Twist 96-Plex Library Preparation Protocol

For Use With Twist NGS Workflow.

The Twist 96-Plex Library Preparation kit enables high-throughput library construction for whole genome sequencing. This protocol details the steps necessary to generate gDNA libraries compatible with Illumina sequencing systems and barcoded for demultiplexing. This library preparation protocol should only be performed with reagents specified.

Twist NGS workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis.

Protocol Components

TWIST 96-PLEX LIBRARY PREPARATION KIT			
CATALOG #	**NAME**	**DESCRIPTION**	**STORAGE**
106541: 480 rxn (Box 1A and 2) 106542: 480 rxn (Box 1B and 2) 106543: 960 rxn (Box 1A, 1B, and 2)	Twist 96-Plex Library Preparation 1A	Enzyme I 10x Enzyme Buffer I dNTP Mix I Primer B Enzyme II 5x Enzyme II Buffer dNTP Mix II 2x PCR Amplification Mix Universal PCR Primer Index PCR Primer bc#1, #2, #3, #4, #5 40% GC Primer A Plate 80% GC Primer A Plate Foil Seals (10x)	-20°C
	Twist 96-Plex Library Preparation 1B	Enzyme I 10x Enzyme Buffer I dNTP Mix I Primer B Enzyme II 5x Enzyme II Buffer dNTP Mix II 2x PCR Amplification Mix Universal PCR Primer Index PCR Primer bc#6, #7, #8, #9, #10 40% GC Primer A Plate 80% GC Primer A Plate Foil Seals (10x)	-20°C
	Twist 96-Plex Library Preparation 2	Capture Beads HS Buffer Bead Wash Buffer DNA Purification Beads	2 to 8°C

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MATERIALS SUPPLIED BY USER

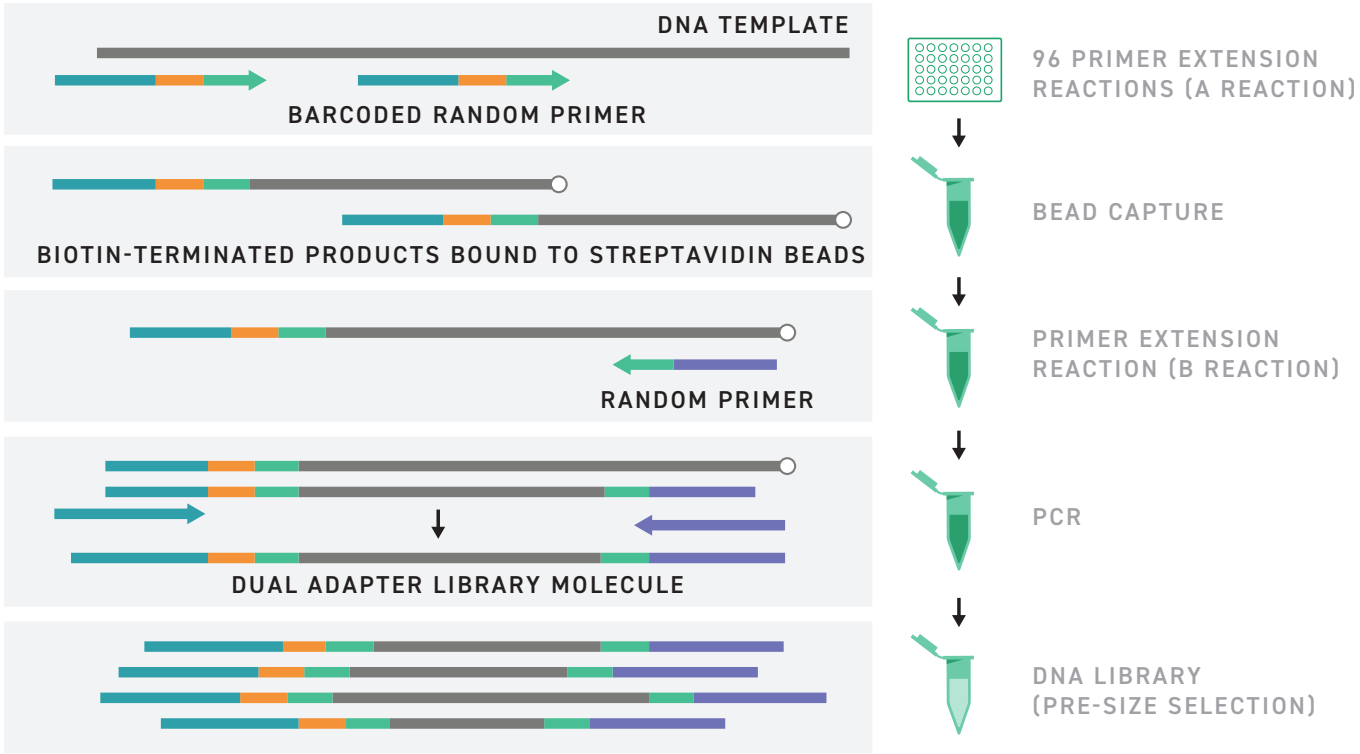
The following materials are required to generate enriched libraries using the Twist Target Enrichment workflow.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
0.5M EDTA	Fisher Scientific
10 mM Tris-HCl pH 8	—
1N NaOH	VWR
1.5 mL DNA LoBind microcentrifuge tubes	VWR/Eppendorf
Thin-walled PCR 0.2 mL strip tubes	Eppendorf
96-well DNA LoBind thermal cycling plates	Eppendorf
1.5 mL compatible magnetic stand	Beckman Coulter
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantification Assay	Thermo Fisher Scientific
Qubit dsDNA High Sensitivity Quantification Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex Mixer	—
Benchtop mini-centrifuge for 0.2 mL tubes	—
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

PROTOCOL OVERVIEW

The Twist 96-Plex Library Preparation kit prepares 96 DNA samples for Illumina-based sequencing at a time. The protocol requires 50 ng of high quality input DNA for optimal sequencing results. The first step of the protocol, a polymerase-mediated primer extension reaction, is performed in a 96-well plate. Following this step, reaction products are combined into one pool for all subsequent steps (see workflow figure below). Final prepared libraries will have barcodes and adapters compatible with Illumina sequencing chemistries. Experienced users can expect to complete one 96 sample prep workflow within four hours.

96-PLEX LIBRARY PREPARATION WORKFLOW		ESTIMATED TIME
STEP 1	Primer Extension and Termination Reaction	1.5 hrs
STEP 2	DNA Capture and Library Conversion	1 hr
STEP 3	Amplification	0.5 hrs
STEP 4	Purification with Size Selection and Library QC	1 hr



*This product is for **research use only**.*

STEP 1 PRIMER EXTENSION AND TERMINATION "A" REACTION

Perform barcoded randomer extension on genomic DNA (gDNA) followed by biotinylated extension termination.

Reagents Required

- gDNA: 50 ng per sample
- Qubit dsDNA Broad Range Quantitation Assay
- Ethanol
- Molecular biology grade water
- 0.5M EDTA
- From the –20°C Reagent Box:
  - 10x Enzyme I Buffer
  - dNTP Mix 1
  - Enzyme I
  - 40% GC Primer A Plate and/or 80% GC Primer A Plate
- From 4°C Reagent Box:
  - Twist DNA Purification Beads

Before You Begin

- Thaw all –20°C Reagents and Primers on ice, pulse-spin to ensure all of the solution is at the bottom of the tube
- Prepare 10 mL of 80% ethanol
- Equilibrate Twist DNA Purification Beads to room temperature for at least 30 minutes

PREPARE THERMAL CYCLER, SAMPLES, AND REAGENTS

1.1 Preheat a thermal cycler to 98˚C for heat denaturing samples.

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

STEP	TEMPERATURE	TIME
1	92˚C	3 minutes
2	16˚C	5 minutes
3	68˚C*	15 minutes
4	4˚C	HOLD

⚠ IMPORTANT: Set ramp rate between steps 2 and 3 to 0.1˚C/s

1.3 Use the Qubit dsDNA Broad Range Quantitation Assay to determine the concentration of your gDNA samples.

1.4 Dilute the gDNA samples to 12.5 ng/µL with water or 10 mM Tris-HCl pH 8. Mix well with gentle pipetting.

1.5 Add 4 µL of each diluted gDNA sample (50 ng total gDNA) into a thin-walled PCR 0.2 mL strip tube or well of a 96-well thermal cycling plate, and place on ice.

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

1.7 Transfer the sample plate to the preheated thermal cycler and incubate for 1 minute at 98˚C.

1.8 Remove the sample plate from the block and place on ice for 3 minutes. Pulse spin sample plate to ensure all solution is at the bottom of the plate.

PERFORM EXTENSION AND TERMINATION REACTION

1.9 Prepare the master mix in a 1.5 mL LoBind microcentrifuge tube on ice. Use the volumes listed below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION
dNTP Mix 1	2 µL
10X Enzyme 1 Buffer	1µL
Enzyme 1	1µL

Extension Master Mix

1.10 Add 4 µL of extension master mix into each sample well of a 96-well LoBind plate.

1.11 Add 2 µL of either Low GC or High GC Primer A into each well of the 96-well LoBind plate, and place on ice.

NOTES: Primer A contains a sample barcode that will label each sample in a well of a 96-well plate with a unique barcode. Primer A choice should be based on the following guidelines:

- Use Low GC Primer A for genomes of low GC content (< 50% GC)
- Use High GC Primer A for genomes of high GC content (> 50% GC)
- Use a combination of both primers in equal proportions for most plasmids or for genomes of intermediate GC content (40%–60% GC)

⚠ IMPORTANT: When removing the plate seal, take caution to avoid cross contamination of the wells. When finished with the Primer A plate, reseal using one of the provided foil seals.

1.12 Transfer 4 µL from Step 1.8 of the heat denatured sample to the corresponding well of the plate containing the extension mix and primer. Mix by pipetting up and down 5–10 times. Pulse spin sample plate to ensure all solution is at the bottom of the plate.

1.13 Transfer plate back to a thermal cycler and begin the thermal cycler program listed at Step 1.2.

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

TERMINATION AND PURIFICATION OF EXTENSION PRODUCTS

1.15 Pulse spin sample plate to ensure all solution is at the bottom of the plate.

1.16 Transfer all of the contents of the sample plate to a single 1.5 mL LoBind microcentrifuge tube.

1.17 Prepare the following EDTA solution using the volumes listed below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION	VOLUME PER 100 REACTIONS
0.5M EDTA	0.3 µL	30 µL
Molecular biology grade water	0.7 µL	70 µL

EDTA Solution

1.18 Add 1 µL of EDTA solution per sample to the sample-pool 1.5mL tube from above and mix well by pipetting.

1.19 Estimate the total volume of the sample pool with EDTA solution, split volume equally between two 1.5 mL LoBind microcentrifuge tubes.

1.20 Add 1.8X volume of homogenized Twist DNA Purification Beads to each 1.5 mL tube. Mix well by pipetting.

1.21 Incubate the samples for 10 minutes at room temperature.

1.22 Place the tubes on a magnetic stand for 5 minutes or until the solution is clear.

1.23 Remove and discard the supernatant without removing the tubes from the magnetic stand. Do not disturb the bead pellet.

1.24 Wash the bead pellet by gently adding 1300 µL freshly prepared 80% ethanol per tube (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

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

1.26 Carefully remove all remaining ethanol with a 200-µL pipet, making sure not to disturb the bead pellet.

1.27 Spin down the tubes. Carefully remove all remaining ethanol with a 10-µL pipette, making sure not to disturb the bead pellet.

1.28 Remove the tubes from the magnetic stand and add 50 µL 10 mM Tris-HCl pH 8 to each tube. Mix by pipetting until homogenized.

1.29 Incubate at room temperature for 10 minutes.

1.30 Place the tubes on the magnetic stand and let stand for 1 minute or until the beads form a pellet.

1.31 Transfer the 50 µL of the clear supernatant containing the terminated extension reactions from each tube to a clean thin-walled PCR 0.2 mL strip-tube, making sure not to disturb the bead pellet. Total volume in new tube should be ~100 µL

STOPPING POINT: If not proceeding immediately store samples at –20°C

STEP 2 DNA CAPTURE AND LIBRARY CONVERSION

Perform capture of extended terminated ssDNA library followed by conversion to dsDNA.

Reagents Required

- Extension products (from Step 1.31)
- Ethanol
- Molecular biology grade water
- 1N Sodium Hydroxide
- From the –20˚C Reagent Box:
  - Primer B
  - 5X Enzyme II Buffer
  - dNTP Mix II
  - Enzyme II
- From 4˚C Reagent Box:
  - Capture Beads
  - HS Buffer
  - Bead Wash Buffer

Before You Begin

- Thaw all –20˚C Reagents on ice.
- Prepare 10 mL of 80% ethanol
- Equilibrate Capture Beads, HS and Bead Wash Buffer to room temperature for at least 30 minutes

PREPARE THERMOCYCLER, SAMPLES, AND REAGENTS

2.1 Preheat a thermal block to 95˚C for incubating samples.

2.2 Prepare the following library conversion master mix in a 1.5 mL LoBind microcentrifuge tube on ice. Use the volumes listed below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION
5X Enzyme II Buffer	8 µL
dNTP Mix II	3 µL
Primer B	4 µL
Molecular biology grade water	24 µL

Library Conversion Master Mix

2.3 Prepare the following sodium hydroxide solution immediately before use in a 1.5 mL LoBind microcentrifuge tube. Use the volumes listed below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION
1N Sodium Hydroxide	20 µL
Nuclease free water	180 µL

Sodium Hydroxide Mix

PERFORM THE HYBRIDIZATION REACTION

2.4 Vortex the pre-equilibrated Capture Beads until mixed.

2.5 Add 40 µL Capture Beads to a clean thin-walled PCR 0.2 mL strip-tube. Prepare one tube for each reaction.

2.6 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

2.7 Add 200 µL HS Buffer to the tube(s) and mix by pipetting.

2.8 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

2.9 Add 40 µL HS Buffer to the tube(s) and mix by pipetting.

2.10 Incubate the tubes containing the samples at 95°C for 3 minutes, immediately place on ice for 2 minutes.

2.11 Pre-heat thermal block to 24°C for incubating samples.

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

2.12 Add samples to tubes containing washed Capture beads, mix the sample by pipetting, incubate the samples for 10 minutes at room temperature.

2.13 Mix the sample by pipetting.

2.14 Incubate the samples for an additional 10 minutes at room temperature.

2.15 Place the tube(s) on a magnetic stand for 1 minute until the solution is clear and remove the supernatant.

2.16 Remove the tube(s) from the magnetic plate and add 100 µL of sodium hydroxide solution to each tube. Mix by pipetting until homogenized.

2.17 Incubate at room temperature for 5 minutes.

2.18 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

2.19 Add 200 µL Bead Wash Buffer to the tube(s) and mix by pipetting.

2.20 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

2.21 Repeat the wash (Steps 2.19–2.20) once, for a total of two washes.

2.22 After the final wash, using a 10 µL pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.

2.23 Remove the tube(s) from the magnetic stand and add 39 µL library conversion master mix. Mix by pipetting until homogenized. Incubate this solution on ice.

LIBRARY CONVERSION REACTION

2.24 Add 1 µL Enzyme II. Mix by pipetting.

2.25 Incubate the tube(s) for 20 minutes at 24°C.

2.26 Transfer back to ice, incubate for 3 minutes.

2.27 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

2.28 Add 200 µL Bead Wash Buffer to the tube(s) and mix by pipetting.

2.29 Repeat the wash (Steps 2.27–2.28) two more times, for a total of three washes.

2.30 After the final wash, using a 10 µL pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.

2.31 Remove the tube(s) from the magnetic stand and add 42 µL water. Mix by pipetting until homogenized.

STEP 3 AMPLIFICATION

Amplify libraries using universal and index specific amplification primers.

Reagents Required

- Converted library products as Capture Bead Solution (from Step 2.31)
- Ethanol
- Molecular biology grade water
- From the –20˚C Reagent Box:
  - 2X PCR Amplification Mix
  - Universal PCR Primer
  - Index PCR Primer

Before You Begin

- Thaw all –20˚C Reagents and Primers on ice.

PREPARE THERMOCYCLER, SAMPLES, AND REAGENTS

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

CYCLE(S)	TEMPERATURE	TIME
1	98˚C	2 minutes
8	98˚C	20 seconds
	60˚C	30 seconds
	72˚C	30 seconds
1	72˚C	5 minutes
1	4˚C	HOLD

3.2 If the Capture Bead Solution from step 2.31 has settled, mix by pipetting.

3.3 Prepare a PCR mixture by adding the following reagents to the tube(s) containing the Capture Bead Solution. Mix by pipetting.

REAGENT	VOLUME PER REACTION
Universal PCR Primer	4 µL
Index PCR Primer (Barcodes 1–12)	4 µL
2X PCR Amplification Mix	50 µL

PCR Master Mix

PCR AMPLIFY

3.4 Pulse spin the tubes, transfer them to the thermal cycler and start the cycling program.

3.5 When the thermal cycler program is complete, remove the tube(s) from the block and place on ice.

3.6 Briefly spin the PCR tube in a microcentrifuge to pellet the beads. Place the tube on a magnetic stand, wait for the solution to clear and transfer the supernatant to a new 200 µL tube. Discard tube with remaining beads.

STOPPING POINT: If not proceeding immediately store samples at –20°C.

STEP 4 PURIFICATION AND SIZE SELECTION

Purification of DNA libraries and double sided size selection

Reagents Required

- Amplified library products (from Step 3.6)
- Ethanol
- Molecular biology grade water
- From 4˚C Reagent Box:
  - Twist DNA Purification Beads

Before You Begin

- Prepare 10 mL of 80% ethanol
- Equilibrate Twist DNA Purification Beads to room temperature for at least 30 minutes

PURIFY AND SIZE SELECT

4.1 Depending on the application, the final size of the library will vary. Refer to the table below to determine which bead ratio to use:

OPTION	BEAD VOLUME 1 (µL)	BEAD VOLUME 2 (µL)	APPROXIMATE LIBRARY FRAGMENT SIZE (BP)	APPROXIMATE INSERT SIZE (BP)
1	65	20	400	200
2	55	15	600	300
3	50	20	700	400

4.2 Add [Bead Volume 1] of homogenized Twist DNA Purification Beads to the tube(s) from Step 3.6. Mix by pipetting.

4.3 Incubate the samples for 5 minutes at room temperature.

4.4 Mix the sample by pipetting.

4.5 Incubate the samples for an additional 5 minutes at room temperature.

4.6 Place the tube(s) on a magnetic stand for 1 minute, then remove the clear supernatant and transfer to a new clean thin-walled PCR 0.2 mL strip-tube. Make sure to not disturb the bead pellet. Remove the tube containing the beads from the magnetic stand and discard.

4.7 Add [Bead Volume 2] of homogenized Twist DNA Purification Beads to the tube(s) from Step 4.6. Mix by pipetting.

4.8 Incubate the samples for 5 minutes at room temperature.

4.9 Mix the sample by pipetting.

4.10 Incubate the samples for an additional 5 minutes at room temperature.

4.11 Place the samples on a magnetic plate for 5 minutes.

4.12 Remove and discard the supernatant without removing the tubes from the magnetic stand. Do not disturb the bead pellet.

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

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

4.15 Carefully remove all remaining ethanol with a 20-µL pipet, making sure not to disturb the bead pellet.

4.16 Remove the tubes from the magnetic stand and add 25 µL 10 mM Tris-HCl pH 8 to each tube. Mix by pipetting until homogenized.

4.17 Incubate the samples for 10 minutes at room temperature.

4.18 Place the tubes on the magnetic stand and let stand for 1 minute or until the beads form a pellet.

4.19 Transfer the 25 µL of the clear supernatant containing the final library from each tube to a clean thin-walled PCR 0.2 mL strip-tube, making sure not to disturb the bead pellet.

PREFORM QC

4.20 Validate and quantify each library using an Agilent Bioanalyzer High Sensitivity DNA Kit and a Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay

STOPPING POINT: If not proceeding immediately to sequencing, store the amplified indexed libraries at –20°C

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LAST REVISED: January 6, 2023

REVISION	DATE	DESCRIPTION
3.0	Jan 6, 2023	
2.0	Mar 21, 2022	• Improvements to language to help improve clarity of workflow.