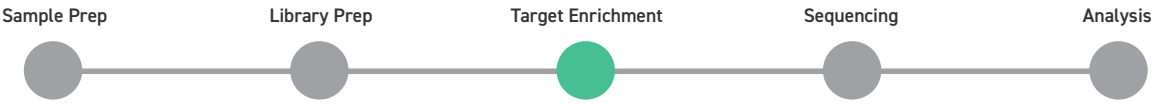
Twist cfDNA Target Enrichment Standard Hybridization Protocol

For use with the Twist NGS Workflow

The Twist cfDNA Target Enrichment protocol generates enriched cfDNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems. This manual details the steps for a 16-hour hybridization in a two-day target enrichment workflow.

A component of the Twist cfDNA Target Enrichment for NGS workflow, this protocol is:

- Designed for single or multiplex (up to 8-plex) hybridization reactions using either Twist fixed or custom panels
- Optimized for use with Twist cfDNA Library Preparation Kit
- Should only be performed with the reagents provided



Twist NGS Workflow. *The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist cfDNA Target Enrichment Standard Hybridization Protocol works in conjunction with the other component protocols.*

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

PROTOCOL COMPONENTS

Read the product packaging and storage recommendations carefully for each component, and store components as recommended below immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
TWIST STANDARD HYBRIDIZATION KIT V2 (FOR TARGET ENRICHMENT WITH STANDARD HYBRIDIZATION)			
101263: 2 rxn 100930: 12 rxn	Twist Hybridization Reagents	· Hybridization Mix · Hybridization Enhancer · Amplification Primer	–20°C
104133: 2 rxn 104135: 12 rxn	Twist Standard Wash Buffers v2	· Binding Buffer · Standard Wash Buffer 1* · Wash Buffer 2	2–8°C
TWIST PROBE PANELS (ORDERED SEPARATELY)			
Choice of panel type and reaction size	Twist Fixed Panel	Fixed content enrichment panel for hybridization reactions	–20°C
	Twist Custom Panel	Custom enrichment panel for hybridization reactions	–20°C
TWIST BLOCKERS & BEADS FOR TARGET ENRICHMENT			
100856: 2 rxn 100578: 12 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: · Universal Blockers · Blocker Solution	–20°C
101262: 2 rxn 100983: 12 rxn	Twist Binding and Purification Beads	For target enrichment and purification: · Streptavidin Binding Beads · DNA Purification Beads	2–8°C

**Buffer component in the Twist Standard Hybridization Kit v2.*

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MATERIALS SUPPLIED BY USER

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

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
1.5-ml compatible magnetic stand	Beckman Coulter
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
Ethanol (200 proof)	—
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer (preferred) or heat block for 1.5-ml tubes x2	Eppendorf
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0 or 4.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies
Vacuum concentrator	—

**KAPA HiFi HotStart ReadyMix or equivalent needs to be ordered prior to preparation and is needed during the target enrichment 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 provided instructions. Twist cannot guarantee the performance of the Twist cfDNA Target Enrichment Workflow if modifications are made to the protocol.

Test the compatibility of your thermal cycler and PCR tubes by incubating them 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.

If using a non-human capture panel, replace the Blocker Solution with a species-specific blocking solution (not provided).

This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.

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.

PROTOCOL OVERVIEW

This protocol is a component of the Twist NGS workflow. It begins with amplified, indexed cell-free DNA (cfDNA) libraries and generates target-enriched cfDNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems.

	HYBRIDIZATION TARGET ENRICHMENT WORKFLOW (AMPLIFIED, INDEXED LIBRARIES)	TIME
STEP 1	**Prepare cfDNA libraries for hybridization** Indexed cfDNA library pool STOPPING POINT	**1 hour**
STEP 2	**Hybridize capture probes with pools** Hybridized targets in solution	**16 hours**
STEP 3	**Bind hybridized targets to streptavidin beads** Captured targets on beads	**1.5 hours**
STEP 4	**Post-capture PCR amplify, purify, and perform QC** Enriched libraries STOPPING POINT	**1 hour**
STEP 5	**Sequence on an Illumina platform** Libraries ready for sequencing on Illumina platform	**—**

STEP 1 PREPARE LIBRARIES FOR HYBRIDIZATION

This step involves aliquoting the appropriate amount of amplified, indexed cfDNA libraries (generated previously in library preparation) and preparing the hybridization reaction solution. To generate cfDNA libraries, use the Twist cfDNA Library Preparation with the Twist UMI Adapter System protocol.

When multiplexing, follow the pooling guidelines found below.

Reagents Required

- Amplified, indexed cfDNA library

ALIQUOT AND DRY DOWN THE LIBRARY

1.1

This protocol supports a singleplex or 8-plex hybridization capture. The library mass used in target enrichment is calculated based on cfDNA sample mass into library preparation. Use the concentration of each amplified, indexed library to calculate the volume (in µl) of each library needed for hybridization when performing a singleplex or 8-plex.

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

Singleplex Target Enrichment

- Minimum recommendations for mass input going into target enrichment are based on mass input into cfDNA library preparation for singleplex samples only (see equation). This is a recommended minimum pass into capture.
- Increasing target enrichment mass up to 12.8 µg can be tolerated.

8-plex Target Enrichment

- Maximum recommended library yield 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 input is 10 ng, pool 800 ng from each library for a total of 6.4 µg.

1.2

Transfer the calculated volumes from each amplified, indexed library to an indexed library pool reaction tube for each hybridization being performed. Clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate are recommended to avoid unnecessary transfers in downstream steps.

NOTE: Check for a proper seal on the tube(s) as evaporation may occur leading to decreased performance.

1.3 Pulse-spin the indexed library pool tube(s) to minimize the amount of bubbles present.

1.4 Dry the indexed library pool(s) using a vacuum concentrator using low or no heat.

STOPPING POINT: If not proceeding immediately to Step 2, store the dried indexed library pool at –20°C for up to 24 hours.

PROCEED TO STEP 2: HYBRIDIZE CAPTURE PROBES WITH POOLS

STEP 2 HYBRIDIZE CAPTURE PROBES WITH POOLS

Use the dried indexed library pool(s) from Step 1 for performing the hybridization reaction.

⚠ IMPORTANT: Before proceeding with this step, test the compatibility of your thermal cycler and PCR tubes or plates by incubating them at 95°C for up to 5 minutes to ensure they 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.

Reagents Required

- Indexed library pool(s) from Step 1.4
- Twist fixed or custom panel
- From Twist Hybridization Reagents:
  - Hybridization Mix
  - Hybridization Enhancer
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution (If using a non-human capture panel, replace with species-specific blocking solution, not provided)

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and then pulse-spin.
- Set a heat block to 65°C.
- Program a 96-well thermal cycler to 95°C and set the heated lid to 105°C.

PREPARE THE PROBE SOLUTION

2.1 Heat the Hybridization Mix at 65°C in the heat block for 10 minutes or until all precipitate is dissolved, then cool to room temperature on the benchtop for 5 minutes.

2.2 Prepare a probe solution in a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate as indicated in the table below. Mix by flicking the tube(s).

REAGENT	VOLUME
Hybridization Mix	20 µl
Twist Fixed or Custom Panel	4 µl
Water	4 µl
Total	**28 µl**

NOTE: Hybridization Mix is very viscous. Pipette slowly to ensure accurate pipetting. Small white particles may be present in the Twist Fixed or Custom Panel tube(s). This will not affect the final capture product.

2.3 Resuspend the dried indexed library pool (from Step 1.4) by adding the reagents described below. Mix by flicking the tube(s).

REAGENT	VOLUME
Dried Indexed Library Pool	—
Blocker Solution*	5 µl
Universal Blockers	7 µl
Total	**12 µl**

*⚠ IMPORTANT: If using a non-human capture panel, replace with a species-specific blocking solution, not provided

PERFORM THE HYBRIDIZATION REACTION

2.4 Heat the probe solution to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.

2.5 While probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool at 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.

2.6 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.

2.7 Pulse-spin the tube(s) to ensure all solution is at the bottom of the tube(s).

2.8 Add 30 µl Hybridization Enhancer to the top of the entire capture reaction.

2.9 Pulse-spin the tube(s) to ensure there are no bubbles present.

⚠ IMPORTANT: Seal the tube(s) tightly to prevent excess evaporation over the 16-hour incubation.

2.10 Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C.

NOTE: Halting hybridization between 15–17 hours will not affect downstream capture quality.

PROCEED TO STEP 3: BIND HYBRIDIZED TARGETS TO STREPTAVIDIN BEADS

STEP 3 BIND HYBRIDIZED TARGETS TO STREPTAVIDIN BEADS

Reagents Required

- Hybridization reactions (from Step 2.10)
- From the Twist Hybridization Reagents:
  - Amplification Primers
- From the Twist Wash Buffers:
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- From Twist Binding and Purification Beads:
  - Streptavidin Binding Beads
  - DNA Purification Beads
- KAPA HiFi HotStart ReadyMix or validated equivalent

Before You Begin

- Preheat the following tubes at 48°C until any precipitate is dissolved:
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- For each hybridization reaction:
  - Equilibrate 800 µl Binding Buffer to room temperature.
  - Equilibrate 225 µl Standard Wash Buffer 1 to 68°C.
  - Preheat 700 µl Wash Buffer 2 at 48°C.
- Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes.

In preparation for Step 4 (Post-Capture PCR Amplify, Purify, and Perform QC):

- Thaw on ice:
  - KAPA HiFi HotStart ReadyMix or validated equivalent
  - Amplification Primers
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes.

PREPARE THE BEADS

3.1 Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.

3.2 Add 100 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

3.3 Add 200 µl Binding Buffer to the tube(s) containing the Streptavidin Binding Beads and mix by pipetting.

3.4 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.

3.5 Repeat the wash (Steps 3.3 and 3.4) two more times for a total of three washes.

3.6 After removing the clear supernatant from the third wash, add a final 200 µl Binding Buffer and resuspend the beads by vortexing until homogenized.

3.7 Heat the resuspended beads at 68°C for at least 10 min before continuing to Step 3.8.

BIND THE TARGETS

3.8 After the hybridization (Step 2.10) is complete, open the thermal cycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of preheated Streptavidin Binding Beads from Step 3.7. Mix by pipetting and flicking.

⚠ IMPORTANT: Rapid transfer directly from the thermal cycler at 70°C is a critical step for minimizing off-target binding. Do not remove the tube(s) of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binding Beads. Allowing to cool to room temperature for less than 5 minutes will result in as much as 10–20% increase in off-target binding.

3.9 Incubate the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 5 minutes at 68°C, agitation is not required.

NOTE: Do not vortex. Aggressive mixing is not required.

3.10 Remove the tube(s) containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube(s).

3.11 Place the tube(s) on a magnetic stand for 1 minute.

3.12 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

NOTE: Some Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product.

3.13 Remove the tube(s) from the magnetic stand and add 200 µl 68°C Standard Wash Buffer 1. Mix by pipetting.

3.14 Incubate the tube(s) for 5 minutes at 68°C.

3.15 Pulse-spin to ensure all solution is at the bottom of the tube(s).

3.16 Transfer the entire volume from Step 3.15 (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.

⚠ IMPORTANT: This step reduces background from non-specific binding to the surface of the tube.

3.17 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube(s) from the magnetic stand and add 200 µl of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube(s).

3.18 Incubate the tube(s) for 5 minutes at 48°C.

3.19 Place the tube(s) on a magnetic stand for 1 minute.

3.20 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

3.21 Repeat the wash (Steps 3.17–3.20) two more times, for a total of three washes.

3.22 After the final wash, use a 10-µl pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.

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

3.23 Remove the tube(s) from the magnetic stand and add 45 µl water. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

PROCEED TO STEP 4: POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

STEP 4 POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

Reagents Required

- Streptavidin Binding Bead slurry (from Step 3.23)
- Ethanol
- Molecular biology grade water
- Reagents thawed and equilibrated in Step 3:
  - DNA Purification Beads
  - KAPA HiFi HotStart ReadyMix or validated equivalent
  - Amplification Primers
- Agilent Bioanalyzer High Sensitivity DNA Kit (or equivalent)
- Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay

Before You Begin

- Prepare 500 µl 80% ethanol for each Streptavidin Binding Bead slurry to be processed.
- Determine the number of PCR cycles needed during post-capture amplification:
  - Follow the example table below for guidance as to how many PCR cycles should be used post-capture. The PCR cycling guidelines rely on a constant that is calculated by multiplying the panel size (kb) by the total library yield going into target enrichment (ng). The total library yield is the mass total of all individual libraries in a single target enrichment.

PCR Cycling Constant = *Panel Size* (kb) X *Total Library Mass Into TE* (ng)

EXAMPLE PANEL SIZE (kb)	EXAMPLE TOTAL LIBRARY MASS INTO T.E. (ng)	EXAMPLE PCR CYCLING CONSTANT
50	80	4,000
50	12,800	640,000
625	3,200	2,000,000
625	640	400,000
1,250	12,800	16,000,000
1,250	6,400	8,000,000
12,500	6,400	80,000,000
12,500	1,600	20,000,000

PREPARE THE BEADS, THERMAL CYCLER, AND PCR MIX

4.1

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

	STEP	TEMPERATURE	TIME	NUMBER 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	—

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

PCR CYCLING CONSTANT	PCR CYCLES
<11,000	20
11,001 – 22,000	19
22,001 – 44,000	18
44,001 – 88,000	17
88,001 – 180,000	16
180,001 – 350,000	15
350,001 – 710,000	14
710,001 – 1,400,00	12
1,400,001 – 2,800,000	11
2,800,001 – 5,700,000	10
5,700,001 – 11,000,000	9
11,000,001 – 20,000,000	8
>20,000,000	6

NOTE: Panel size and library yield into target enrichment dictate how many PCR cycles should be performed post target enrichment. Multiply both variables to get an estimate of how many PCR cycles should be used. Slight variation in PCR cycling will not drastically impact final Picard HS metrics.

4.2

If the Streptavidin Binding Bead slurry has settled, mix by pipetting.

4.3

Transfer 22.5 µl of the Streptavidin Binding Bead slurry to a 0.2-ml thin-walled PCR strip-tube(s). Keep on ice until ready to use in the next step.

NOTE: Store the remaining 22.5 µl Streptavidin Binding Bead slurry at –20°C for future use.

4.4

Prepare a PCR mixture by adding the following reagents to the tube(s) containing the Streptavidin Binding Bead slurry. Mix by pipetting.

REAGENT	VOLUME
Streptavidin Binding Bead Slurry	22.5 µl
Amplification Primers, ILMN	2.5 µl
KAPA HiFi HotStart ReadyMix or validated equivalent	25 µl
Total	**50 µl**

PCR AMPLIFY

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

4.6 When the thermal cycler program is complete, remove the tube(s) from the block and immediately proceed to the Purify step.

PURIFY

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

4.8 Add 90 µl (1.8x) homogenized DNA Purification Beads to the tube(s) from Step 4.6. Mix well by vortexing.

NOTE: It is not necessary to recover supernatant or remove Streptavidin Binding Beads from the amplified PCR product.

4.9 Incubate for 5 minutes at room temperature.

4.10 Place the tube(s) on a magnetic plate for 1 minute or until the supernatant is clear.

4.11 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube(s) from the magnetic plate, remove and discard the clear supernatant.

4.12 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.

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

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

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.

4.15 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.

4.16 Remove the tube(s) from the magnetic plate and add 32 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each capture reaction. Mix by pipetting until homogenized.

4.17 Incubate at room temperature for 2 minutes.

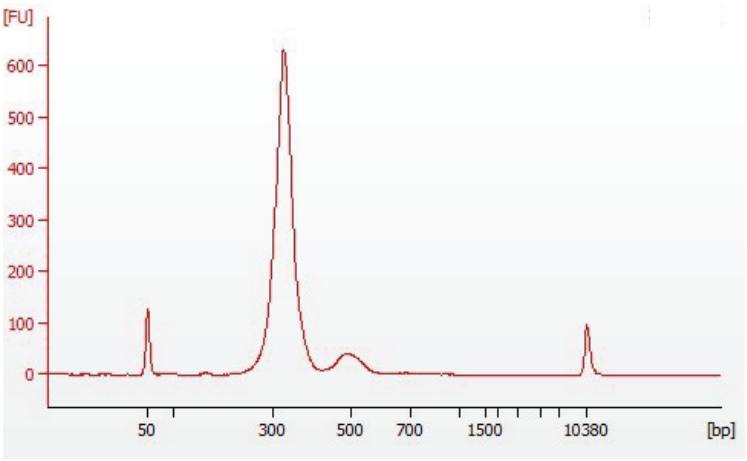
4.18 Place the plate or tube(s) on a magnetic plate and let stand for 3 minutes or until the beads fully pellet.

4.19 Transfer 30 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure to not disturb the bead pellet.

PERFORM QC

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

NOTE: When using the Agilent Bioanalyzer High Sensitivity DNA Kit, load 0.5 µl of the final sample. Average fragment length should be 375–450 bp using a range setting of 150–1,000 bp. Final concentration may vary and is dependent on panel size, library input, hybridization reaction size, and the number of PCR cycles.



Electropherogram generated by an Agilent High Sensitivity DNA analysis of the enriched gDNA library samples that were prepared as described. Note the single prominent peak with later consecutive smaller peaks.

STOPPING POINT: If not proceeding immediately to sequencing, store the enriched library sample at –20°C for up to 24 hours.

STEP 5 SEQUENCING ON AN ILLUMINA PLATFORM

Sequence the enriched libraries on an Illumina platform. Sequencing protocols and settings depend on the application and instrumentation used. Please contact **customersupport@twistbioscience.com** for recommendations.

END OF WORKFLOW