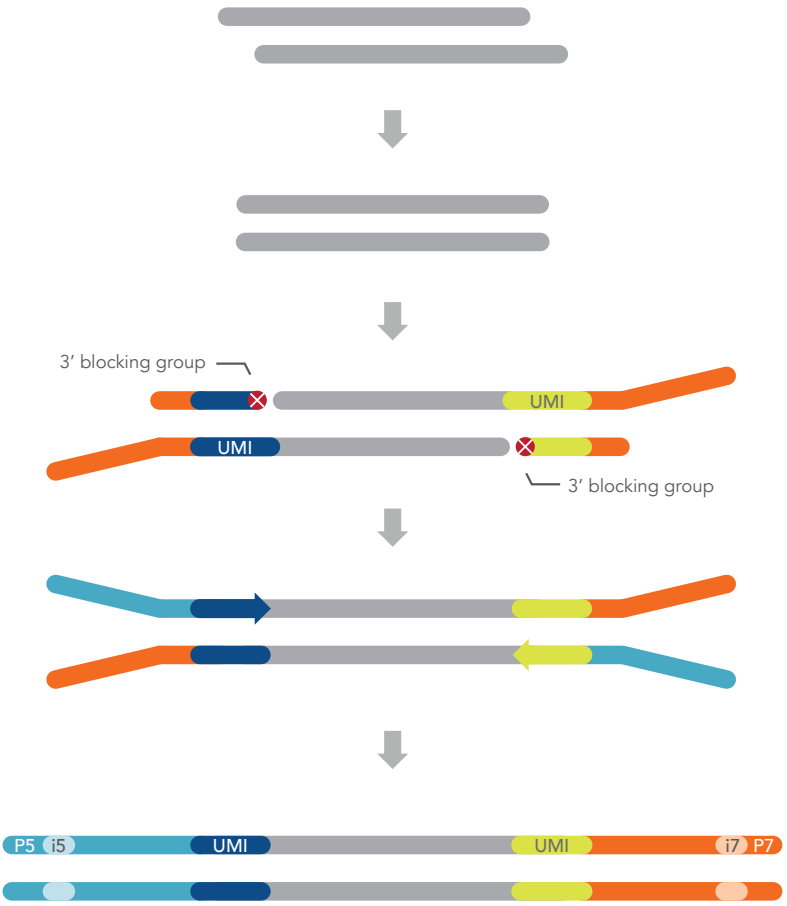

Figure 1. xGen cfDNA & FFPE DNA Library Prep v2 MC Kit process. Illustration of the library construction method.

WORKFLOW

The xGen cfDNA & FFPE DNA Library Prep v2 MC Kit workflow takes between 4–5 hours. There are four major steps to perform in this protocol:

- **End repair.** When the End Repair Enzyme Mix converts cfDNA, or sheared input DNA, into blunt-ended DNA ready for ligation.
- **Ligation 1.** When the Ligation 1 Enzyme catalyzes the single-stranded addition of the Ligation 1 Adapter to the 3′ ends of the insert DNA. This novel enzyme is unable to ligate inserts together, which minimizes the formation of chimeras. The 3′ end of the Ligation 1 Adapter also contains a blocking group to prevent adapter-dimer formation.
- **Ligation 2.** When the Ligation 2 Adapter acts as a primer to gap-fill the bases complementary to the UMI, followed by ligation to the 5′ end of the DNA insert to create a fully double-stranded product.
- **PCR amplification.** When the xGen 2x HiFi PCR Mix is added to perform indexing PCR for Illumina® sequencing.
- Indexing primers are sold separately (see Consumables from IDT—Reagents).

1	End repair		Total time: 35 min
2	End repair cleanup	2.5X AMPure	Total time: 30 min
3	Ligation 1		Total time: 40 min
4	Ligation 2		Total time: 45 min
5	Ligation 2 cleanup	2.5X PEG/NaCl	Total time: 45 min
6	PCR amplification		Total time: 15–30 min (variable depending on input)
7	PCR cleanup	1.3X AMPure	Total time: 40 min

CONSUMABLES AND EQUIPMENT

Consumables from IDT—Kit contents

xGen cfDNA & FFPE DNA Library Prep v2 MC Kit components		16 rxn (µL)	96 rxn (µL)	Storage
End repair module	End Repair Buffer	108	645	–20°C
	End Repair Enzyme Mix	54	323	
Ligation 1 module	Ligation 1 Buffer	448	2688	
	Ligation 1 Adapter	36	215	
	Ligation 1 Enzyme	54	323	
Ligation 2 module	Ligation 2 Buffer	81	484	
	Ligation 2 Adapter	72	430	
	Ligation 2 Enzyme A	9	54	
	Ligation 2 Enzyme B	18	108	
PCR Module	2x HiFi PCR Mix	450	2700	
Other reagents	PEG/NaCl	1.7 mL	10.7 mL	–20°C, or room temperature

Consumables from IDT—Reagents

Item	Catalog #
IDTE pH 8.0 (1X TE Solution), 300 mL	11-05-01- 13
Nuclease-free Water	11-05-01-04, 11-04-02-01, 11-05-01-14
Indexing Primers (choose one):	
xGen UDI Primers, 16 rxn	10005975
xGen UDI Primer Pairs, Plate 1, 8 nt	10005922
Custom indexing options	Contact us

*For additional indexing primer solutions, visit idtdna.com/pages/products/next-generation-sequencing/workflow/xgen-ngs-library-preparation/ngs-adapters-indexing-primers or www.idtdna.com/ContactUs.

Consumables—Other suppliers

Item	Supplier	Catalog #
Buffer EB (10 mM Tris-HCl, pH 8.5), 250 mL	Qiagen	19086
Absolute ethanol (200 proof)	Various	Varies
Purification beads		
AMPure® XP-PCR Purification Beads 5 mL (22 rxn) or 60 mL (270 rxn)	Beckman Coulter	A63880 or A63881
Digital electrophoresis chips and associated reagents (choose one):		
Experion® DNA 1K Analysis Kit	Bio-Rad	700-7107
High Sensitivity DNA Kit	Agilent	5067-4626
High Sensitivity D1000 ScreenTape®	Agilent	5067-5584
twin.tec® 96 Well LoBind® PCR Plates	Eppendorf	0030129504
KAPA Biosystems Library Quantification Kit- Illumina®/Universal	Roche	KK4824
DNA LoBind® Tubes, 1.5 mL	Eppendorf	022431021
Filtered low bind pipette tips	Various	Varies
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	032851 or 032854

Equipment

Item	Supplier	Catalog #
Microcentrifuge	Various	Varies
Thermal cycler	Various	Varies
Qubit 4 Fluorometer, or similar DNA quality analyzer	Thermo Fisher Scientific	033226
Magnet options (choose one):		
Permagen 96-well side pull magnet	TMO	NC1568572
Magnum® EX Universal Magnet Plate	Alpaqua	A000380
Magnetic Stand-96, or similar magnetic stand.	Thermo Fisher Scientific	AM10027

GUIDELINES

Reagent storage and handling

Always store the xGen cfDNA & FFPE DNA Library Prep v2 MC Kit reagents at –20°C, except for PEG/ NaCl, which can be stored either at room temperature, or at –20°C.

Note: The enzymes provided in this kit are temperature-sensitive and appropriate care should be taken during storage and handling.

Note: The xGen 2x HiFi PCR Mix may be in a liquid state after storage in –20°C condition; this is expected and will not impact product integrity.

For all non-enzyme reagents, thaw on ice, then briefly vortex to mix well. Remove enzyme tubes from –20°C storage and place on ice just before use. Briefly centrifuge the tubes in a microcentrifuge to collect contents before opening.

To create master mixes, scale reagent volumes as appropriate, using 10% excess volume to compensate for pipetting loss. Add reagents in the order listed when preparing master mixes, then pulse-vortex to mix and briefly centrifuge.

Note: You may observe precipitation in the End Repair Buffer. Continue vortexing until no precipitate can be observed.

Important: Use extra caution when handling Ligation 1 and Ligation 2 adapter tubes. Never handle Ligation 2 adapter prior to, or during Ligation 1 Master Mix setup and handling. Trace contamination of Ligation 2 adapter into Ligation 1 adapter has been shown to induce adapter-dimer formation.

Avoid cross-contamination

To reduce the risk of DNA and library contamination, physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed.

Follow these instructions to avoid cross-contamination:

- Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change tips between each sample.

Size selection during cleanup steps

This protocol has been tested with AMPure XP beads for whole genome sequencing (WGS) and targeted sequencing applications but can also be used with SPRIselect® beads (Beckman Coulter) or similar bead-based DNA purification products.

Caution: If other beads are used, solutions and conditions for DNA binding may differ.

DNA input considerations

This kit works with a wide range of DNA inputs, ranging from 1–250 ng. Input DNA should be stored in IDTE buffer (10 mM Tris-Cl, pH 8.0; 0.1 mM EDTA) or Buffer EB (Qiagen, 10 mM Tris HCl, pH 8.5).

Important: Input quantities recommended in this protocol refer to the total DNA quantified after fragmentation.

DNA should be an appropriate size before library construction. For genomic DNA, or DNA derived from FFPE samples, using a Covaris® or similar DNA shearing instrument can create fragments with an average insert size of 150–300 base pairs. As cfDNA typically has an average size of 160 base pairs, no further fragmentation is required.

For FFPE samples, use standard quality control methods, such as Q-ratio with qPCR or the DNA Integrity Number (DIN) using size distribution (i.e., Bioanalyzer instrument (Agilent) or similar DNA quality analyzer).

These methods can help you choose the appropriate number of PCR cycles for your DNA sample. For cfDNA, we suggest assessing the size distribution with electrophoresis. Note, if large molecular weight DNA is present, an additional cleanup may be necessary to remove genomic DNA contamination. However, this additional cleanup can reduce sample complexity and mass.

In our tests, we have successfully generated libraries from 1–250 ng of high-quality genomic DNA, 1–25 ng of cfDNA, and 25–250 ng of DNA extracted from FFPE samples. Although the xGen cfDNA & FFPE DNA Library Prep v2 MC is designed for degraded and damaged DNA, the quality of the DNA can still have an impact on yield and sequencing metrics, particularly for low-quality FFPE samples. We have successfully generated and sequenced libraries with DINs as low as 1.6.

Tip: We recommend determining post-shear dsDNA concentration using a Qubit Fluorometer, or a similar fluorometric method.

PROTOCOL

End repair

Before starting the protocol, ensure that the AMPure and PEG/NaCl reagents are at room temperature (20–25°C). Also, prepare a fresh 80% ethanol solution in nuclease-free water.

1. Add 50 µL of each sample into a low-bind PCR plate that resists nucleic acid adsorption.

   Note: If sample volume <50 µL, use IDTE buffer (10 mM Tris-Cl, pH 8.0; 0.1 mM EDTA) or Buffer EB (10 mM Tris-HCl, pH 8.5) to bring the volume up to 50 µL.

2. For each sample, make the following End Repair Master Mix:

End Repair Master Mix	
Component	Volume per reaction (µL)
End Repair Buffer	6
End Repair Enzyme	3
Total volume	9

   Tip: If there is precipitate in the End Repair Buffer, vortex until the precipitate becomes clear in solution. The resulting Master Mix is viscous and requires careful pipetting.

3. Pulse-vortex the master mix for 10 sec, then briefly centrifuge. Keep the master mix on ice.
4. Add 9 µL of End Repair Master Mix to each well. Using a pipette set to 40 µL, pipette 10 times to mix, then seal the plate. Alternatively, seal the plate and vortex mix for minimum of 10 seconds.
5. Run the following thermal cycler program:

End Repair program		
Step	Temperature* (°C)	Time
End repair	20	30 min
Hold	4	Hold

   * Set the lid temperature to OFF, or to 40°C.

   While the end repair program runs, make the Ligation 1 Master Mix in preparation for the Perform post end repair cleanup steps.

Ligation 1 Master Mix	
Component	Volume per reaction (µL)
Ligation 1 Buffer	25
Ligation 1 Adapter	2
Ligation 1 Enzyme	3
Total volume	30

   Important: Use extra caution when handling Ligation 1 and Ligation 2 adapter tubes: never handle Ligation 2 adapter before or during Ligation 1 Master Mix setup and handling. Trace contamination of Ligation 2 adapter into Ligation 1 adapter has been shown to induce adapter-dimer formation.

6. Pulse vortex the master mix for 10 seconds, then briefly centrifuge. Keep the master mix on ice until ready to use.
7. After the End Repair program reaches 4°C, proceed immediately to End repair cleanup.

End repair cleanup

Note: Before starting cleanup, make sure the Ligation 1 Master Mix has already been prepared.

1. Thoroughly resuspend AMPure XP beads before use, then add 147.5 µL of AMPure beads (2.5X volume) to each well and pipette 10 times to thoroughly mix.
2. Incubate the plate at room temperature for 10 minutes.
3. Place the plate on a magnet and wait for the liquid to clear completely or at least for 2 minutes.

   Important: If the solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, sequencing library yield and quality may suffer.

4. Remove and discard the cleared supernatant making sure not to remove any beads. With a clean P20 pipette tip, remove and discard any trace amount of supernatant that remains.

   Important: Be careful not to remove any beads.

5. Keeping the plate on the magnet, add 160 µL of 80% ethanol and incubate for 30 seconds.
6. Remove and discard the supernatant.
7. Use a P20 pipette tip to remove any residual ethanol.
8. Dry the beads at room temperature for 1–3 minutes.

   Important: Never allow the beads to dry for >5 minutes; over-dry beads will decrease final library yield.

9. Proceed immediately to Ligation 1.

Ligation 1

1. Remove the plate from the magnet, then add 30 µL Ligation 1 Master Mix.
2. Pipette mix a minimum of 10 times, then seal the plate.

   Important: Make sure the samples are thoroughly mixed and that the beads are fully resuspended before proceeding.

3. Run the following thermal cycler program:

Ligation 1 program		
Step	Temperature* (°C)	Time
Ligation	20	15 min
Inactivation	65	15 min
Hold	4	Hold

*If possible, set lid temperature to 70°C. If lid cannot be programmed, set the thermal cycler heat block to 105°C.

Safe Stop: The plate can temporarily remain at 4°C (no more than 2 hours). It is normal for beads to settle during this reaction.

4. Proceed to Ligation 2.

Ligation 2

1. For each sample, prepare the Ligation 2 Master Mix.

Ligation 2 Master Mix	
Component	Volume per reaction (µL)
Ligation 2 Buffer	4.5
Ligation 2 Adapter	4
Ligation 2 Enzyme A	0.5
Ligation 2 Enzyme B	1
Total volume	10

2. Pulse-vortex the master mix for 10 seconds, then briefly centrifuge. Keep the master mix on ice until ready to use.
3. Add 10 µL of the Ligation 2 Master Mix to each well.
4. Using a pipette set to 35 µL, pipette 10 times to mix, then seal the plate.

   Important: Ensure the samples are thoroughly mixed, and make sure the beads are fully resuspended before proceeding.

   Note: If necessary, briefly centrifuge to collect contents to the bottom of the wells.

5. Run the following thermal cycler program:

Ligation 2 program		
Step	Temperature* (°C)	Time
Ligation	65	30 min
Hold	4	Hold

*If possible, set lid temperature to 70°C. If lid cannot be programmed, set the thermal cycler heat block to 105°C.

6. After thermal cycler program is completed, proceed immediately to Ligation 2 cleanup.

Ligation 2 cleanup

1. Add 100 µL of PEG/NaCl (2.5X volume) to each well, then pipette 10 times to mix.
2. Incubate the plate at room temperature for 10 minutes.
3. Place the plate on a magnet and wait for the liquid to clear completely or at least for 2 minutes.

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, sequencing library yield and quality may suffer.
4. Remove and discard the cleared supernatant making sure not to remove any beads. With a clean P20 pipette tip, remove and discard any trace amount of supernatant that remains.

   Important: Be careful not to remove any beads.
5. Keeping the plate on the magnet, add 160 µL of 80% ethanol and incubate for 30 seconds.
6. Remove and discard the supernatant.
7. Use a P20 pipette tip to remove any residual ethanol.

   Important: Make sure all the ethanol has been removed before proceeding.
8. Dry the beads at room temperature for 1–3 minutes.

   Important: Never allow the beads to dry for >5 minutes; over-dry beads will decrease final library yield.
9. Remove the plate from the magnet, then add 20 µL of Buffer EB.
10. Seal the plate, then gently vortex (use 70% vortex capacity) to resuspend beads.
11. Allow the plate to incubate at room temperature for 5 minutes to elute DNA off the beads.
12. Place the plate on a magnet and wait for the beads to be cleared from the liquid (approximately 1–2 minutes).

    Note: Depending on the strength of your magnet, you may need to wait longer.
13. Carefully transfer 20 µL of the cleared liquid containing the eluted DNA into a new well. Proceed to PCR amplification or pause here.

    Safe Stop: The plate can be stored at –20°C overnight.

PCR amplification

Note: Sample index barcodes are introduced during PCR; double check that a unique primer pair is used for each sample.

1. Add 5 µL of xGen UDI Primer Pairs to each well.

   Note: If using alternative indexing primers, contact us to check that they are compatible with this protocol.

2. Add 25 µL of xGen 2X HiFi PCR Mix to each well, then pipette 10 times to mix.
3. Seal the plate, then briefly centrifuge.
4. Run the following thermal cycler program:

   Note: Only use standard cycling conditions for the PCR program. Fast cycling has been shown to negatively impact library yields.

PCR program			
Step	Temperature* (°C)	Time	Cycles
Polymerase activation	98	45 sec	Varies based on sample input (see Table 1)
Denature, Anneal, Extend	98	15 sec	
	60	30 sec	
	72	30 sec	
Final Extension	72	1 min	
Hold	4	Hold	

*Set lid temperature to 105°C. If the lid cannot be programmed, set the thermal cycler block to 105°C.

Table 1. Recommended PCR cycling parameters to yield >500 ng.

Number of cycles		
Input mass (ng)	gDNA or dsDNA	FFPE*
1	11–13	14–16
10	9–11	11–13
25	7–9	9–11
100	5–7	7–9
250	4–6	6–8

* For lower-quality FFPE samples, we recommend using the higher number of recommended PCR cycles from Table 1. For very low quality FFPE (DIN 1–2), you may not obtain 500 ng, but we do not recommend increasing the number of PCR cycles.

5. After the program completes, proceed to post-PCR cleanup.

PCR cleanup

1. Add 65 µL of AMPure beads (1.3X volume), or similar bead-based DNA purification product, to each well, then pipette 10 times to thoroughly mix.
2. Incubate the plate at room temperature for 5 minutes.
3. Place the plate on a magnet and wait for the liquid to clear completely or at least for 2 minutes.

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, sequencing library yield and quality may suffer.

4. Remove and discard the cleared supernatant; make sure not to remove any beads.

   Important: Be careful not to remove any beads.

5. Keeping the plate on the magnet, add 160 µL of 80% ethanol, then incubate for 30 seconds.
6. Remove and discard the supernatant.
7. Use a P20 pipette tip to remove any residual ethanol.

   Important: Make sure all the ethanol has been removed before proceeding.

8. Dry the beads at room temperature for 1–3 minutes.

   Important: Never allow the beads to dry for >5 minutes; over-dry beads will decrease final library yield.

9. Remove the plate from magnet, then add 31 µL of Buffer EB.
10. Seal the plate and gently vortex (use 70% vortex capacity) to resuspend beads.
11. Allow the plate to incubate at room temperature for 5 minutes to elute DNA off beads. Then place the plate on a magnet and wait for the liquid to clear completely for 1–2 minutes.

    Note: Depending on the strength of your magnet, you may need to wait longer.

12. Carefully transfer 30 µL of eluted DNA into a new well.

Library quantification

The library is now ready for quantification, which can be performed using fluorometric methods (i.e., Qubit Fluorometer) or a quantitative method such as qPCR. Digital electrophoresis, such as the Agilent TapeStation system (Figure 2) or Agilent Bioanalyzer system can also be used to assess the library size.

Note: For use in targeted sequencing, see the xGen Hybridization Capture of DNA Libraries protocol.

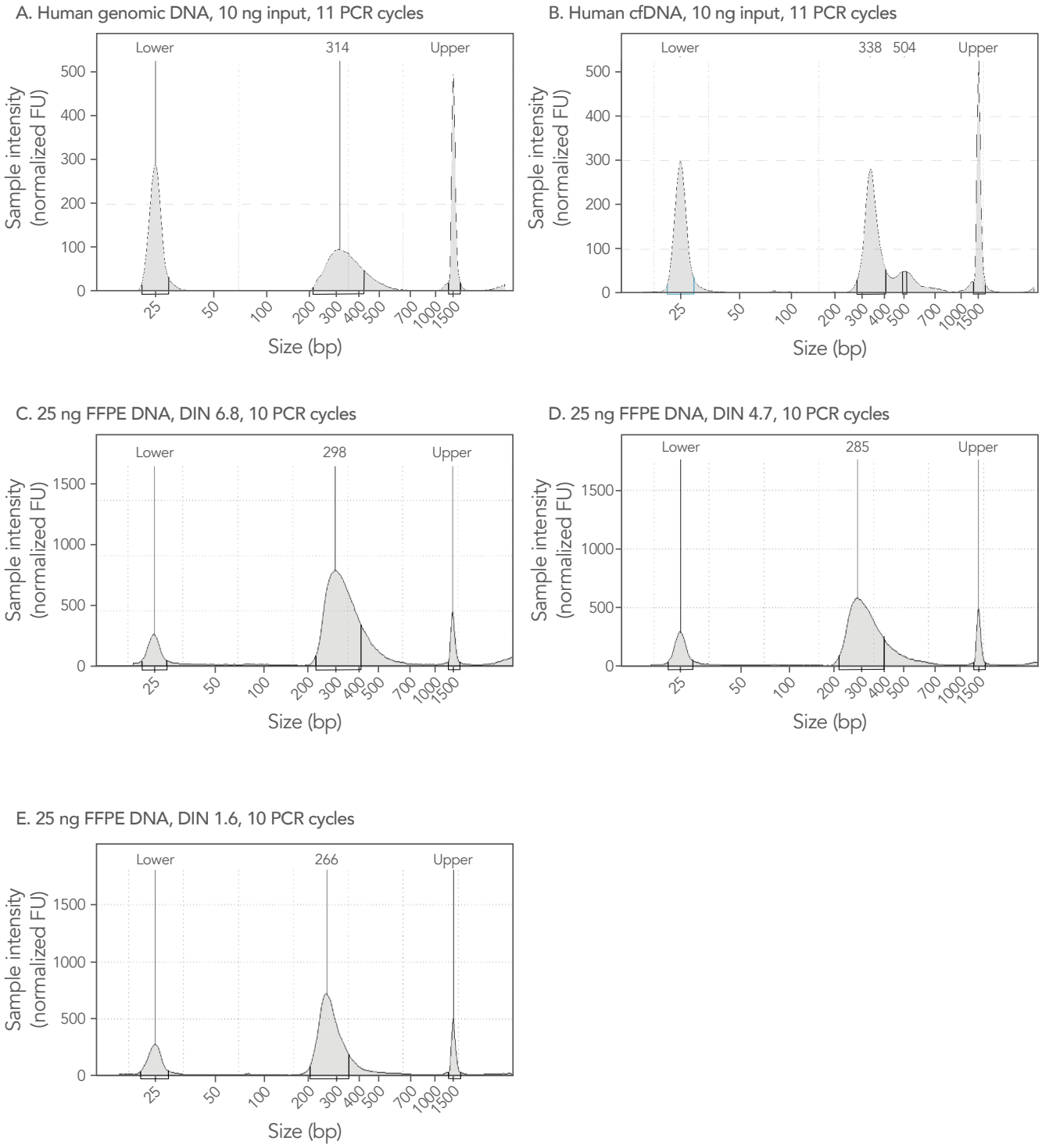

Figure 2. Representative electropherograms from libraries prepared with the xGen cfDNA & FFPE DNA Library Prep v2 MC Kit. Electropherograms were generated from 1:10 diluted final library with Agilent DNA ScreenTape on the TapeStation 2200 using the manufacturer's recommendations. (**A**) A library prepared from 25 ng of Coriell genomic DNA was sheared to 150 bp using the Covaris S2 instrument and amplified with 11 cycles of PCR. (**B**) A library was prepared from 10 ng of cell free DNA (Biochain) and amplified using 11 cycles of PCR. 100 ng of DNA extracted from FFPE (Horizon FFPE standard) was sheared to 150 bp using the Covaris S2 instrument. Libraries were amplified using (**C**) 10 cycles for FFPE DNA with DIN 6.8, (**D**) 10 cycles for FFPE DNA with DIN 4.7, and (**E**) 10 cycles for FFPE DNA with DIN 1.6.

Final sequence read structure

A schematic of the final sequencing libraries generated with the xGen cfDNA & FFPE DNA Library Prep v2 MC Kit and amplified with IDT xGen UDI Primer Pairs is shown in Figure 3. The libraries contain fixed, in-line 8 bp UMIs at the beginning of reads 1 and 2. These UMIs consist of 32 optimized, fixed sequences that can aid in deduplication or error correction. Since these sequences are fixed, even when there are sequencing or PCR errors in the UMI sequence, it is possible to identify the correct UMI sequence. See Appendix A for the sequences of these UMIs. In addition, these libraries contain 8 bp unique, dual index sequences added during PCR amplification with xGen UDI Primer Pairs.

Figure 3. Sequencing read structure.

APPENDIX A: FIXED UMI SEQUENCES

The Ligation 1 Adapter contains 32 optimized, fixed UMI sequences that are 8 base pairs in length.

Adapter name	UMI sequence	Adapter name	UMI sequence
UMI_1	GAGACGAT	UMI_17	GCACAACT
UMI_2	TTCCAAGG	UMI_18	GCGTCATT
UMI_3	CGCATGAT	UMI_19	GAAGGAAG
UMI_4	ACGGAACA	UMI_20	ACTGAGGT
UMI_5	CGGCTAAT	UMI_21	TGAAGACG
UMI_6	GCTATCCT	UMI_22	GTTACGCA
UMI_7	TGGACTCT	UMI_23	AGCGTGTT
UMI_8	ATCCAGAG	UMI_24	GATCGAGT
UMI_9	CTTAGGAC	UMI_25	TTGCGAAG
UMI_10	GTGCCATA	UMI_26	CTGTTGAC
UMI_11	TCGCTGTT	UMI_27	GATGTGTG
UMI_12	TTCGTTGG	UMI_28	ACGTTCAG
UMI_13	AAGCACTG	UMI_29	TTGCAGAC
UMI_14	GTCGAAGA	UMI_30	CAATGTGG
UMI_15	ACCACGAT	UMI_31	ACGACTTG
UMI_16	GATTACCG	UMI_32	ACTAGGAG

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For more information, go to: **www.idtdna.com/ContactUs**

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