Twist cfDNA Library Preparation with the Twist UMI Adapter System

For use with the Twist NGS Workflow

This protocol details the steps needed to prepare cell-free DNA (cfDNA) libraries with unique molecular identifiers (UMIs). The Twist cfDNA Library Preparation Kit contains new enzymes and buffers that have been reformulated and optimized to provide best-in-class amplified, indexed libraries that can be used in whole genome sequencing (WGS) experiments or downstream with Twist target enrichments kits and sequenced on Illumina next-generation sequencing (NGS) systems. The Twist UMI Adapter System consists of Twist UMI Adapters and Twist Unique Dual Indexed (UDI) Primers. This cfDNA library preparation protocol is optimized for use with Twist target enrichment kits and should only be performed with reagents specified or their equivalents.

Twist NGS Workflow. *This NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist cfDNA Library Preparation Protocol, works in conjunction with the other component protocols.*

For Research Use Only. *Not intended for use in diagnostic procedures.*

PROTOCOL COMPONENTS

Please read the product packaging and storage recommendations carefully for each kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
107603: 16 rxn 107604: 96 rxn	Twist cfDNA Library Preparation Kit	Reagents for library construction	—
	Twist cfDNA Library Preparation Kit, 16/96 Samples	· 5X Twist ERAT Enzyme Mix · 10X Twist ERAT Buffer · 20X Twist DNA Ligation Mix 2.0 · 4X Twist DNA Ligation Buffer 2.0	–20°C
	Twist Purification Beads, 16/96 Samples	DNA Purification Beads	2-8°C
105040: 16 rxn 105041 105042 105043 105044: 96 rxn	Twist UMI Adapter System— TruSeq Compatible	Twist UMI Adapters and Twist UDI Primers provide UMI labeling and unique dual-indexed combinations with 1 reaction per index pair	–20°C

For kits that include the Twist cfDNA Target Enrichment components, use the following catalog numbers:
107609: Twist cfDNA Library Preparation and Hyb Mix Kit–16 Samples and 2 Reactions
107610: Twist cfDNA Library Preparation and Hyb Mix Kit–96 Samples and 12 Reaction

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist cfDNA Library Preparation Kit and the Twist UMI Adapter System.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
KAPA HiFi HotStart ReadyMix or validated equivalent*	Roche (PN KK2601 or KK2602)
Molecular biology grade water	—
10 mM Tris-HCl ph 8 (optional)	—
Buffer EB (optional)	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
Agilent DNA 7500 Kit	Agilent Technologies
Ethanol (200 proof)	—
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96 well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

**KAPA HiFi HotStart ReadyMix or validated equivalent needs to be ordered prior to preparation and is needed during the library generation process.*

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol and follow the instructions provided. Twist cannot guarantee the performance of the Twist cfDNA Library Preparation Kit with the Twist UMI Adapter System if modifications are made to the protocol.

This library preparation method may yield more material than needed for target enrichment. Excess product can be stored at –20°C for later use.

Test the compatibility of your thermal cycler and PCR tubes by incubating at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

An amplification polymerase must be ordered in order to perform this protocol. During the development of this protocol, Twist validated several amplification polymerases. For best results and performance metrics, Twist recommends using KAPA HiFi HotStart ReadyMix. If a different polymerase is used, it is recommended to perform validation experiments. The thermal cycler program and cycling conditions in this protocol are specific to KAPA HiFi HotStart ReadyMix.

GUIDELINES FOR SAMPLES

cfDNA SAMPLES

- For optimum performance, analyze the size distribution and quantification of the input cfDNA with an Agilent High Sensitivity DNA Kit or similar electropherogram assay before proceeding with end repair and dA-tailing reactions. Ensure the mode of the fragment size distribution is free from high molecular weight DNA contamination. To quantify, assess the concentration between the size range of 100 bp and 1 kb.
- Measuring cfDNA concentration by absorbance at 260 nm or fluorescence is not recommended.
- The recommended DNA input is 1-20 ng of cfDNA.
- Input DNA should be suspended in molecular biology grade water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- Use any desired cfDNA extraction method to prepare the cfDNA sample for use with this protocol.
- Using a lower cfDNA mass input will decrease library diversity and increase duplication rate but can be performed if a low cfDNA yield is obtained from your plasma extraction. Optimization of the following steps may be required to achieve optimal performance:
  - Amount of Twist UMI Adapter (Step 2.2, page 12)
  - Number of PCR Cycles (Step 3.1, page 14)

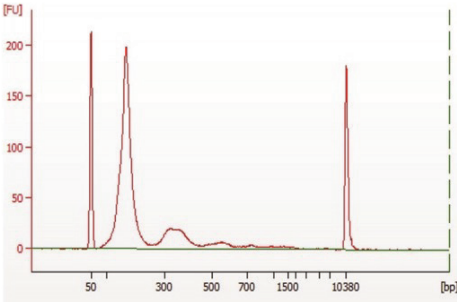
PROTOCOL OVERVIEW

This protocol begins with input cfDNA and generates amplified, indexed libraries for subsequent target enrichment or WGS. It features native cfDNA and Twist UMI Adapters with UDI Primers. This protocol allows you to perform cfDNA library preparation (Steps 1–3) in 2.5 hours.

cfDNA LIBRARY PREPARATION WITH TWIST UMI ADAPTER SYSTEM (cfDNA, 1 – 20 NG STARTING DNA MATERIAL)	TIME
STEP 1 **Perform End Repair and dA-Tailing** dA-Tailed cfDNA fragments	**1 hour**
STEP 2 **Ligate Twist UMI Adapters and Purify** cfDNA libraries ready for indexing	**30 minutes**
STEP 3 **PCR Amplify Using Twist UDI Primers, Purify, and Perform QC** Amplified, indexed libraries	**1 hour**

STEP 1 PERFORM END REPAIR AND DA-TAILING

The first step in this kit is to convert native cfDNA into dA-tailed fragments. Use any desired cfDNA extraction method to prepare the DNA input. For optimum performance, analyze the size distribution and quantification of the input cfDNA with an Agilent High Sensitivity DNA Kit or similar before proceeding with end repair and dA-tailing reactions. Ensure the input sample is free from high molecular weight gDNA and contains a primary peak at approximately 170 bp. To quantify, assess the concentration between the size range of 100 bp and 1 kb.



DNA fragment size distribution of a cfDNA fragment library, as analyzed using an Agilent High Sensitivity DNA Assay

Reagents Required

- Native cfDNA: 1 - 20 ng per sample
- Molecular biology grade water (chilled)
- Optional 10 mM Tris-HCl pH 8 or Buffer EB
- Agilent High Sensitivity DNA Kit (or equivalent)
- From the Twist cfDNA Library Preparation Kit, 16/96 Samples:
  - 5X Twist ERAT Enzyme Mix
  - 10X Twist ERAT Buffer

Before You Begin

- Thaw 5X Twist ERAT Enzyme Mix on ice, then mix by flicking the tube with a finger.
- Thaw 10X Twist ERAT Buffer on ice, then mix by pulse vortexing for 2 seconds. If the buffer contains a white precipitate, vigorously vortex the buffer until the precipitate dissolves.
- Place molecular biology grade water, 10 mM Tris-HCl pH 8, or Buffer EB on ice.

PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

1.1

Program the thermal cycler with the following conditions. Set the temperature of the heated lid to 70°C. Start the program to pre-chill the thermal cycler.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	32°C	22 min
3	65°C	30 min
4	4°C	HOLD

1.2 Use the Agilent High Sensitivity DNA Kit to determine the concentration of your cfDNA samples.

⚠ IMPORTANT: Ensure the mode of the fragment size distribution is free from high molecular weight contamination. Assess the concentration between the size range of 100 bp and 1 kb.

1.3 Bring the cfDNA samples with the desired concentration to a total volume of 35 µl in water, 10 mM Tris-HCl pH 8, or buffer EB. Mix well with gentle pipetting.

1.4 Add 35 µl of each diluted cfDNA sample (1 - 20 ng total cfDNA) into a 0.2-ml thin-walled PCR strip-tube or well of a 96-well thermal cycling plate and place on ice.

1.5 Pulse-spin to ensure all of the solution is at the bottom of the tube.

PERFORM END REPAIR AND dA-TAILING (ERAT)

1.6 Prepare an ERAT reaction master mix in a 1.5-ml microcentrifuge tube on ice. Use the volumes listed below. Mix thoroughly by gentle pipetting.

REAGENT	VOLUME PER REACTION*
10X Twist ERAT Buffer	5 µl
5X Twist ERAT Enzyme Mix	10 µl
Total	**15 µl**

**Prepare a master mix for multiple reactions.*

1.7 Add 15 µl ERAT reaction master mix (from Step 1.6) to each 35 µl cfDNA sample well or tube and mix well by gentle pipetting. Cap the tube and keep the reaction on ice.

1.8 Pulse-spin the sample plate or tubes and immediately transfer to the pre-chilled thermal cycler.

1.9 Proceed with steps 2–4 of the thermal cycler program in Step 1.1 above (32°C step of the thermal cycler program).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 2 Ligate Twist UMI Adapters and Purify (see Before You Begin).

1.10 When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

PROCEED IMMEDIATELY TO STEP 2: LIGATE TWIST UMI ADAPTERS AND PURIFY

STEP 2 LIGATE TWIST UMI ADAPTERS AND PURIFY

Ligate Twist UMI Adapters to the dA-tailed DNA fragments from Step 1 and purify to generate cfDNA libraries ready for index introduction through amplification in Step 3.

This protocol is compatible with Twist Full Length UDI Adapters. Please see Appendix C: Twist Full Length UDI Adapters.

Reagents Required

- dA-tailed DNA fragments (from Step 1.10)
- Ethanol
- Molecular biology grade water (chilled)
- Optional 10 mM Tris-HCl pH 8.0 of Buffer EB
- From the Twist cfDNA Library Preparation Kit, 16/96 Samples:
  - 20X Twist DNA Ligation Mix 2.0
  - 4X Twist DNA Ligation Buffer 2.0
- From the Twist UMI Adapter System:
  - Twist UMI Adapters
- From the Twist Purification Beads, 16/96 Samples:
  - DNA Purification Beads

Before You Begin

- Thaw on ice, then mix by vortexing
  - Molecular biology grade water
  - Twist UMI Adapters (tube; utilized for all samples)
  - 20X Twist DNA Ligation Mix 2.0
  - 4X Twist DNA Ligation Buffer 2.0
- Prepare 1 ml 80% ethanol for each sample (for use in Steps 2 and 3 of the protocol).
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (for use in both Steps 2 and 3 of the protocol).

LIGATE TWIST UMI ADAPTERS

2.1

Program the thermal cycler with the following conditions. Turn off the heated lid or set to minimum temperature. Start the program to pre-chill the thermal cycler

STEP	TEMPERATURE	TIME
1	25°C	HOLD
2	25°C	15 min
3	4°C	HOLD

2.2 Add 3 µl Twist UMI Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.

2.3 Prepare the ligation master mix in a 1.5-ml microcentrifuge tube on ice as indicated below. Mix well by vortexing.

REAGENT	VOLUME PER REACTION*
Water (chilled)	17 µl
4X Twist DNA Ligation Buffer 2.0	25 µl
20X Twist DNA Ligation Mix 2.0	5 µl
Total	**47 µl**

**Prepare a master mix for multiple reactions. Do not add Twist UMI adapters to the ligation master mix.*

2.4 Add 47 µl of the ligation master mix to the sample(s) from Step 2.2 and mix well by gentle pipetting.

2.5 Seal or cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.

2.6 Incubate the ligation reaction at 25°C by proceeding with steps 2–3 of the thermal cycler program in Step 2.1 above (25°C step of the thermal cycler program).

⚠ IMPORTANT: Turn off the heated lid or set to minimum temperature.

NOTE: While the thermal cycler program is running, prepare the reagents for Step 3 (see Step 3: PCR Amplify Using Twist UDI Primers, Purify, and Perform QC).

PURIFY

2.7 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

2.8 Add 90 µl (0.9x) of homogenized DNA Purification Beads to each ligation sample from Step 2.6. Mix well by vortexing.
NOTE: The ligase works well in low PEG conditions and the buffer contains less PEG to decrease viscosity for better automation handling. A higher SPRI ratio can be used to recover shorter cfDNA fragments.

2.9 Incubate the samples for 5 minutes at room temperature.

2.10 Place the samples on a magnetic plate for 1 minute or until supernatant clears.

2.11 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

2.12 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

2.13

2.14 Repeat the wash once, for a total of two washes, while keeping the samples on the magnetic plate.

Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.

2.15 NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

2.16 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

Remove the plate or tubes from the magnetic plate and add 15 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.

2.17 NOTE: For high-throughput or automated workflows, consider eluting in 17 µl for ease of use.

2.18 Incubate at room temperature for 2 minutes.

2.19 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

Transfer 15 µl of the clear supernatant containing the ligated and indexed libraries to a clean thin-walled PCR 0.2-ml strip-tube or 96-well thermal cycling plate. Bead carryover will not impact performance.

PROCEED TO STEP 3: PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC

STEP 3 PCR AMPLIFY USING UDI PRIMERS, PURIFY, AND PERFORM QC

Amplify the cfDNA libraries with Twist UDI Primers, purify them, and perform quality control (QC) analysis to complete the protocol.

Reagents Required

- Ligated, adapted libraries (from Step 2.19)
- KAPA HiFi HotStart ReadyMix or validated equivalent
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- Optional 10 mM Tris-HCl pH 8 or Buffer EB
- From the Twist UMI Adapter System:
  - Twist UDI Primers

Before You Begin

- Thaw on ice, then mix by vortexing:
  - Twist UDI Primers (plate with single use primers)
  - KAPA HiFi HotStart ReadyMix or validated equivalent

PREPARE THE THERMAL CYCLER AND PCR MIX

3.1

Program a thermal cycler with the following conditions.* Set the temperature of the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	*Varies. Use the table to the right.*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

MASS INPUT	# OF CYCLES
>1 – 3 ng	12
4 – 6 ng	10
7 – 10 ng	9
10+ ng	8

**Thermal cycle program and cycling have been optimized using KAPA HiFi HotStart ReadyMix. Slight adjustments may be needed if different polymerase is used.*

PERFORM PCR

3.2 Add 10 µl of Twist UDI Primer from the provided 96-well plate to each of the cfDNA libraries from Step 2.19.

NOTE: For index selection and multiplexing refer to the pooling guidelines in the Appendix A.

3.3 Add 25 µl of KAPA HiFi HotStart ReadyMix or validated equivalent to the cfDNA libraries from Step 3.2 and mix well by gentle pipetting.

3.4 Pulse-spin sample plate or tube and immediately transfer to the thermal cycler. Start the program.

3.5 Remove the sample(s) from the block when the thermal cycler program is complete. Proceed to the Purify step.

PURIFY

3.6 Vortex the pre-equilibrated DNA Purification Beads until mixed.

3.7 Add 50 µl (1x) of homogenized DNA Purification Beads to each ligation sample from Step 3.5. Mix well by vortexing.

3.8 Incubate the samples for 5 minutes at room temperature.

3.9 Place the samples on a magnetic plate for 1 minute.

3.10 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

3.11 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.

3.12 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.

3.13 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.

NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

3.14 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

3.15 Remove the plate or tubes from the magnetic plate and add 22 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.

3.16 Incubate at room temperature for 2 minutes.

3.17 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

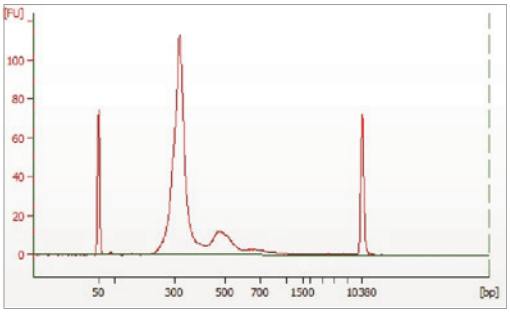
3.18 Transfer 20 µl of the clear supernatant containing the amplified, indexed libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

PERFORM QC

3.19 Quantify and validate the size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 7500 Assay.

Amplification with the recommended number of PCR cycles using KAPA HiFi HotStart ReadyMix should result in final concentration values of ≥80 ng/µl. Concentrations below 80 ng/µl may reflect inefficient sample preparation and can result in reduced library diversity after hybridization.

For cfDNA libraries, a primary library peak should be observed at around 320 bp and a secondary peak should be observed around 480 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of a cfDNA library sample that was prepared as described. Note the prominent peak at ~320 bp and secondary peak at ~480 bp.

Depending on mass input and quality of cfDNA, some libraries may produce an overabundance of heteroduplexes, which can interfere with final library quantification and downstream NGS analysis. To more accurately quantify, qPCR assays may be necessary.

NOTE: For library mass into downstream target enrichment experiments, refer to Appendix B.

STOPPING POINT: If not proceeding immediately to a Twist target enrichment system, store the amplified, indexed libraries at –20°C.

END OF WORKFLOW

APPENDIX A: UDI ADAPTER SEQUENCES AND POOLING GUIDELINES

UDI SEQUENCES

For a complete guide of the Twist UDI sequences used in the Twist Universal Adapter System, please refer to the UDI Sequences Reference Spreadsheet and the Sample Sheet Template. All files are available for download at twistbioscience.com/resources/protocol/Unique%20Dual-Index-Sequences-reference-spreadsheets-and-sample-sheet-templates.

POOLING GUIDELINES

Twist UDI primers are base balanced for next-generation sequencing on a column basis. When pooling unique dual-indexed libraries for 8-plex hybridization, it is recommended that libraries be selected from a single column. Multiple columns may be selected in any desired combination across a single plate or multiple plates for sequencing.

Twist UDI primer plate layouts and pooling guidelines.

Twist UMI Adapter System: TruSeq Compatible, 16 Samples (105040)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9										
B	2	10										
C	3	11										
D	4	12										
E	5	13										
F	6	14										
G	7	15										
H	8	16										

NOTE: The indexes in the 16 sample plate are not the same in 96 samples, Plate A.

APPENDIX A: UDI ADAPTER SEQUENCES AND POOLING GUIDELINES

Twist UMI Adapter System: TruSeq TruSeq Compatible, 96 Samples, Plates A to D (105041, 105042, 105043, 105044)

Plate A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Plate B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Plate C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Plate D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

APPENDIX B: LIBRARY MASS INTO TWIST TARGET ENRICHMENT

cfDNA MASS INPUT AND DIVERSITY

Library mass going into target enrichment is calculated based on sample mass input into library preparation for optimal downstream target enrichment complexity metrics. To calculate the amount of library mass going into target enrichment, multiply the sample mass input into library preparation by 80x. Follow the formula below for library mass into target enrichment.

$$\textit{Minimum Mass Into TE} = 80 \times \textit{Mass Into Library Prep}$$

GUIDELINES FOR MULTIPLEX SAMPLES

Maximum recommended library mass into target enrichment should not be greater than 12.8 µg. When pooling libraries from variable starting mass inputs, each library should have the same mass added to the pool. To calculate that mass, multiply the highest mass input by 80x.

For example, for an 8-plex where the highest sample mass input is 20 ng, pool 1,600 ng from each library for a total of 12.8 µg. For an 8-plex where the highest sample mass input is 10 ng, pool 800 ng from each library for a total of 6.4 µg.

APPENDIX C: TWIST FULL LENGTH UDI ADAPTERS

This workflow is compatible with Twist Full Length Unique Dual Index (UDI) Adapters. Twist provides 1536 unique indexes (SKUs 107376-107379, and 107383-107394). These adapters are typically used in PCR-free library prep workflows, where sample indexes are added in the ligation step. No UMI will be introduced when using Twist full-length UDI adapters.

FULL LENGTH UDI SEQUENCES

For a complete guide to the Twist Full Length UDI adapter indexes, please refer to the Full Length UDI Sequences Reference Spreadsheet and Sample Sheet Template.

All files are available for download at: www.twistbioscience.com/resources/data-files/full-length-unique-dual-index-sequences-reference-spreadsheets-and-sample

ADAPTER INPUT

To obtain high conversion and minimize adapter dimer product when using the Twist Full Length UDI Adapter System, 1 µl of adapter with 0.9x SPRI ratio is recommended for 1-20 ng of cfDNA.

MODIFIED STEPS

The following steps in Step 2 of this protocol are impacted (see bolded and italicized values):

2.2 Add ***1 µl*** Twist Full Length UDI Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.

2.3 Prepare the ligation master mix in a 1.5-ml microcentrifuge tube on ice as indicated below. Mix well by vortexing.

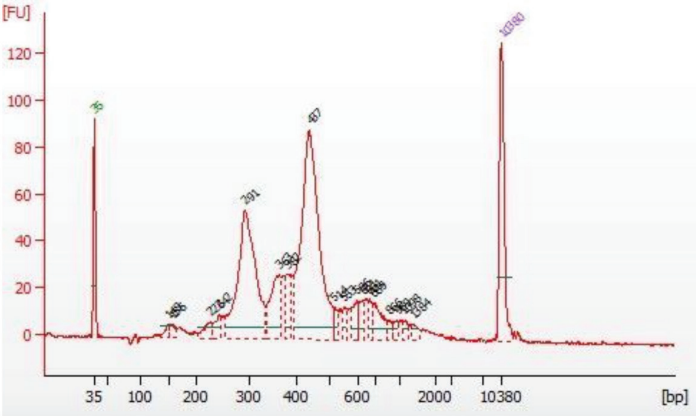
REAGENT	VOLUME PER REACTION*
Water (chilled)	***19 µl***
4X Twist DNA Ligation Buffer 2.0	25 µl
20X Twist DNA Ligation Mix 2.0	5 µl
Total	***49 µl***

**Prepare a master mix for multiple reactions. Do not add Twist Full Length UDI adapters to the ligation master mix.*

2.4 Add ***49 µl*** of the ligation master mix to the sample(s) from Step 2.2 and mix well by gentle pipetting.

LIBRARY QC

For cfDNA libraries with Full Length UDI adapters, a primary library peak should be observed at around 430 bp and a secondary peak should be observed around 620 bp. Other peaks observed in the electropherogram are artifacts from single adapter ligations. Due to the structure of the Full Length UDI adapters, the fragments may migrate slower in the capillary gel electrophoresis assay causing the sizes to appear slightly skewed.



Electropherogram generated by an Agilent 7500 DNA analysis of a cfDNA library sample that was prepared as described. Note the prominent peak at ~430 bp and the secondary peak at ~620 bp.

END OF APPENDIX