Target Enrichment Standard Hybridization v2 Protocol for MRD Application

For use with the Twist NGS Workflow

For Research Use Only (RUO). Not for use in diagnostic procedures

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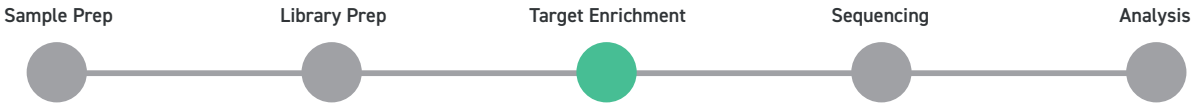
The Twist Target Enrichment protocol generates enriched DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems. This manual details the steps for a 16-hour hybridization in a two-day target enrichment workflow.

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

- Designed for single hybridization reactions using either Twist MRD Rapid 500 panel, 12 tests; optional secondary panels (spike-ins) can also be added for additional content
- Optimized for use with Library Preparation with the Twist UMI and Universal Adapter Systems
- 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. A component of this workflow, the Twist Target Enrichment Protocol works in conjunction with the other component protocols.

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
104445: 2 rxn*	Twist Standard Hyb and Wash Kit v2 [SKU 101263]	• Hybridization Mix • Hybridization Enhancer • Amplification Primers	–20°C
	Twist Standard Hyb and Wash Kit v2 with Amp Mix [SKU 104133]		
105560: 12 rxn	Twist Hybridization Reagents, 12 reactions [SKU 100930]	• Hybridization Mix • Hybridization Enhancer • Amplification Primers • Binding Buffer • Standard Wash Buffer 1+ • Wash Buffer 2 • Equinox Library Amp Mix (2x)	–20°C
	Twist Standard Wash Buffers, 12 reactions [SKU 104135]		2–8°C
	Equinox Library Amp Mix, 16 samples [SKU 104107]		-20°C
105561: 96 rxn	Twist Hybridization Reagents, 96 reactions [SKU 100982]	• Hybridization Mix • Hybridization Enhancer • Amplification Primers • Binding Buffer • Standard Wash Buffer 1+ • Wash Buffer 2 • Equinox Library Amp Mix (2x)	–20°C
	Twist Standard Wash Buffers, 12 reactions [SKU 104137]		2–8°C
	Equinox Library Amp Mix, 16 samples [SKU 104108]		-20°C

** Catalog # 104445 does not contain Equinox Library Amp Mix. Sufficient Equinox Library Amp Mix for the 2 rxn enrichment workflow is included with the 16 sample Twist Library Prep Kit.*

+ Buffer component in the new Standard Hyb kit v2.

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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 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.

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

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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
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 High Sensitivity Quantitation 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	—
Theromomixer (preferred) or heat clock for 1.5-ml tubes x2	Eppendorf
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer or equivalent	Agilent Technologies
Vacuum concentrator	—

PROTOCOL OVERVIEW

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

	LIBRARY PREPARATION WORKFLOW	TIME
STEP 1	**Prepare Libraries for Hybridization** Indexed library pool	**1 hour**
	STOPPING POINT	
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	**1 hour**
	STOPPING POINT	
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 libraries (generated previously with Library Preparation with the Twist UMI Adapter System) and preparing the hybridization reaction solution.

- 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

ALIQUOT AND DRY DOWN THE LIBRARY

⚠ IMPORTANT: This protocol supports a single plex hybridization capture.

1.1 Use the concentration of each amplified, indexed library to calculate the volume (in µl) of each library needed for 500 ng hybridization

- Divide the amount of each indexed library per pool by the concentrations measured in ng/µl from the library preparation QC.
- If the amount of library you have is insufficient, you can use a smaller amount; using less, however, may result in decreased library complexity.

1.2 Transfer the calculated volumes from each amplified indexed library to an indexed library pool reaction tube (clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate) for each hybridization reaction being performed.

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
- Twist MRD Rapid 500 panel, 12 tests
- Twist custom secondary (spike-in) panel(s) (optional)
- From Twist Hybridization Reagents:
  - Hybridization Mix
  - Hybridization Enhancer
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution

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 PROBE SOLUTION

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

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
Optional: Secondary Panel	4 µl
Water (up to total volume)	(0 –4) µl
Total	**28 µl**

2.2 (continued)

NOTE:

- If using optional Secondary Panel (spike-in) content, add 4 µl 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.

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**

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

Carefully mix the probe solution by flicking the tube, then add all 28 µl 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

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 840 µl Binding Buffer to room temperature
  - Equilibrate 225 µl Standard Wash Buffer 1 to 68°
  - Preheat 700 µl Wash Buffer 2 to 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):
  - Equilibrate DNA Purification Beads (from the Twist Binding and Purification Beads) to room temperature for at least 30 minutes
- Thaw on ice:
  - Equinox Library Amp Mix (2x)
  - Amplification Primers
- Once these reagents are thawed, pulse-vortex for 2 seconds to mix.

PREPARE THE BEADS

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

3.2 Add 40 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) 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 resususpened beads at 68°C for at least 10min before continuing to step 3.8.

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 washed Streptavidin Binding Beads from Step 3.6. 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.

BIND THE TARGETS

3.9 Place 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 of 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 3.13 (~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.

3.18 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).

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

3.20 Transfer the entire volume from Step 3.18 (~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.

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

3.22 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).

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

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

3.25 Repeat the wash (Steps 3.22–3.24) one more time, for a total of two washes.

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

3.27 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 3.27)
- Ethanol
- Molecular biology grade water
- Reagents thawed and equilibrated in Step 3:
  - DNA Purification Beads
  - Equinox Library Amp Mix (or 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

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	# OF CYCLES
1	Initialization	98˚C	45 seconds	1
2	Denaturation	98˚C	15 seconds	17
	Annealing	60˚C	30 seconds	
	Extension	72˚C	30 seconds	
3	Final Extension	72˚C	1 minute	1
4	Final Hold	4˚C	HOLD	–

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. Store the remaining 22.5 µl water/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
Equinox Library Amp Mix	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.

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 Pulse spin and carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet.

4.15 Air-dry the bead pellet on a magnetic plate for 5–10 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.

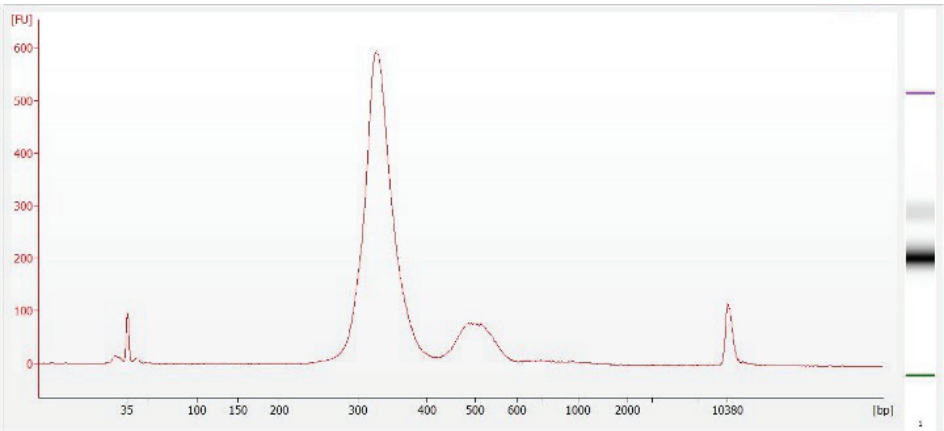
4.19 Transfer 30 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure to not disturb the bead pellet.

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 350–450 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 electrophoretic separations of cfDNA often display three types of fragments: mononucleosome (~325 bp), dinucleosome (~490 bp), and tri-nucleosome (~650 bp).

STOPPING POINT: If not proceeding immediately, 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