Ancient Human DNA Target Enrichment Standard Hybridization Protocol

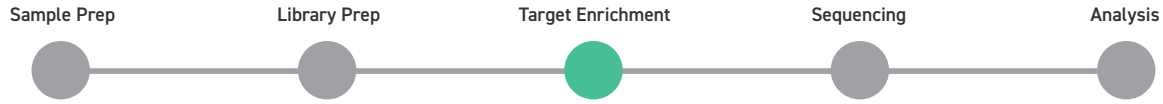
For use with the Twist NGS Workflow

NOTE: This is a Twist-compatible protocol that has been developed with partner institutions and laboratories using Twist products. This protocol has not undergone internal validation at Twist Bioscience, but the methods have been successfully demonstrated and are detailed in a peer-reviewed publication.†

This Ancient Human DNA Target Enrichment Standard Hybridization protocol generates enriched DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems. This protocol details the steps for a 16-hour hybridization in a two-day target enrichment workflow and is optimized for use with the Twist Ancient Human DNA Panel.

The protocol is:

- Designed for single-plex hybridization reactions using the Twist Ancient DNA Panel.
- Optimized for use with Twist Library Preparation Kits.
- Intended to be performed with the specified reagents or their equivalents.



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

†Rohland, N., Mallick, S., Mah, M., et al. Three assays for in-solution enrichment of ancient human DNA at more than a million SNPs. Genome Res 32, 2068-2078 (2022). https://doi.org/10.1101/gr.276728.122

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 PROBE PANELS (ORDERED SEPARATELY)			
107479: 12 rxn 106658: 96 rxn	Twist Ancient Human DNA Panel	Target Enrichment panel for hybridization reaction with ancient DNA samples	–20°C
104562: 96 rxn	*(Optional)* Twist High Concentration Mitochondrial Panel	Fixed enrichment panel for coverage of the mitochondrial genome	–20°C
TWIST HYBRIDIZATION REAGENTS			
100578: 12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: · Universal Blockers · Blocker Solution	–20°C
100983: 12 rxn 100984: 96 rxn 3 units	Twist Binding and Purification Beads	For target enrichment and purification: · Streptavidin Binding Beads · DNA Purification Beads	2–8°C
100930: 12 rxn 100982: 96 rxn 2 units	Twist Hybridization and Wash Kit (2 Boxes)	For target enrichment with standard hybridization: · Twist Hybridization Reagents (Box 1 of 2): Hybridization Mix, Hybridization Enhancer, Amplification Primers · Twist Wash Buffers (Box 2 of 2): Binding Buffer, Wash Buffer 1, Wash Buffer 2	–20°C 2–8°C
100985: 12 rxn 100846: 96 rxn 3 units	Twist Wash Buffers	For target enrichment	2–8°C
104107: 12 rxn 104108: 96 rxn	Equinox Library Amp Mix*	For target enrichment	2–8°C

**Can substitute with KAPA HiFi HotStart ReadyMix from Kapa Biosystems.*

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate enriched libraries using the Ancient Human DNA Target Enrichment Standard Hybridization workflow.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8	—
Buffer EB	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates (optional)	VWR
1.5-ml compatible magnetic stand	Beckman Coulter
96-well compatible magnetic plate	Alpaqua
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
KAPA HiFi HotStart ReadyMix, if using	Kapa Biosystems
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96-well) with heated lid	—
Lab shaker, rocker, rotator	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	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 provided instructions. Twist cannot guarantee the performance of the Ancient Human DNA Target Enrichment Standard Hybridization 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, tapping, or 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 has been optimized for use with the Twist Ancient Human DNA panel to produce high coverage and superior on-target uniformity across 1.24M SNPs.

The protocol begins with amplified, indexed genomic DNA (gDNA) libraries and generates target-enriched DNA libraries for sequencing on Illumina NGS systems. It features a 16-hour hybridization time.

More information about the panel content can be found within the publication:
Rohland et al., Genome Research, 2022 (doi: 10.1101/gr.276728.122).

	HYBRIDIZATION TARGET ENRICHMENT WORKFLOW (AMPLIFIED INDEXED LIBRARIES)	TIME
STEP 1	**Prepare libraries for hybridization** Indexed library pool	**1 hour** *(may vary by library concentration)*
STEP 2	**Hybridize capture probes with pools** Hybridized targets in solution	**16 hours**
STEP 3	**Bind hybridized targets to streptavidin beads** Capture targets on beads	**1.5 hours**
STEP 4	**Post-capture PCR amplify, purify, and perform QC** Enriched libraries	**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 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.

This protocol supports a single-plex hybridization capture.

Reagents Required

• Amplified, indexed library

ALIQUOT AND DRY DOWN THE LIBRARY

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

- 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 1,000 ng (1 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 a clean 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.

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

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

1.4 Dry the indexed library with a vacuum concentrator using low or no heat.

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

PROCEED TO STEP 2: HYBRIDIZE CAPTURE PROBES WITH LIBRARIES

STEP 2 HYBRIDIZE CAPTURE PROBES WITH LIBRARIES

Use the dried indexed library from Step 1 to perform 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 libraries from Step 1
- Twist Ancient Human DNA Panel
- Twist High Concentration Mitochondrial Panel (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 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 PER REACTION*
Hybridization Mix	5 µl
Twist Ancient Human DNA Panel	1 µl
Optional Twist High Concentration Mitochondrial Panel	0.04 µl
Water (up to total volume)	0-0.04 µl
Total	**6.04 µl**

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

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

REAGENT	VOLUME
Dried Indexed Library	—
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 the probe solution is cooling on ice, heat the tube containing the resuspended indexed libraries 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 libraries to room temperature on the benchtop for 5 minutes.

2.6 Carefully mix the probe solution by flicking the tube, then add all 6.04 µl of the probe solution to the resuspended indexed libraries. 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 62°C for 16 hours in a thermal cycler with the lid at 65°C.

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
  - 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
  - Wash Buffer 1
  - Wash Buffer 2
- For each hybridization reaction:
  - Equilibrate 200 µl Wash Buffer 1 to room temperature.
  - 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
  - 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 300 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

3.3 Add 600 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 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 62°C is a critical step for minimizing off-target binding. Do not remove the tube(s) of the hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 62°C before transferring the solution to the washed Streptavidin Binding Beads. Allowing it 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

3.8 Mix the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 30 minutes at room temperature on a shaker, rocker, or rotator at a speed sufficient to keep the solution mixed.

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

3.9 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).

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

3.11 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.

3.12 Remove the tube(s) from the magnetic stand and add 200 µl Wash Buffer 1. Mix by pipetting.

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

3.14 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.

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

3.16 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).

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

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

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

3.20 Repeat the wash (Steps 3.16–3.19) two more times, for a total of three washes.

3.21 After the third 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.

3.22 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 3.22)
- Ethanol
- Molecular biology grade water
- Reagents thawed and equilibrated in Step 3:
  - DNA Purification Beads
  - Equinox Library Amp Mix (2x) or KAPA HiFi HotStart ReadyMix
  - 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	NUMBER OF CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	23
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

NOTE: Number of amplification cycles may be reduced depending on the complexity of the library.

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 (2x) or KAPA HiFi HotStart ReadyMix	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 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.

When using the Agilent Bioanalyzer High Sensitivity DNA Kit, load 0.5 µl of the final sample. Average fragment length should be 50–150 bp but this will depend on the size of the input indexed libraries. Final concentration should be ≥15 ng/µl but this depends on the hybridization reaction size and number of PCR cycles used.

STOPPING POINT: If you are not proceeding immediately, store the enriched library sample at –20°C for 24 hours or more.

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. Contact customersupport@twistbioscience.com for recommendations.

END OF WORKFLOW