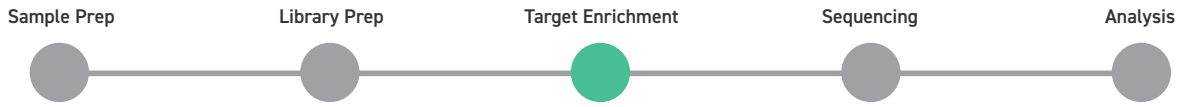
Twist High-Throughput Target Enrichment Standard Hybridization v2 Protocol

For use with the Twist NGS Workflow

The Twist High-Throughput Target Enrichment protocol generates enriched DNA libraries for sequencing on a preferred short-read next-generation sequencing (NGS) platform. This manual details the steps for a 16-hour hybridization in a two-day target enrichment workflow.

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

- Designed for single or multiplex hybridization reactions using either Twist fixed or custom panels; optional secondary panels (spike-ins) can also be added for additional content
- Optimized for use with Twist Library Preparation Kits
- Should only be performed with the reagents specified or their equivalents



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

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
TWIST STANDARD HYB AND WASH KIT V2 WITH TRUEAMP MIX (FOR TARGET ENRICHMENT WITH STANDARD HYBRIDIZATION)			
116613: 2 rxn 116614: 12 rxn 116615: 96 rxn	Twist Hybridization Reagents	· Hybridization Mix · Hybridization Enhancer · Amplification Primer	–20°C
	Twist Standard Wash Buffers v2	· Binding Buffer · Standard Wash Buffer 1 · Wash Buffer 2	2-8°C
	Twist TrueAmp Polymerase Mix	Twist TrueAmp Polymerase Mix	–20°C
TWIST PROBE PANELS (ORDERED SEPARATELY)			
Varies based on choice of panel and reaction size.	Twist Fixed Panel	Fixed content enrichment panel for hybridization reactions (for example, Twist Human Core Exome Panel)	–20°C
	Twist Custom Panel	Custom enrichment panel for hybridization reactions	–20°C
	(Optional) Additional Twist Probe Panel(s)	Custom or fixed enrichment panel for adding content to a fixed or custom panel	–20°C
TWIST BLOCKERS & BEADS FOR TARGET ENRICHMENT (ORDERED SEPARATELY)			
100856: 2 rxn 100578: 12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: · Universal Blockers · Blocker Solution	–20°C
101262: 2 rxn 100983: 12 rxn 100984: 96 rxn	Twist Binding and Purification Beads	For target enrichment and purification: · Streptavidin Binding Beads · DNA Purification Beads	2-8°C
104324: 2 rxn 104325: 12 rxn 104326: 96 rxn	Twist Dry Down Beads NOTE: Not needed if using a vacuum concentrator for library dry down.	For target enrichment and purification: · Streptavidin Binding Beads · DNA Purification Beads (contains additional volume for Alternate Pre-Hybridization DNA Concentration Protocol)	2-8°C

The following catalog numbers for Twist Standard Hyb and Wash Kit v2 include the Equinox Library Amp Mix instead of the Twist TrueAmp Polymerase Mix:

105559: Twist Std Hyb and Wash Kit v2 with amp mix, 2 rxn
105560: Twist Std Hyb and Wash Kit v2 with amp mix, 12 rxn
105561: Twist Std Hyb and Wash Kit v2 with amp mix, 96 rxn

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INTENDED USE

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent 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	—
10 mM Tris-HCl pH 8 (optional)	—
Buffer EB (optional)	Qiagen
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
Qubit dsDNA Broad Range Quantification Assay	Thermo Fisher Scientific
Agilent DNA 5000 ScreenTape Assay	Agilent Technologies
PCR plate seals (MicroAmp or PolarSeal)	Thermo Fisher Scientific
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
96-well compatible magnetic plate	Alpaqua, Permagen Labware
1.5-ml compatible magnetic stand	Beckman Coulter
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer (preferably) or heat block	Eppendorf
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
TapeStation	Agilent Technologies
Vacuum concentrator	—

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 High-Throughput Target Enrichment Standard Hybridization v2 Workflow if modifications are made to the protocol.

Do NOT mix or combine the same reagents from different lots.

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.

FOR TECHNICAL SUPPORT, CONTACT CUSTOMERSUPPORT@TWISTBIOSCIENCE.COM.

PROTOCOL OVERVIEW

This protocol is a component of the Twist NGS workflow. It begins with amplified, indexed DNA libraries and generates target-enriched DNA libraries for sequencing on NGS systems.

STEP	HYBRIDIZATION TARGET ENRICHMENT WORKFLOW (AMPLIFIED, INDEXED LIBRARIES)	TIME
1	**Prepare Libraries for Hybridization** Indexed library pool	**1 hour**
2	**Hybridize Capture Probes With Pools** Hybridized targets in solution	**16 hours**
3	**Bind Hybridized Target to Streptavidin Beads** Captured targets on beads	**1.5 hours**
	STOPPING POINT	
4	**Post-Capture PCR Amplify, Purify, and Perform QC** Enriched libraries	**1 hour**
	STOPPING POINT	
5	**Sequencing** Libraries ready for sequencing on your preferred platform	–

STEP 1 PREPARE LIBRARIES FOR HYBRIDIZATION

⚠ IMPORTANT: If planning to use a vacuum concentrator for drying down libraries rather than SPRI beads, please refer to Appendix A. If planning to use 3 or 4 panels during hybridization, please refer to Appendix B for alternative protocol steps and guidance.

This step involves aliquoting the appropriate amount of amplified, indexed libraries (generated previously in library preparation) and preparing the hybridization reaction solution. For a list of Twist Library Preparation Kit options, see twistbioscience.com/products/ngs.

- When multiplexing, follow the pooling guidelines included in the Appendix of the Twist Library Preparation Protocol used.
- If using another library preparation method, use the pooling guidelines specific to that method.

Reagents Required

- Amplified, indexed library pool(s)
- Ethanol
- Molecular biology grade water
- From Twist Dry Down Beads:
  - DNA Purification Beads
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution

Before You Begin

- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes.
- Vortex the pre-equilibrated DNA Purification Beads until well mixed.
- Prepare 500 µl fresh 80% ethanol for each sample to be processed.

CONCENTRATE THE DNA LIBRARIES

This protocol supports a single or multiplex (up to 8-plex) hybridization capture. The amount of indexed library to use depends on the number of indexed samples per pool. If using more than two Twist panels per reaction, refer to Appendix B.

1.1

Use the concentration of each amplified, indexed library to calculate the volume (in µl) of each library needed for hybridization:

- Determine the amount of each indexed library per pool from the table below.
- Divide the amount of each indexed library per pool by the concentrations measured in ng/µl from the library preparation QC.

For example: If multiplexing eight libraries per hybridization reaction, the amount of each library will be 187.5 ng and the total mass of the pool will be 1,500 ng.

NUMBER OF INDEXED SAMPLES PER POOL	AMOUNT OF EACH INDEXED LIBRARY PER POOL	TOTAL MASS PER POOL
1	500 ng	500 ng
2	500 ng	1000 ng
3	500 ng	1500 ng
4	375 ng	1500 ng
8	187.5 ng	1500 ng

NOTES:

- If the amount of library you have is insufficient, you can use a smaller amount; using less, however, may result in decreased library complexity.
- More than 1,500 ng (1.5 µg) total DNA can be used; do not, however, use more than 4 µg total DNA as this might lead to reduced performance of the enrichment.

1.2

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

NOTE: If using a vacuum concentrator for dry down instead, refer to Appendix A after completing Steps 1.1 and 1.2.

1.3

Add 1.8X homogenized DNA Purification Beads to the tube/plate(s) containing the DNA library(ies) from Step 1.2. Mix well by vortexing. Incubate for at least 5 minutes at room temperature.

NOTE: For amplified, indexed library pool(s) with a volume of less than 10 µl, bring volume up to 10 µl with water.

⚠IMPORTANT: The volume of the total library pool cannot exceed 70 µl.

PROCEED TO STEP 2: HYBRIDIZE CAPTURE PROBES WITH POOLS

STEP 2 HYBRIDIZE CAPTURE PROBES WITH POOLS

Use the SPRI-concentrated indexed library pool(s) started in Step 1 or dried down indexed library pool(s) from Appendix A for performing the hybridization reaction.

⚠IMPORTANT: Before proceeding with this step, test the compatibility of your thermal cycler and PCR tubes/plates and seals by incubating them at 95°C for up to 5 minutes to ensure they do not crack or unseal 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/Appendix A
- Twist fixed or custom panel
- Twist custom secondary (spike-in) panel(s) (optional)
- 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 for 2 seconds.
- Set a heat block or incubator to 65°C to heat the Hybridization Mix.

RESUSPEND THE PRE-HYBRIDIZATION SOLUTION

2.1 Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 85°C.

STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
1	95°C	5 minutes	1
2	70°C	HOLD	—

2.2 Heat the Hybridization Mix at 65°C in the heat block or incubator for 10 minutes, or until all precipitate is dissolved.

2.3 Prepare the hybridization reaction master mix as indicated in the table below.

REAGENT	VOLUME PER REACTION*
Hybridization Mix	20 µl
Twist Fixed or Custom Panel	4 µl
Blocker Solution*	5 µl
Universal Blockers	7 µl
Optional: Secondary Panel (in place of water)	4 µl
Water (up to total volume)	(0-4) µl
Total	**40 µl**

NOTES:
- If using optional Secondary Panel (spike-in) content, add 4 µl of probes in place of water.
- 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.

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

2.4 Pulse-spin the indexed library pool(s) from Step 1.3 for 2 seconds to ensure all the solution is at the bottom of the wells and place the tube/plate(s) on a magnetic stand for 3 minutes or until the solution is clear.

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

2.6 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.

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

2.8 Carefully remove all remaining ethanol using a 10-µl pipette, making sure to not disturb the bead pellet.
NOTE: Pulse-spin for 2 seconds, if necessary, to ensure complete removal of ethanol.

2.9 Air-dry the bead pellet on a magnetic stand for 1–5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

2.10 Add 40 µl of the master mix from Step 2.3 to each concentrated sample. Maintain the SPRI beads in the reaction during hybridization unless using a vacuum concentrator.

2.11 Pulse-vortex for 2 seconds, then pulse-spin for 2 seconds.

PERFORM THE HYBRIDIZATION REACTION

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

2.13 Pulse-spin the tube/plate(s) for 2 seconds to ensure there are no bubbles present.

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

2.14 Transfer the Hybridization Reactions into the thermal cycler and start the thermal cycler program. Incubate for ~16 hours at the 70°C hold step.
NOTE: Halting hybridization between 15–20 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 2.14)
- 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 or Twist Dry Down Beads:
  - Streptavidin Binding Beads
  - DNA Purification Beads

Before You Begin

- Preheat Standard Wash Buffer 1 at 48°C until all precipitate is dissolved. Precipitate occurs during 4°C storage of Standard Wash Buffer 1.
- For each hybridization reaction:
  - Equilibrate 650 µl of Binding Buffer to room temperature.
  - Equilibrate 175 µl of precipitate-free Standard Wash Buffer 1 to room temperature. Precipitation will not occur at room temperature.
  - Equilibrate 500 µl of Wash Buffer 2 to room temperature.
- Set one thermal cycler to 66°C with the lid set to 68°C. This is the standard wash 1 temperature. Other wash 1 temperatures can be used; for further guidance, refer to Appendix C.
- Set one thermal cycler to 48°C with the lid set at 50°C. This is the wash 2 temperature and should not be changed.
- Both wash thermal cyclers should be set to the highest possible volume setting. A volume setting of 100 µl is sufficient for high-performance washes.
- Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes.
- We recommend using PCR plate seals that are compatible with wide temperature ranges like MicroAmp or PolarSeal from Thermo Fisher Scientific. Always ensure a tight seal during incubations.

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

- Thaw on ice:
  - Twist TrueAmp Polymerase Mix or validated equivalent
  - Amplification Primers
- Equilibrate DNA Purification Beads (from the Twist Binding and Purification Beads or Twist Dry Down 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 clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate. Prepare one well for each hybridization reaction.

3.3 Add 100 µl Binding Buffer to the tube(s) and mix by pipetting or gentle vortexing.

3.4 Place the tube/plate(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 100 µl Binding Buffer and resuspend the beads by vortexing until homogenized.

3.7 Heat the resuspended beads in the wash 1 thermal cycler for at least 10 minutes before continuing to Step 3.8.

BIND THE TARGETS

3.8 After the hybridization is complete, open the two thermal cycler lids and directly transfer the volume of each hybridization reaction from Step 2.14 into a corresponding tube of preheated Streptavidin Binding Beads from Step 3.7. Mix by pipetting.

⚠ 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/plate(s) of the hybridization reaction with the Streptavidin Binding Beads for 5 minutes in the wash 1 thermal cycler; agitation is not required.
NOTE: Do not vortex. Aggressive mixing is not required.

3.10 Remove the tube/plate(s) containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin for 2 seconds to ensure all solution is at the bottom of the tube(s).

3.11 Place the tube/plates(s) on a magnetic stand until all beads separate (~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/plates(s) from the magnetic stand and add 150 µl precipitate-free Standard Wash Buffer 1. Mix by pipetting.

3.14 Incubate the tube(s) for 5 minutes in the wash 1 thermal cycler.

3.15 Pulse-spin for 2 seconds to ensure all solution is at the bottom of the tube(s).

3.16 Transfer the entire volume from Step 3.15 (~150 µl) into a new clean thin-walled PCR 0.2-ml strip tube or well of a 96-well thermal cycling plate. Place the tube(s) on a magnetic stand until all beads separate (~1 minute).

⚠ IMPORTANT: This transfer step reduces background from non-specific binding to the surface of the tube. It is important to perform Step 3.15, Step 3.16, and the removal of wash 1 supernatant in Step 3.17 quickly to prevent off-target.

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 150 µl of Wash Buffer 2. Mix by pipetting, then pulse-spin for 2 seconds to ensure all solution is at the bottom of the tube(s).

3.18 Incubate the tube(s) for 5 minutes in the 48°C wash 2 thermal cycler.

3.19 Place the tube(s) on a magnetic stand until all beads separate (~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.
NOTE: Alternatively, elute in 22.5 µl water instead of the full 45 µl water. Using all the bead slurry in the post-capture PCR leads to a ~15% increase in complexity (HS Library Size) compared to using half. If using all bead slurry in the post-capture PCR, reduce the number of PCR cycles in Step 4.5 by one. The advantage of eluting in 45 µl and using half for the post-capture PCR is that it enables a retain of the post-capture material.

STOPPING POINT: If not proceeding immediately, store the bead slurry sample at –20°C.

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
  - Twist TrueAmp Polymerase Mix or validated equivalent
  - Amplification Primers
- Agilent DNA 5000 ScreenTape Assay (or equivalent)
- Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay

Before You Begin

- Prepare 500 µl 80% ethanol for each Streptavidin Binding Bead slurry to be processed.

PREPARE THE BEADS, THERMAL CYCLER, AND PCR MIX

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 pool.

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	640	400,000
625	3,200	2,000,000
1,250	6,400	8,000,000
1,250	12,800	16,000,000
12,500	1,600	20,000,000
12,500	6,400	80,000,000

4.1 Program a thermal cycler with the following conditions. **Set 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 table to the right.*
	Annealing	60°C	30 seconds	
	Extension	68°C*	30 seconds	
3	Final Extension	68°C*	1 minute	1
4	Final Hold	4°C	HOLD	—

**If desired, instead of the Twist TrueAmp Polymerase Mix, alternative polymerases can be used. Modification to PCR temperatures may be required. For example, with Equinox Library Amp Mix, extension and final extension temperatures should be changed to 72°C. For other polymerases, use the manufacturer's suggested extension temperature as a starting point. Contact customersupport@twistbioscience.com for further guidance.*

***If using the entire Streptavidin Binding Bead slurry, reduce the recommended cycle count in this column by 1.*

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 – 1,400,000	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

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) or 96-well thermal cycling plate(s). Keep on ice until ready to use in the next step.
NOTE: If not using the full slurry, 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 PER REACTION
Streptavidin Binding Bead Slurry	22.5 µl
Amplification Primers, ILMN	2.5 µl
Twist TrueAmp Polymerase Mix	25 µl
Total	**50 µl**

PCR AMPLIFY

4.5 Pulse-spin the tubes for 2 seconds, then transfer them to the thermal cycler and start the cycling program (see Step 4.1).

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 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.

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 22 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each capture reaction. Mix by pipetting until homogenized.

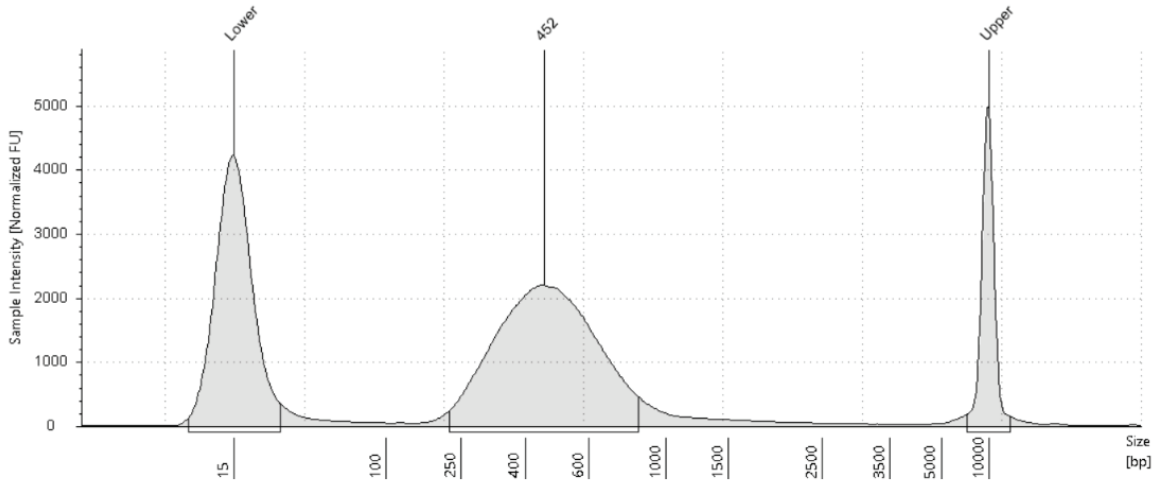
4.17 Incubate at room temperature for 2 minutes.

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 20 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 TapeStation D5000 ScreenTape Assay and a Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay.



Representative electropherogram generated by an Agilent D5000 ScreenTape Assay of the enriched library samples that were prepared as described. Note the single prominent peak.

STOPPING POINT: If not proceeding immediately, store the enriched library sample at –20°C.

STEP 5 SEQUENCING ON NGS PLATFORM

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

END OF WORKFLOW

APPENDIX A: ALTERNATE PRE-HYBRIDIZATION DNA CONCENTRATION PROTOCOL

Reagents Required

- Amplified, indexed library pool(s) from Step 1.2
- Ethanol
- Molecular biology grade water
- From Twist Binding and Purification Beads:
  - DNA Purification Beads
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution

Before You Begin

- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes.
- Vortex the pre-equilibrated DNA Purification Beads until well mixed.
- Prepare 500 µl fresh 80% ethanol for each sample to be processed.

CONCENTRATE THE DNA LIBRARIES

A.1 Pulse-spin the indexed library pool tube/plate(s) from Step 1.2 for 2 seconds.

A.2 Dry the indexed library pool(s) using a vacuum concentrator with no heat.

A.3 Once fully dry, proceed to Step A.4 and continue the protocol.

STOPPING POINT: If not proceeding immediately, store the dried sample at –20°C.

A.4 Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 85°C.

STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
1	95°C	5 minutes	1
2	70°C	HOLD	—

A.5 Heat the Hybridization Mix at 65°C in the heat block or incubator for 10 minutes, or until all precipitate is dissolved.

A.6 Prepare the hybridization reaction master mix as indicated in the table below.

REAGENT	VOLUME PER REACTION
Dried Indexed Library Pool	—
Hybridization Mix	20 µl
Twist Fixed or Custom Panel	4 µl
Blocker Solution*	5 µl
Universal Blockers	7 µl
Optional: Secondary Panel (in place of water)	4 µl
Water (up to total volume)	(0-4) µl
Total	**40 µl**

NOTES:
- If using optional Secondary Panel (spike-in) content, add 4 µl of probes in place of water.
- 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.

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

A.7 Add 40 µl of the master mix from Step A.6 to each dried down sample.

A.8 Pulse-vortex for 2 seconds, then pulse-spin for 2 seconds.

A.9 Proceed to Step 2.12 and continue the protocol.

APPENDIX B: ADDING ADDITIONAL PANELS TO THE PROBE SOLUTION

The following steps and modifications are necessary when adding a third and/or fourth panel to the probe solution in Step 2: Hybridize Capture Probes With Pools.

Detailed below are two methods for using an additional third or fourth panel in the hybridization reaction:

- Vacuum Concentrator
- Bead Concentration

Performance of capture with additional panels should be tested empirically.

Alternatively, custom blended panels can be ordered to avoid using multiple panels. Contact Twist customer support for more details.

VACUUM CONCENTRATOR

B.V.1 Pulse-spin the indexed library pool tube/plate(s) from Step 1.2 for 2 seconds.

B.V.2 Add the third and/or fourth panels to each pool. Use 4 µl of each Twist panel.

B.V.3 Dry the indexed library pool(s) using a vacuum concentrator with no heat.
NOTE: Ensure the indexed library pools are completely dry before proceeding with the protocol.

STOPPING POINT: If not proceeding immediately, store the dried sample at –20°C.

B.V.4 Program a thermal cycler with the following conditions. **Set the temperature of the heated lid to 85°C.**

STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
1	95°C	5 minutes	1
2	70°C	HOLD	—

B.V.5 Heat the Hybridization Mix at 65°C in the heat block or incubator for 10 minutes, or until all precipitate is dissolved.

B.V.6 Prepare the hybridization reaction master mix as indicated in the table below.

REAGENT	VOLUME PER REACTION
Dried Indexed Library Pool	–
Hybridization Mix	20 µl
Twist Fixed or Custom Panel	4 µl
Blocker Solution*	5 µl
Universal Blockers	7 µl
Optional: Secondary Panel (in place of water)	4 µl
Water (up to total volume)	(0-4) µl
Total	**40 µl**

NOTES:
- If using optional Secondary Panel (spike-in) content, add 4 µl of probes in place of water.
- 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.

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

B.V.7 Add 40 µl of the master mix from Step B.V.6 to each dried down sample.

B.V.8 Pulse-vortex for 2 seconds, then pulse-spin for 2 seconds.

B.V.9 Proceed to Step 2.12 and continue the protocol.

BEAD CONCENTRATION

B.B.1 Pulse-spin the indexed library pool tube/plate(s) from Step 1.2 for 2 seconds.

B.B.2 Add the third and/or fourth panels to the pooled libraries. Use 4 µl of each panel to be tested.

B.B.3 Proceed to Step 1.3 and continue the protocol.

APPENDIX C: WASH 1 TEMPERATURE'S IMPACT ON UNIFORMITY

Off-Bait and uniformity metrics are primarily tuned by the wash 1 temperature after hybridization. A higher wash 1 temperature results in lower Off-Bait, higher AT Dropout, and lower GC Dropout. A lower wash 1 temperature results in the opposite. Typical wash 1 temperatures are between 64°C and 70°C.

When comparing the traditional Twist Standard Hybridization v2 Protocol (using 1.5-ml tubes) to this updated high-throughput protocol (using PCR strip tubes or plates), Twist has found that the geometry of the tube affects the optimal wash 1 temperature. Since the strip tube/plate plastic is thinner than the 1.5-ml tubes, the thermal conduction is more efficient, and the reaction runs hotter. To get comparable performance to the traditional protocol, use a wash 1 temperature 2°C lower in the high-throughput protocol. For example, 68°C in the traditional protocol is equivalent to 66°C in the high-throughput protocol.

NOTE: Refer to the Twist Tech Note 'Evaluation of a Streamlined High-Throughput Hybridization Protocol for Target Enrichment' for more information regarding Wash 1 temperatures.

APPENDIX D: KIT COMPONENTS

The following table details part numbers for each component provided in the kits required for this protocol.

BOX	COMPONENT	COMPONENT PART NUMBER
Twist Hybridization Reagents	Amplification Primers	100287 (2 rxn) 100586 (12 rxn) 100770 (96 rxn)
	Hybridization Mix	100212 (2 rxn) 100587 (12 rxn) 100528 (96 rxn)
	Hybridization Enhancer	101266 (2 rxn) 100937 (12 rxn) 100986 (96 rxn)
Twist Standard Wash Buffers v2	Binding Buffer	100214 (2 rxn) 100588 (12 rxn) 100771 (96 rxn)
	Standard Wash Buffer 1	104454 (2 rxn) 104455 (12 rxn) 104456 (96 rxn)
	Wash Buffer 2	100216 (2 rxn) 100590 (12 rxn) 100530 (96 rxn)
Twist TrueAmp Polymerase Mix	Twist TrueAmp Polymerase Mix	116475 (2 and 12 rxn)
		116476 (96 rxn)