xGen Prism DNA Library Prep Kit

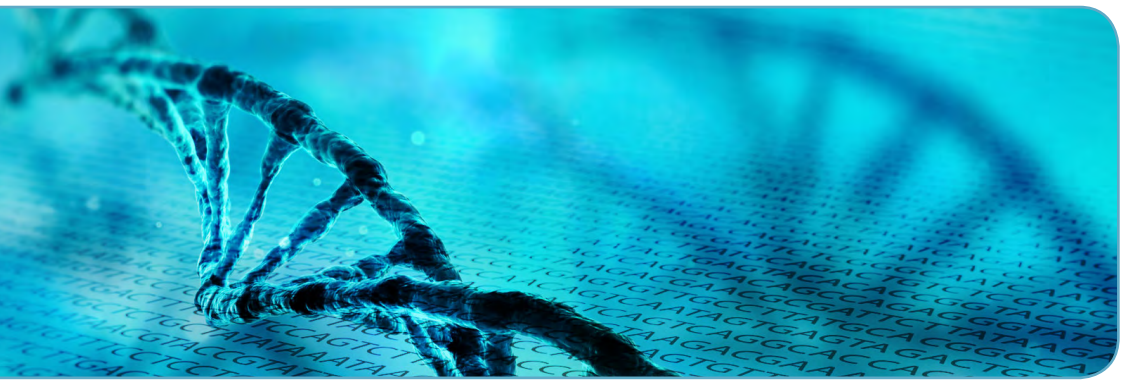

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Table of contents

Overview

The xGen Prism DNA Library Prep 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 method features a proprietary single-stranded ligation strategy that maximizes conversion, suppresses adapter-dimer formation, and reduces chimera rates (Figure 1). Since dimer formation is negligible, a fixed concentration of adapter can be used, and aggressive size-selection is no longer required post-ligation. Altogether, this strategy delivers higher conversion and library complexity than conventional TA ligation–based methods, enabling highly sensitive, low-frequency variant detection. In addition, fixed single-stranded unique molecular index (UMI) sequences are added to the insert during Ligation 1 (shown in dark blue and green in Figure 1). This unique single-stranded ligation to fixed UMIs enables strand-specific molecular indexing by independently tagging the top and bottom strands. After conversion to fully double-stranded products, libraries are PCR amplified (Figure 1).

Because the UMI sequences are fixed, even when there are sequencing or PCR errors in the UMI, it is possible to identify and correct these errors. This prevents artificial inflation of library complexity due to errors in the UMI sequence. Depending on the application, these fixed UMIs can be used for various deduplication and error correction strategies.

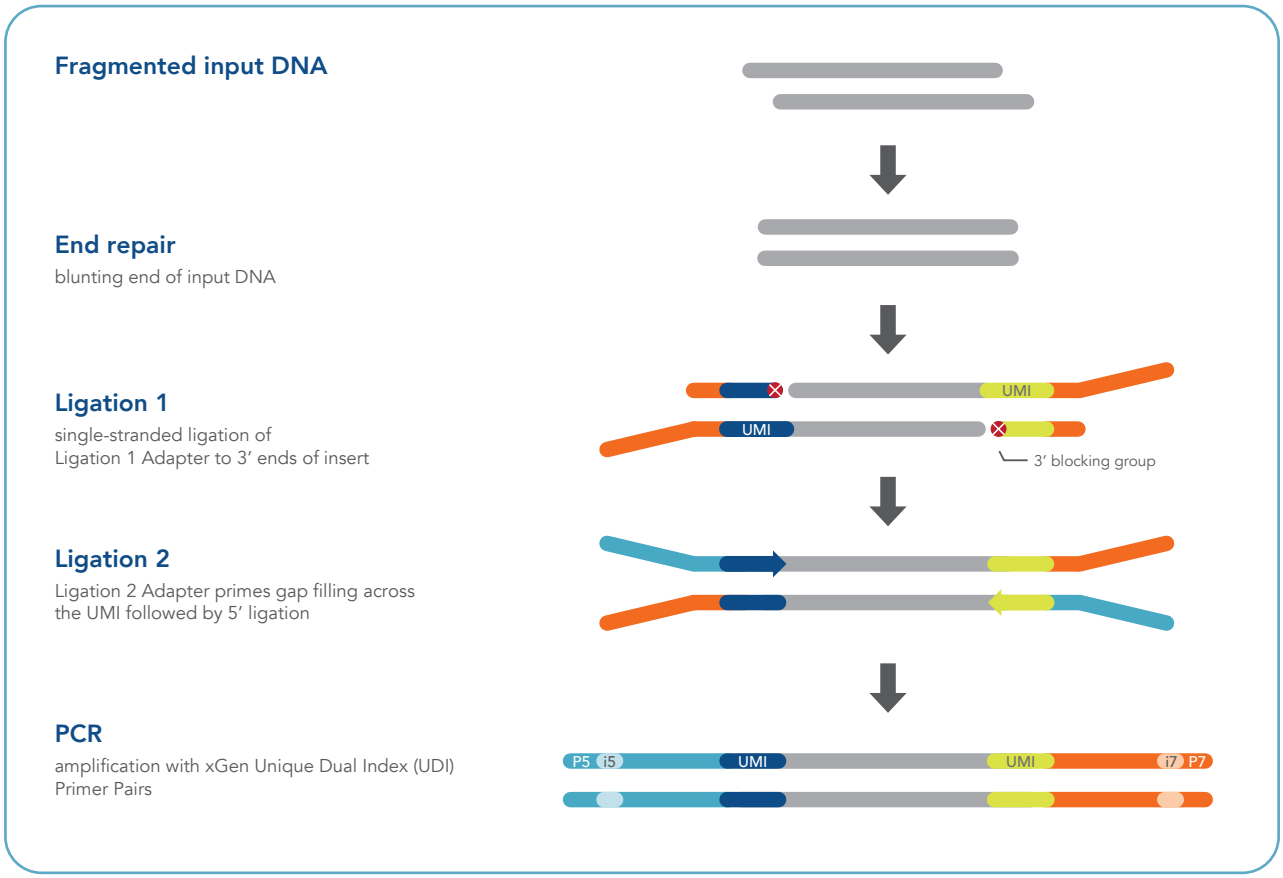

Figure 1: xGen Prism DNA Library Prep Kit process. Illustration of the library construction method.

Workflow

The xGen Prism DNA Library Prep Kit workflow takes about 2 hours before PCR amplification. There are 4 major steps to perform in this protocol:

- **End repair—**The End Repair Enzyme Mix converts cfDNA or sheared input DNA into blunt-ended DNA ready for ligation.
- **Ligation 1—**The Ligation 1 Enzyme catalyzes the single-stranded addition of the Ligation 1 Adapter to just the 3′ ends of the insert. 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—**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—**PCR is used to incorporate sample indexes and incorporate sequences needed for Illumina sequencing. These reagents are not included in this kit and should be purchased separately. (See Consumables from IDT—Reagents and Consumables from other suppliers)

1	Perform end repair		Total time: 35 min
2	Perform post-end repair cleanup	2.5X AMPure	Total time: 20 min
3	Perform Ligation 1		Total time: 40 min
	Pause		
4	Perform Ligation 2		Total time: 45 min
5	Perform post-ligation 2 cleanup	2.5X PEG/NaCl	Total time: 20 min
	Stop		
6	Run PCR amplification		Total time: 15–30 min (variable depending on input)
7	Perform post-PCR cleanup	1.3X AMPure	Total time: 20 min

Consumables and equipment

Consumables from IDT—Kit contents

xGen Prism DNA Library Prep Kit components		16 rxn	96 rxn	Storage
End repair module	End Repair Buffer	108 µL	645 µL	–20°C
	End Repair Enzyme Mix	54 µL	323 µL	
Ligation 1 module	Ligation 1 Buffer	448 µL	2688 µL	
	Ligation 1 Adapter	36 µL	215 µL	
	Ligation 1 Enzyme	54 µL	323 µL	
Ligation 2 module	Ligation 2 Buffer	81 µL	484 µL	
	Ligation 2 Adapter	72 µL	430 µL	
	Ligation 2 Enzyme A	9 µL	54 µL	
	Ligation 2 Enzyme B	18 µL	108 µL	
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
Indexing Primers (choose one):	
xGen UDI Primer Pairs, Index 1–16	10005975
xGen UDI Primer Pairs, Index 1–96	10005922
Custom indexing options	applicationsupport@idtdna.com

Consumables from other suppliers

Item	Supplier	Catalog #
Buffer EB (10 mM Tris-HCl, pH 8.5), 250 mL	Qiagen	19086
Absolute ethanol (200 proof)	Various suppliers	Varies
Polymerase master mix: KAPA Biosystems® HiFi HotStart ReadyMix, 1.25 mL (50 rxn) or 6.25 mL (250 rxn)	Roche	KK2601 or KK2602
Purification beads: Agencourt® 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®, or equivalent	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
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851 or Q32854

Equipment

Item	Supplier	Catalog #
Microcentrifuge	Various suppliers	Varies
Thermal cycler	Various suppliers	Varies
Qubit 4 Fluorometer, or equivalent	Thermo Fisher Scientific	Q33226
Magnet options (choose one):		
Magnum™ EX Universal Magnet Plate	Alpaqua	A000380
Magnetic Stand-96	Thermo Fisher Scientific	AM10027

Guidelines

Reagent handling

Always store the xGen Prism DNA Library Prep Kit reagents at –20°C, with the exception of 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.

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. Spin all 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.

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:

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

Size selection during clean up steps

This protocol has been optimized with AMPure XP beads for whole genome sequencing (WGS) and targeted sequencing applications but can also be used with SPRIselect beads (Beckman Coulter).

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, we recommend using Covaris shearing to achieve average insert sizes of 150–300 base pairs. As cfDNA typically has an average size of 160 base pairs, no further fragmentation is required.

For FFPE samples, we recommend using standard quality control methods, such as Q-ratio with qPCR or the DNA Integrity Number (DIN) using size distribution (i.e., Bioanalyzer instrument). These methods can help you choose the appropriate number of PCR cycles for your sample. For cfDNA, we recommend 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 can reduced sample complexity and mass.

We have successfully generated libraries from 1–250 ng of high-quality genomic DNA,1–50 ng of cfDNA, and 25–250 ng of DNA extracted from FFPE samples. Although we have optimized xGen Prism DNA Library Prep Kit for degraded and damaged DNA, DNA quality can still have an impact on yield and sequencing metrics, particularly for low-quality FFPE samples. Still, we have successfully generated and sequenced libraries with DINs of 1.5 to 9.0.

Tip: We recommended determining dsDNA concentration using a Qubit Fluorometer, or a similar fluorometric method.

Protocol

Perform end repair

Before starting the protocol, make sure the AMPure and PEG/NaCl reagents are at room temperature (20–25°C). Also, prepare a fresh 80% ethanol solution.

1. Add 50 µL of each sample into a low, nucleic acid binding PCR plate.

   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 (µL):	**9**

   Note: If there is precipitate in the End Repair Buffer, vortex until the precipitate becomes clear in solution.

3. Pulse vortex the master mix for 10 seconds, then briefly centrifuge. Keep the master mix on ice.

4. Add 9 µL of End Repair Master Mix to each well and using a pipette set to 40 µL, pipette 10 times to mix.

5. Seal the plate and briefly centrifuge.

6. Run the following thermal cycler program:

End repair program		
Step	**Temperature***	**Time**
1	20°C	30 min
2	4°C	Hold

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

7. While the end program runs, make the Ligation 1 Master Mix in preparation for the Post-end repair cleanup.

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

8. Pulse vortex the master mix for 10 seconds, then briefly centrifuge. Keep the master mix on ice until ready to use.

9. After the End repair program reaches 4°C, proceed immediately to Post-end repair cleanup.

Perform post-end repair cleanup

Note: Before starting cleanup, make sure you have prepared the Ligation 1 Master Mix.

1. 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, up to 5 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; make sure 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; overdry beads will decrease final library yield.
9. Proceed immediately to Ligation 1.

Perform 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 that the beads are fully resuspended.
3. Run the following thermal cycler program:

Ligation 1 program		
Step	**Temperature***	**Time**
1	20°C	15 min
2	65°C	15 min
3	4°C	hold

*If possible, set lid temperature to 70°C. If lid cannot be programmed, set 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.

Perform 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 (µL):	**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! Make sure the beads are fully resuspended.

   Note: If you notice droplets on the sides of the well, gently centrifuge.

5. Run the following thermal cycler program:

Ligation 2 program		
Step	**Temperature***	**Time**
1	65°C	30 min
2	4°C	hold

*If possible, set lid temperature to 70°C. If lid cannot be programmed, set to 105°C.

6. After program completes, proceed **immediately** to Post-ligation 2 cleanup.

Perform post-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, up to 5 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.
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; overdry 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 liquid to clear completely for 1–2 minutes.

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

13. Carefully transfer 20 µL of eluted DNA into a new well. Proceed to Run PCR amplification or pause here.

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

Run 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 IDT application support to ensure that they are compatible with this protocol.

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

PCR program			
Step	**Temperature***	**Time**	**Cycles**
1	98°C	45 sec	Based on sample input (see Table 1)
2	98°C	15 sec	
	60°C	30 sec	
	72°C	30 sec	
3	72°C	1 min	
4	4°C	∞	

*Set lid temperature to 105°C.

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

	Number of cycles	
Input mass (ng)	gDNA or cfDNA	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.

Perform post-PCR cleanup

1. Add 65 µL of AMPure beads (1.3X volume) 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, up to 5 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.
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.
8. Dry the beads at room temperature for 1–3 minutes.

   Important! Never allow the beads to dry for >5 minutes; overdry 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.
12. 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.

13. Carefully transfer 30 uL of eluted DNA into a new well.

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

Note: For use in target sequencing, see the xGen hybridization capture of DNA libraries protocol.

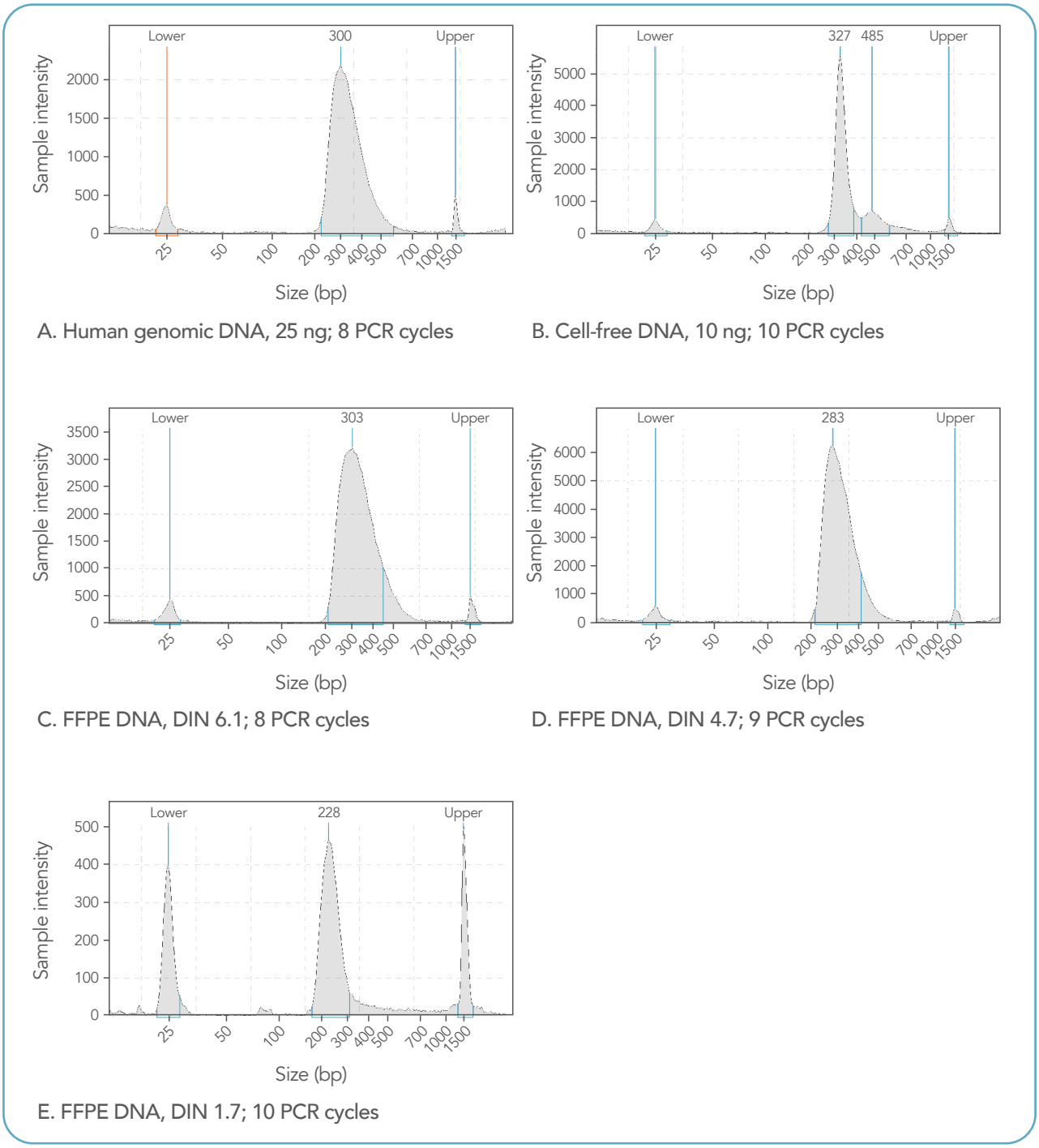

Figure 2: Representative electropherograms from libraries prepared with the xGen Prism DNA Library Prep Kit. Electropherograms were generated from 1:10 diluted final library with Agilent DNA ScreenTape on the TapeStation 2200 using the manufacturer's protocol. (**A**) 25 ng of human genomic DNA was sheared to 150 bp using the Covaris S2 instrument and amplified with 8 cycles of PCR. (**B**) Libraries were prepared from 10 ng of cell free DNA and amplified using 10 cycles of PCR. 100 ng of DNA extracted from FFPE was sheared to 150 bp using the Covaris S2 instrument. Libraries were amplified using (**C**) 8 cycles for FFPE DNA with DIN 6.1, (**D**) 9 cycles for FFPE DNA with DIN 4.7, and (**E**) 10 cycles for FFPE DNA with DIN 1.7.

Appendix A: Sequencing read structure

A schematic of the final sequencing libraries generated with the xGen Prism DNA Library Prep 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. Because 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 B for the sequences of these UMIs. In addition, these libraries contain 8 bp unique dual index sequences added during PCR amplification with IDT xGen UDI Primer Pairs. These UDIs can minimize sample misassignment from cross-contamination or index hopping.

Note: If you would like custom indexing primers, contact applicationsupport@idtdna.com for design recommendations that will be compatible with the xGen Prism DNA Library Prep Kit.

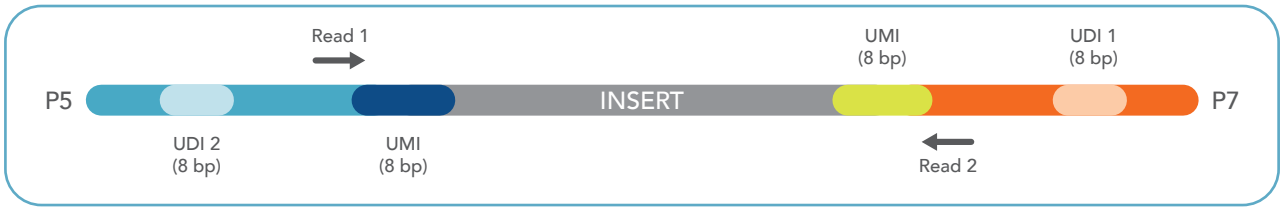

Figure 3: Sequencing read structure.

Appendix B: Fixed UMI sequences

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

Adaptor name	UMI sequence	Adaptor name	UMI sequence
PrismUMI_1	GAGACGAT	PrismUMI_17	GCACAACT
PrismUMI_2	TTCCAAGG	PrismUMI_18	GCGTCATT
PrismUMI_3	CGCATGAT	PrismUMI_19	GAAGGAAG
PrismUMI_4	ACGGAACA	PrismUMI_20	ACTGAGGT
PrismUMI_5	CGGCTAAT	PrismUMI_21	TGAAGACG
PrismUMI_6	GCTATCCT	PrismUMI_22	GTTACGCA
PrismUMI_7	TGGACTCT	PrismUMI_23	AGCGTGTT
PrismUMI_8	ATCCAGAG	PrismUMI_24	GATCGAGT
PrismUMI_9	CTTAGGAC	PrismUMI_25	TTGCGAAG
PrismUMI_10	GTGCCATA	PrismUMI_26	CTGTTGAC
PrismUMI_11	TCGCTGTT	PrismUMI_27	GATGTGTG
PrismUMI_12	TTCGTTGG	PrismUMI_28	ACGTTCAG
PrismUMI_13	AAGCACTG	PrismUMI_29	TTGCAGAC
PrismUMI_14	GTCGAAGA	PrismUMI_30	CAATGTGG
PrismUMI_15	ACCACGAT	PrismUMI_31	ACGACTTG
PrismUMI_16	GATTACCG	PrismUMI_32	ACTAGGAG

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