Manual

NxSeq HybCap Kits

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

IMPORTANT
Store each box at the appropriate temperature as indicated immediately upon receipt.

BIOSEARCH™ TECHNOLOGIES
GENOMIC ANALYSIS BY LGC

Manual

NxSeq HybCap Kits

Contents

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1. Technical support

LGC, Biosearch Technologies™ is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user are of the highest quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Email: techsupport@lgcgroup.com
Phone: +1 (888) 575-9695

Stability: Biosearch Technologies guarantees that this product is stable for one year from the date of shipment, if stored at the appropriate temperatures.

2. Product description

The NxSeq™ HybCap™ Kits are custom-designed, in-solution, hybridisation-based NGS library target enrichment kits compatible with libraries constructed using LGC, Biosearch Technologies, Illumina®, Ion Torrent®, and many other NGS DNA fragment library preparation kits. Our custom biotinylated RNA enrichment probes, HybCap Oligo Probes, are built on a highly flexible and efficient platform, based on your application, target regions and sample quality. Each kit contains all the reagents necessary, including your custom HybCap Oligo Probes, for hybridisation-based enrichment of your NGS libraries.

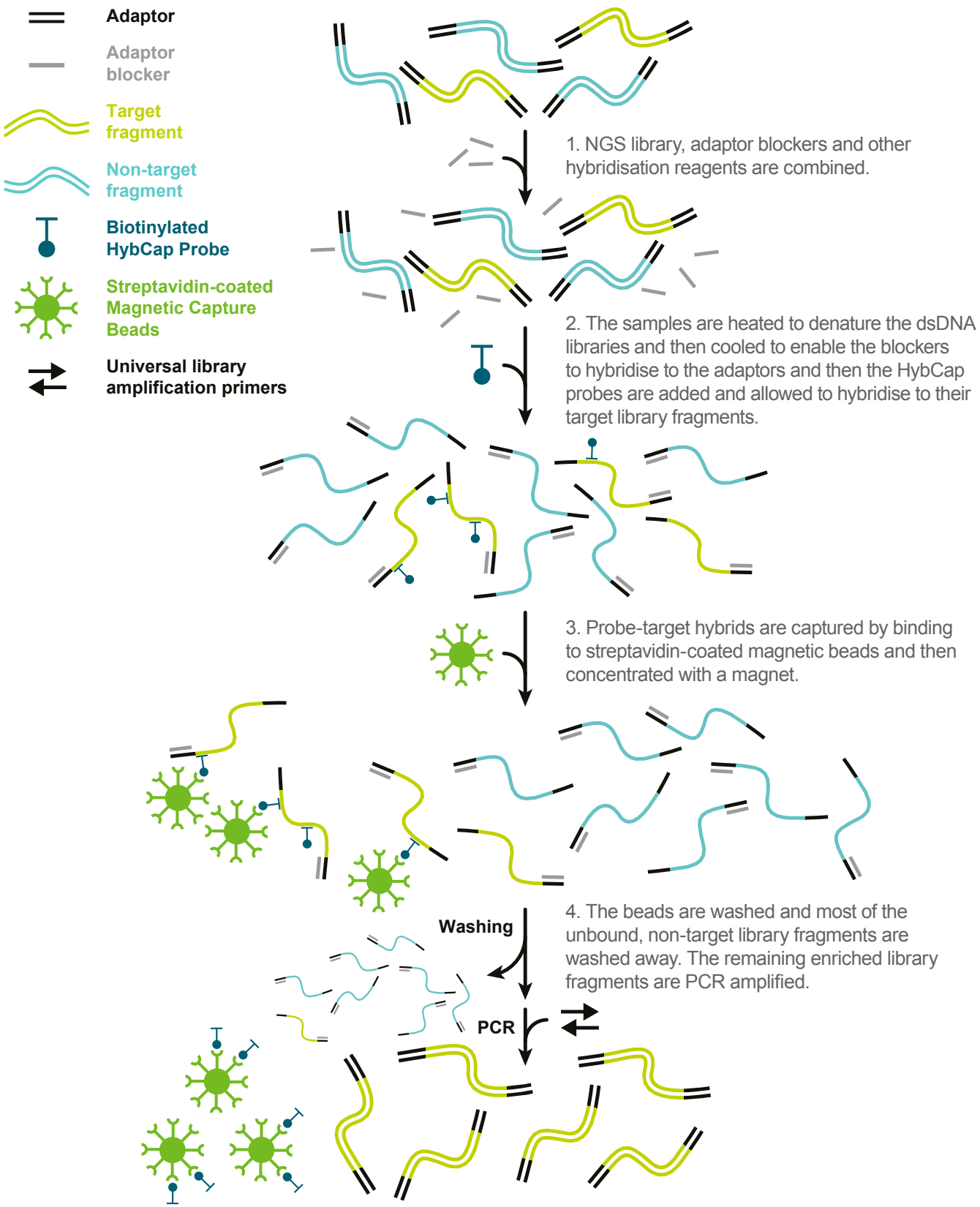

Figure 1. NxSeq HybCap Kit method overview

3. Product designations, kit components and storage conditions

3.1. NxSeq HybCap Kits covered by this manual

Part No.	Description
GEN-HC020K-016	NxSeq HybCap Kit, 1-20K probes, 16 reactions
GEN-HC020K-024	NxSeq HybCap Kit, 1-20K probes, 24 reactions
GEN-HC020K-096	NxSeq HybCap Kit, 1-20K probes, 96 reactions
GEN-HC020K-384	NxSeq HybCap Kit, 1-20K probes, 384 reactions
GEN-HC040K-016	NxSeq HybCap Kit, 21-40K probes, 16 reactions
GEN-HC040K-024	NxSeq HybCap Kit, 21-40K probes, 24 reactions
GEN-HC040K-096	NxSeq HybCap Kit, 21-40K probes, 96 reactions
GEN-HC040K-384	NxSeq HybCap Kit, 21-40K probes, 384 reactions
GEN-HC060K-016	NxSeq HybCap Kit, 41-60K probes, 16 reactions
GEN-HC060K-024	NxSeq HybCap Kit, 41-60K probes, 24 reactions
GEN-HC060K-096	NxSeq HybCap Kit, 41-60K probes, 96 reactions
GEN-HC060K-384	NxSeq HybCap Kit, 41-60K probes, 384 reactions
GEN-HC080K-016	NxSeq HybCap Kit, 61-80K probes, 16 reactions
GEN-HC080K-024	NxSeq HybCap Kit, 61-80K probes, 24 reactions
GEN-HC080K-096	NxSeq HybCap Kit, 61-80K probes, 96 reactions
GEN-HC080K-384	NxSeq HybCap Kit, 61-80K probes, 384 reactions
GEN-HC100K-016	NxSeq HybCap Kit, 81-100K probes, 16 reactions
GEN-HC100K-024	NxSeq HybCap Kit, 81-100K probes, 24 reactions
GEN-HC100K-096	NxSeq HybCap Kit, 81-100K probes, 96 reactions
GEN-HC100K-384	NxSeq HybCap Kit, 81-100K probes, 384 reactions
GEN-HC120K-016	NxSeq HybCap Kit, 101-120K probes, 16 reactions
GEN-HC120K-024	NxSeq HybCap Kit, 101-120K probes, 24 reactions
GEN-HC120K-096	NxSeq HybCap Kit, 101-120K probes, 96 reactions
GEN-HC120K-384	NxSeq HybCap Kit, 101-120K probes, 384 reactions
GEN-HC140K-016	NxSeq HybCap Kit, 121-140K probes, 16 reactions
GEN-HC140K-024	NxSeq HybCap Kit, 121-140K probes, 24 reactions
GEN-HC140K-096	NxSeq HybCap Kit, 121-140K probes, 96 reactions
GEN-HC140K-384	NxSeq HybCap Kit, 121-140K probes, 384 reactions
GEN-HC160K-016	NxSeq HybCap Kit, 141-160K probes, 16 reactions
GEN-HC160K-024	NxSeq HybCap Kit, 141-160K probes, 24 reactions
GEN-HC160K-096	NxSeq HybCap Kit, 141-160K probes, 96 reactions
GEN-HC160K-384	NxSeq HybCap Kit, 141-160K probes, 384 reactions
GEN-HC180K-016	NxSeq HybCap Kit, 161-180K probes, 16 reactions
GEN-HC180K-024	NxSeq HybCap Kit, 161-180K probes, 24 reactions
GEN-HC180K-096	NxSeq HybCap Kit, 161-180K probes, 96 reactions
GEN-HC180K-384	NxSeq HybCap Kit, 161-180K probes, 384 reactions
GEN-HC200K-016	NxSeq HybCap Kit, 181-200K probes, 16 reactions
GEN-HC200K-024	NxSeq HybCap Kit, 181-200K probes, 24 reactions
GEN-HC200K-096	NxSeq HybCap Kit, 181-200K probes, 96 reactions
GEN-HC200K-384	NxSeq HybCap Kit, 181-200K probes, 384 reactions

3.2. Kit components and storage conditions

			Kit by size					
			16 rxn		24-96 rxn	24 rxn	96 rxn	384 rxn
Box, storage	**Reagent**	**Cap color**	**Vol.**	**Qty**	**Vol.**	**Qty**		
Box 1, NxSeq HybCap Reagents Store at 4 °C	Hyb Reagent 1	Red	175 µL	1	500 µL	1	2	8
	Hyb Reagent 2	Teal	750 µL	1	750 µL	1	2	8
	Magnetic Capture Beads	Clear	550 µL	1	1600 µL	1	2	8
	Binding Solution	-	12 mL	1	36 mL	1	2	8
	Wash Solution	-	20 mL	1	60 mL	1	2	8
Box 2, NxSeq HybCap Reagents Store at -20 °C	Hyb Reagent 3	Yellow	70 µL	1	190 µL	1	2	8
	Hyb Reagent 4	Purple	25 µL	1	70 µL	1	2	8
	Blocker C	Green	50 µL	1	130 µL	1	2	8
	Blocker B	Blue	50 µL	1	130 µL	1	2	8
	Blocker A	Orange	30 µL	1	30 µL	1	2	8
Box 3, NxSeq HybCap Oligo Probes Store at -80 °C	HybCap Oligo Probes	White	50 µL	2	50 µL	3	12	48

The NxSeq HybCap reagents are stable for 1 year from date of shipment. If you will be using each tube of HybCap Oligo Probes over multiple, small experiments, we recommend thawing upon receipt, making sub-aliquots best suited to your experimental plans and refreezing at -80 °C. This approach minimises freeze-thaw cycles, which may negatively affect performance.

4. Additional materials and equipment needed

Materials and equipment needed	Vendor
Conical tubes, 50 mL, nuclease-free	Various
1.7 mL low-bind microcentrifuge tubes	Eppendorf (Cat. # 022431021), Axygen (Cat. # AXYMCT175LC)
0.2 mL low-bind PCR strip tubes with lids (optional)	Various – for 96-well format binding and washing
0.2 mL low-bind, thin wall PCR tubes	Various
Pipettors and tips (0.5 µL - 500 µL range)	Various
Multichannel pipettors (≤20 µL and ≤200 µL, optional but recommended)	Various
Magnetic rack	Various (e.g. Thermo Fisher Scientific, DynaMag™-2, #123-210) and/or 96-well magnetic particle concentrator, Permagen® 96-well Ring Magnet Plate S500 or similar
PCR thermal cycler with heated lid*	Various – must be compatible with 0.2 mL tubes
Minifuge (compatible with 0.2 mL and 1.5 – 1.7 mL tubes)	Various
Vortex mixer	Various
Water bath or hybridisation oven (65 °C)	Various
Heat block (65 °C)	Various
Nuclease-Free Water	Various, e.g. Thermo Fisher Scientific (Cat. #AM9938)
Resuspension Solution (10 mM Tris-Cl, 0.05% TWEEN®-20 solution, pH 8.0-8.5) (Need 30 µL per enrichment reaction)	User-made
PCR primers for post-enrichment library amplification	Various depending on library/sequencing platform (e.g. for Illumina libraries see primers described in Meyer & Kircher 2010 (doi:10.1101:pdb.prot5448) for Illumina libraries)
Hot start PCR master mix (for post-enrichment library amplification)	Various (e.g. LGC, Biosearch Technologies, 2X PCR Master Mix v2 – contact us, or Roche, KAPA™ HiFi HotStart ReadyMix)
PCR clean-up system (column-based or bead-based)	Various (e.g. LGC, Biosearch Technologies, sbeadex™ SAB Cat. # NAP45001/NAP45002/NAP45003/NAP45004, Beckman Coulter, AMPure® XP Beads, Cat. # A63880, A63881, A63882)

***Caution:** Ensure that you observe no more 4 µL of 30 µL volume evaporation overnight at 65 °C when using your preferred combination of 0.2 mL PCR tubes and thermal cycler.

5. Before you begin: experimental design considerations

Due to the diversity of sample and library types, quality, and type of targets that are compatible with the NxSeq HybCap Kits, it is impossible to provide target enrichment conditions that will be optimal for all libraries and custom HybCap Oligo Probes. If optimal performance is desired, it will require empirical testing to identify the best experimental conditions. Based on thousands of enrichments with the NxSeq HybCap Kits, the following experimental guidelines have proven effective over a range of sample, target and library types.

5.1. General recommendations

- The NxSeq HybCap Kits are compatible with most sequencing libraries, including LGC, Biosearch Technologies NxSeq UltraLow DNA Library Kits, Illumina TruSeq-style and Ion Torrent kits. **It is important to note, that each custom kit includes Blocker A, which is specific to the library type/kit and its index configuration used to build the libraries that will be input for each enrichment.** When using your custom kit for multiple target enrichments, be certain to use the same library kit and indexing strategy to build all the input libraries as outlined during the initial HybCap Oligo Probes design phase of project. Incorrect Blocker A may lead to more non-specific capture.
- When using libraries prepared with the Illumina Nextera® XT kits as input, please see Appendix 7.2 for important pre-treatment instructions. Libraries constructed with Nextera Flex Kits do not require pre-treatment.
- Using PCR-amplified libraries, as opposed to PCR-free libraries, as input for enrichment is strongly recommended. Generally, PCR-free libraries tend to perform poorly.
- Up to 7 µL of library can be used as input into each enrichment, and 100-500 ng total library per reaction is recommended. It may be necessary to concentrate the input libraries prior to hybridisation in order to be within this concentration range of ~14-70 ng/µL. If necessary, be certain to concentrate your libraries in advance of setting up the hybridisation reactions.
- We recommend quantifying each library using either a fluorescent intercalating dye system, such as Qubit®, or qPCR.
- As stated, we recommend using 100-500 ng of input library per capture. However, less than 1 ng and as much as 3 µg of total library may produce acceptable results.
- Multiple libraries can be pooled into a single capture reaction. We recommend starting with 4 pooled libraries per capture reaction using 125 ng of each library, for a total of 500 ng. When pooling libraries from larger genomes (>10 Gb), we recommend starting with pools of 4 libraries using 250 ng of each library for a total of 1 µg per pool/capture.
- We generally recommend using dual-indexed libraries and especially when pooling multiple libraries per capture reaction, to reduce the rate of mis-indexing induced by jumping events during pooled amplification. Unique dual indexed libraries are optimal although combinatorial dual indexed libraries can be pooled by carefully combining combinations that do not share any indexes.
- For most applications, **use 65 °C for all relevant steps** in the NxSeq HybCap Kit protocol. Do not exceed 68 °C. When the expected complementarity between the HybCap Oligo Probes and targets is below 95%, we recommend reducing the temperature from 65 °C to **62 °C** for all relevant steps.
- For most applications, maximum capture performance is usually obtained with 16-24 hour hybridisations.

5.2. Recommendations for degraded and/or contaminated libraries and low-target copy number libraries

- For libraries with a substantial anticipated amount of non-target templates (e.g., host/pathogen, saliva, ancient, forensic, or environmental samples), use a maximal amount of input library – up to 2 µg as input.
- When working with these challenging samples/libraries, we do not recommend pooling multiple libraries per enrichment reaction. If libraries must be pooled, normalise the input amount of each library based on the fraction of target in each library and not total mass. E.g. If “Library 1” is 50% target/50% other and “Library 2” is 25% target/75% other and your target is host, you would pool twice as much of “Library 2” with “Library 1.”

- When most of the library inserts are shorter than 80 bp and/or heavily damaged, use 60 °C temperatures for all relevant steps of the NxSeq HybCap Kit protocol. Improved results, if desired, will require additional optimisation experiments.
- For libraries with very low proportions of target DNA, such as transgene mapping samples/libraries or heavily contaminated ancient DNA libraries, a 36-48 hour hybridisation time, or longer in some cases, may be beneficial.
    - Additionally, two rounds of capture are also strongly recommended for these types of enrichments; consult with us for experiment design advice.

6. Protocol

6.1. Hybridisation set up

In this first part of the protocol, the DNA fragment sequencing libraries are mixed with blocking nucleic acids, denatured, and then combined with a mixture of hybridisation reagents (including your custom HybCap Oligo Probes). These hybridisation reactions are then incubated for several hours to allow the HybCap Oligo Probes to hybridise to their specific target library molecules.

6.1.1. Gather the required materials and thaw at room temperature unless noted otherwise

Reagents:

- Hyb Reagents 1-4 (Boxes 1 and 2)
- Blockers A-C (Box 2)
- HybCap Oligo Probes (Box 3) ***Keep on ice***
- Input sequencing libraries, generally 100-500 ng of total library in a final volume of 7 µL per hybridisation reaction

Equipment:

- 1.7 mL low-bind, nuclease-free microcentrifuge tubes (×2 per hyb reaction)
- Low-bind 0.2 mL tubes with individual caps (×2 per hyb reaction)
- Pipettors and tips; **multichannel pipettor for pipetting up to 20 µL recommended**
- Vortex mixer
- Thermal cycler; 2 blocks recommended for 24 or more reactions

6.1.2. Program thermal cycler

- **Important:** Use a heated lid for all steps to keep evaporation to a minimum.
- The following 65 °C temperature for steps 2 and 3 is the standard hybridisation temperature. Based on your sample type, targets, etc, you may adjust these temperatures as outlined in section 5.1 and 5.2.

Step	Temperature	Time
1	95 °C	5 min
2	65 °C	5 min
3	65 °C	∞

6.1.3. Set up hybridisation mix

1. Once the Hyb Reagents have thawed, vortex them to homogenise, and briefly centrifuge.
   Note: If Hyb Reagent 1 and/or 2 have visible precipitate after thawing, heat them to 60 °C and vortex until the precipitate dissolves.
2. Assemble the hybridisation mix in a 1.7 mL tube as follows, briefly vortex and centrifuge to collect contents in the bottom of the tube.
   - **Important:** Do not adjust these volumes per reaction because extra volume of each component is already included to account for downstream pipetting errors.
   - When performing more than one hybridisation reaction, we recommend scaling the volumes of each component in the hybridisation mix based on the number of reactions and then dispensing 18.5 µL aliquots from that bulk hybridisation mix (step 4 below).

Hybridisation mix

Component	Volume per reaction (µL)
Hyb Reagent 1	9.25
Hyb Reagent 2	0.5
Hyb Reagent 3	3.5
Hyb Reagent 4	1.25
HybCap Oligo Probes	5.5
TOTAL	**20**

Note: Addition of Hyb Reagent 2 will cause cloudiness; the mixture will clear after step 3.

3. Incubate the hybridisation mix at 60 °C for 10 minutes in a heat block with occasional vortexing to collect condensation from the tube lid. Remove the mix from the heat block and incubate at room temperature for 5 minutes.
4. For each capture reaction, aliquot 18.5 µL of hybridisation mix into a 0.2 mL low-bind tube. These aliquots of hybridisation mix are now named “HMXs.”

6.1.4. Set up blocker mix

1. Assemble the blocker mix as follows in an appropriately sized tube and mix by pipetting.
   - **Important:** Do not adjust these volumes per reaction because extra volume of each component is already included to account for downstream pipetting errors.
   - When performing more than one hybridisation reaction, we recommend scaling the volumes of each component in the blocker mix based on the number of reactions and then dispensing 5 µL aliquots from that bulk blocker mix (step 2 below).

Blocker mix

Component	Volume per reaction (µL)
Blocker A	0.5
Blocker B	2.5
Blocker C	2.5
TOTAL	**5.5**

2. For each capture reaction, aliquot 5 µL of blocker mix into a low-bind 0.2 mL tube.
3. Add 7 µL of library (100 – 500 ng recommended) to each blocker mix aliquot and mix by pipetting. These libraries/blocker mixes are now named "**LBMXs**."

6.1.5. Assemble hybridisation reaction and hybridise

Important: Verify that the thermal cycler is programmed correctly. Standard hybridisation conditions are:

Step	Temperature	Time
1	95 °C	5 min
2	65 °C	5 min
3	65 °C	∞

1. Place the **LBMXs** in the thermal cycler, close the lid, and start the thermal program.

2. Once the cycler reaches the hybridisation temperature, 65 °C (standard), during step 2, pause the program, place the **HMXs** in the thermal cycler, close the lid, and resume the program.

3. After step 2 of the program is complete, leave all tubes in the thermal cycler and **pipette 18 µL of each HMX into one of the LBMX tubes**. Use a multichannel pipettor to simplify transfer. Gently homogenise the HMX + LBMX mixes by pipetting up and down 5 times.

4. Dispose of the **HMX** tubes. Briefly spin down the **LBMXs**, return to the thermal cycler, close the lid, and allow the reactions to incubate at the hybridisation temperature, 65 °C (standard), for your preferred incubation time (e.g., 16-24 hours).

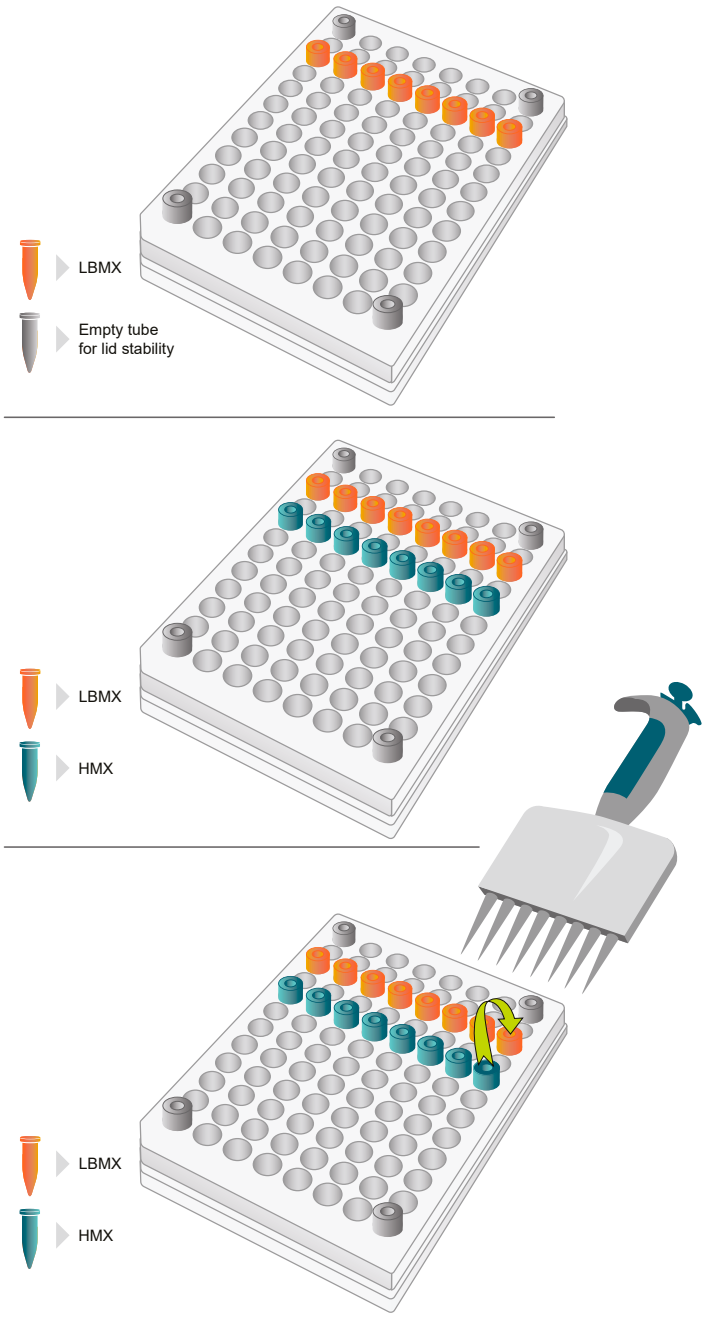

Figure 2. Hybridisation reaction mixing and overnight hybridisation steps in the thermal cycler.

6.2. Capture and washing (clean-up)

In this portion of the protocol, the biotin-labeled HybCap Oligo Probe-target hybrid molecules (probes hybridised to their target library fragments) are bound to streptavidin-coated magnetic beads, and the non-specific library fragments are removed with several wash steps. This portion of the protocol is performed after the overnight hybridisation step in section 6.1 is completed.

6.2.1. Gather the following materials and warm to room temperature if needed

Important: Start this step at least 90 minutes before the overnight hybridisation reactions are stopped.

Reagents:

- Hyb Reagent 2
- Binding Solution
- Wash Solution

  Bring these solutions to room temperature prior to use; warm gently to dissolve precipitate if necessary
- Magnetic Capture Beads
- Nuclease-free Water (need up to 900 µL per reaction clean-up)
- Resuspension Solution (User made: 10 mM Tris-Cl, 0.05% TWEEN-20 solution, pH 8.0-8.5)

Equipment:

- Water bath or hybridisation oven set to the intended bind and wash temperature (standard temperature is 65 °C)
- Vortex mixer
- Microfuge for 2 mL and 0.2 mL size tubes/strips
- Magnetic rack/concentrator for ~1.8mL and/or 0.2 mL PCR strips/plates

When using only a microfuge tube-compatible magnetic rack	When using a 0.2 mL tube- or strip-tube-compatible magnetic rack
• Low-bind, nuclease-free 1.7 mL tubes, 1 tube per clean-up • Heat block set to the bind and wash temperature(s) – normally 65 °C • Pipettors and tips for 20 – 500 µL volumes • 50 mL conical tube, nuclease-free - 1 per 44 clean-ups needed	• Nuclease-free 0.2 mL PCR strip tubes with individually-attached lids, 1 tube per clean-up • Thermal cycler set to the bind and wash temperature(s) – normally 65 °C • Pipettors and tips for 20 – 200 µL volumes; multichannel pipettor strongly recommended • 50 mL conical tube, nuclease-free - 1 per 68 clean-ups needed

6.2.2. Prepare Wash Solution F

This step generates enough Wash Solution F for 44 reactions in microcentrifuge ("MC") tube clean-up format, and 68 reactions in 0.2 mL clean-up format; scale up or down if needed. Wash Buffer F can be stored at 4 °C for 1 month.

1. Thaw and thoroughly homogenise the Wash Solution and Hyb Reagent 2 prior to aliquoting in order to dissolve any visible precipitate; warm slightly if necessary.
2. Prepare Wash Solution F by combining the following in a 50 mL conical tube and mix thoroughly by vortexing.

Wash Solution F

Component	Volume
Nuclease-free water	39.6 mL
Hyb Reagent 2	400 µL
Wash Solution	10 mL
TOTAL	**50 mL**

3. Label this mix as "Wash Solution F."
4. Heat Wash Solution F to the hybridisation temperature, normally 65 °C, in the water bath or hybridisation oven for at least 30 minutes before use.

6.2.3. Prepare Magnetic Capture Beads

Important: Prepare beads fresh immediately before use

1. For each capture reaction, aliquot 30 µL of Magnetic Capture Beads into a low-bind, 1.7 mL microfuge tube.
2. Pellet the beads by placing them in the appropriate magnetic rack until the suspension is clear (1-2 minutes). After the pellet has formed and with the tubes on the magnet, carefully remove and discard the supernatant.
3. Add 200 µL Binding Solution to each bead pellet, remove from the magnetic rack, vortex to resuspend the beads and then centrifuge briefly. Pellet the beads by placing the tubes in the magnetic rack, and once the pellet has formed (1-2 minutes), carefully remove and discard the supernatant.
4. Repeat Step 3 above two additional times for a total of three washes.
5. Resuspend each washed bead pellet in 70 µL Binding Solution. If proceeding to washing in 0.2 mL format, transfer the aliquots to PCR strips.

TIP: *Bead preparation can be scaled to simplify the process. Beads can be prepared in batches of 8 (or fewer) reactions in a 1.7 mL tube. Multiply all reagent volumes by the number of reactions in the batch; i.e., for 8 reactions-worth (240 µL beads), wash with 1.6 mL and resuspend in 560 µL Binding Buffer, then aliquot 70 µL suspension to individual tubes.*

6.2.4. Bind beads and hybrids

1. Heat the bead aliquots to the hybridisation temperature (e.g., normally 65 °C) for at least 2 minutes in the heat block or thermal cycler.
2. Transfer each capture reaction (from step 4 of section 7.1.5) to the heated bead aliquots. Mix by pipetting.
3. Incubate the libraries + beads in the hot block or thermal cycler for 5 minutes. Agitate the mix after 2.5 minutes by flicking or inverting the tubes to keep the beads suspended, followed by briefly centrifuging to collect and placing the tubes back in the heat block or thermal cycler.

6.2.5. Wash beads

1. Place the bead tubes on a magnetic rack to pellet the beads until the solution is clear. Carefully remove and discard the supernatant while the tubes are still on the magnetic rack.
2. Add 375 µL (microcentrifuge tube format) or 180 µL (0.2 mL tube format) of warmed Wash Solution F (prepared in 6.2.2) to the beads, remove from the magnetic rack, and briefly vortex or mix by pipetting. Briefly centrifuge to collect all contents in the bottom of the tube.
3. Incubate for 5 minutes at the hybridisation temperature, normally 65 °C, in the heat block or thermal cycler. Mix after 2.5 minutes by gently vortexing and briefly centrifuge to collect contents in the bottom of the tube.
4. Repeat steps 1 through 3 two times for microcentrifuge tube format (three washes total), or three times for 0.2 mL tube format (four washes total). After the last wash and pelleting, **remove as much fluid as possible without touching the bead pellet.**

   Tip: *A quick spin after the last wash may help to collect any remaining fluid in the bottom of the tube, which can then be removed with the tubes on the magnetic rack.*

6.3. Library resuspension and amplification

Within this section of the protocol, the Magnetic Capture Bead-bound enriched library is resuspended in Resuspension Solution, a simple Tris-based solution, and then either taken directly to PCR amplification while bound to beads or heat-denatured from the HybCap Oligo Probes and then PCR amplified.

6.3.1. Gather and prepare the following materials

Reagents:

- Resuspension Solution (User-made: 10 mM Tris-Cl, 0.05% TWEEN-20 solution, pH 8.0-8.5)
- Reagents for library amplification using universal primers
- PCR purification system, e.g., silica columns or SPRI beads

Equipment:

- Tubes appropriate for PCR master mix assembly
- Tubes for 50 µL PCR amplification reactions, e.g., 0.2 mL PCR strips or plates
- Pipettors and tips capable of 5 – 100 µL volumes
- Vortex mixer
- Mini centrifuge for 2 mL and 0.2 mL size tubes/strips
- Thermal cycler

6.3.2. Resuspend the enriched library

1. Add 30 µL of Resuspension Solution to the washed beads and thoroughly resuspend the bead pellet by pipetting. Then, depending on your amplification system:

When using KAPA HiFi HotStart ReadyMix	When using a different HiFi PCR master mix for amplification
2. Proceed directly to section 6.3.3 using these resuspended beads as template in the PCR amplification step	2. Incubate the resuspended beads at 95 °C for 5 minutes. 3. Immediately pellet the beads by placing in a magnetic rack. Once the pellet forms, carefully remove only the supernatant containing the denatured, enriched library fragments.

6.3.3. PCR amplify each enriched library

The following protocol is a library amplification example using KAPA HiFi HotStart ReadyMix and Illumina libraries. If you are using libraries constructed with a different library preparation kit or a different PCR master mix, please adjust the parameters appropriately.

1. Assemble the following PCR reaction mix in PCR tubes or strips of your choice:

   Tip: *If you are amplifying multiple enriched libraries, you can scale this reaction mix without library as needed with some overage to cover pipetting errors. Then combine 35 µL of this reaction mix with 15 µL of each enriched library to be amplified in PCR tubes.*

Component	Final Concentration	Volume per reaction (µL)
Nuclease-free water	-	5
2X KAPA HiFi HotStart ReadyMix	1 X	25
Forward library primer (at 10 µM)	500 nM	2.5
Reverse library primer (at 10 µM)	500 nM	2.5
Enriched library (on- or off-bead)	-	15*
TOTAL		**50**

*Remaining bead-bound library can be stored at -20 °C for several months and amplified later if necessary.

2. Place the tubes in thermal cycler set to the following cycling conditions and cycle. Use a heated lid to avoid evaporation.

   Important notes on cycling and total cycling number:

   - For libraries <500 bp average size use a 30 sec extension time
   - For libraries ranging from 500-700 bp average size use a 45 sec extension time
   - For libraries >700 bp average size, use a 1 min extension time
   - **Use the fewest cycles required for minimum sequencing requirements. Some situations, for example when using very low input amounts per enrichment, may require >14 PCR cycles.**

Step	Temperature	Time	
1	98 °C	2 minutes	
2	98 °C	20 seconds	× 8 to 14 cycles
3	60 °C	30 seconds	
4	72 °C	length-dependent, see above	
5	72 °C	5 minutes	
6	8 °C	∞	

3. **If the Magnetic Capture Beads were included in the amplification reaction and you intend to use silica columns for purification,** pellet the beads first by placing the PCR reactions on a magnetic rack, remove the supernatant and purify the amplified, enriched libraries from the supernatant. Otherwise, purify the amplified, enriched libraries using your preferred clean-up method/system (e.g., silica columns, sbeadex SAB or AMPure XP beads).
4. The enriched libraries are now ready for sequencing.

7. APPENDIX

7.1. Troubleshooting and anticipated results

During hybridisation, my thermal cycler dropped below the required hybridisation temperature. What should I do?

You can expect a lower proportion of on-target reads proportion and lower read complexity for these libraries than libraries hybridised at the correct temperature. You will not observe a complete failure of the enrichment and sequencing. We would recommend a shallow, preliminary sequencing run with these libraries to determine if your targets will ultimately be sequenced at sufficient coverage and within your budget.

My amplified enriched library is not visible on an electrophoresis gel or similar

After enrichment and amplification, the yields, and therefore concentrations, of some libraries will be too low to visualise with standard electrophoresis methods. This visualisation failure does not always indicate that the enrichment procedure failed. Successful enrichments frequently yield just a few nanograms of library even after amplification of the enriched libraries. Invisible enriched libraries are typically the result of (1) capturing very small targets (<100 bp), (2) targets that were present at low frequency in the starting library (like those in transgene mapping experiments or degraded/ancient/environmental DNA samples), or (3) under-amplification of the library post-enrichment. Often a few more amplification cycles of the enriched library will increase the yield high enough such that the enriched library will be visible by electrophoresis. However, we recommend enriched library quantification by qPCR, and visualisation of the qPCR product prior to reaching amplification plateau, in order to determine sequenceable mass and length distribution of the enriched library. This added step will also tell you whether it is necessary to amplify the library with more cycles before sequencing. Consult with your sequencing provider for their library concentration and volume requirements.

I am observing a high ratio of PCR duplicates in my enriched library sequence data. Why and what can I do?

The total number of unique sequences available in an enriched library is mainly driven by the starting complexity of the DNA fragment library used as input and the capture sensitivity of the custom HybCap Oligo Probe set. Each PCR cycle used during indexing and post-capture amplifications can induce representation bias between templates. **Percent duplicates** (aka "duplication rate") in the sequencing results, on the other hand, is tightly linked to sequencing depth, and can only be fairly compared between experiments when the sequencing depth is normalised before analysis. Investigate whether you have simply over-sequenced the libraries by building a 2D plot with raw sequencing reads obtained on the X-axis, and unique on-target reads observed on the Y-axis. If this **complexity curve** has plateaued, but you achieved sufficient unique reads, you sequenced more deeply than necessary. If it has not plateaued, or you need to increase the total potential unique read yield of the library, **use more DNA per library preparation and/or more library per enrichment reaction. Avoid diluting the HybCap Oligo Probes before enrichment**. If you are working with heavily contaminated or damaged DNA target molecules, consider reducing temperatures used in all steps to improve enrichment sensitivity. Reducing PCR cycles where you can might also improve target coverage uniformity and complexity for a given sequencing depth, and in some cases, this cycle reduction may have an indirect effect on observed duplication rates. For more information about library complexity for any NGS application, we recommend Daley & Smith 2013 (doi: 10.1038/nmeth.2375).

7.2. Pre-treating libraries made with Nextera kits

We have observed that unmodified, fully indexed and amplified libraries made with the Nextera kits will bind the streptavidin Magnetic Capture Beads. This binding renders these libraries incompatible with the HybCap Oligo Probes and this protocol. The following procedure solves this problem by doing additional amplification of the Nextera libraries with a few PCR cycles and universal primers. This addition PCR amplification produces library fragments that will no longer bind to the streptavidin Magnet Capture Beads unless they are complexed with HybCap Oligo Probe, making them compatible with these kits and protocol.

7.2.1. Prepare and gather the required materials

Reagents:

- Universal PCR library amplification primers for Illumina (e.g. Nextera) libraries after enrichment. e.g., the "reamp" primers described in Meyer & Kircher 2010 (doi:10.1101:pdb.prot5448) for Illumina libraries
- High fidelity, hot start PCR master mix (e.g., Roche, KAPA HiFi HotStart ReadyMix or the LGC, Biosearch Technologies 2X PCR Master Mix v2 – contact us)
- Dynabeads® MyOne™ Streptavidin C1 Beads (require 30 µL per library amplification)
- Salt Solution: 1M NaCl, 10 mM Tris-HCl, pH 7.5 (require 200 µL per library amplification)
- PCR purification materials of your choice (e.g., silica columns, sbeadex SAB or AMPure XP beads, etc.)

Equipment:

- Tubes for 50 µL PCR amplification reactions, e.g., 0.2 mL PCR tubes/strips
- Thermal cycler
- Magnetic rack compatible with tubes or strips (1 tube per library)
- Pipettors and tips for 20 µL – 200 µL volume ranges
- Vortex mixer, mini centrifuge, etc.

7.2.2. Amplify indexed Nextera libraries

We recommend using a polymerase with reduced GC- and length-bias, such as our custom 2X PCR Master Mix v2 (contact us) or the KAPA HiFi HotStart ReadyMix (Roche) and the universal primers designed to amplify Illumina sequencing libraries without overwriting the index sequences (see Reagents section above).

The following amplification protocol example uses the KAPA HiFi HotStart ReadyMix and Nextera libraries. If you are using a different PCR master mix, please adjust the parameters appropriately.

1. Assemble the following PCR reaction mix in PCR tubes or strips of your choice:

Component	Final conc.	Volume per Reaction (µL)
2X KAPA HiFi HotStart ReadyMix	1 X	25
P5-side universal primer (10 µM)	500 nM	2.5
P7-side universal primer (10 µM)	500 nM	2.5
200 ng Indexed Nextera Library		≤20
Nuclease-free water		Bring up to 50 total
TOTAL		**50**

2. Place the tubes in thermal cycler set to the following cycling conditions and cycle. Use a heated lid to avoid evaporation.

Step	Temperature	Time	
1	98 °C	2 min	
2	98 °C	20 sec	× 4 cycles
3	60 °C	30 sec	
4	72 °C	60 sec	
5	72 °C	5 min	
6	8 °C	∞	

7.2.3. Prepare streptavidin magnetic beads

While the reactions in 7.2.2 are cycling, prepare streptavidin-coated magnetic beads, which will be used to remove the residual/original streptavidin-binding molecules still present in the Nextera library after PCR amplification in step 7.2.2.

1. For each amplification reaction, aliquot 30 µL Dynabeads MyOne Streptavidin C1 beads into a tube compatible with your magnetic rack.
2. Place the tube of beads in the magnetic rack and allow the bead pellet to form until the suspension is clear (~1-2 minutes). Leave the tubes in the magnetic rack, then remove and discard the supernatant.
3. Add 100 µL of Salt Solution to each bead pellet. Take off the magnetic rack and vortex 3 seconds, followed by brief centrifugation. Pellet the beads on the magnetic rack until clear, and then remove and discard the supernatant.
4. Repeat step 3 one more time for a total of two washes.

7.2.4. Deplete residual streptavidin-binding molecules

In this section of the Nextera clean-up protocol, we will remove the residual/original streptavidin-binding molecules still present in the Nextera library after PCR amplification in step 7.2.2 and used the cleaned Nextera library as input into the standard NxSeq HybCap Kit enrichment protocol.

1. Once the PCR program (7.2.2) is complete, remove the reactions from the thermal cycler.
2. Transfer each PCR reaction to a washed aliquot of Dynabeads MyOne Streptavidin C1 beads prepared above.
3. Mix the beads and PCR reaction by pipetting up and down until all the beads are suspended.
4. Incubate the suspension at room temperature for 15 minutes.
5. Place the tube in a magnetic rack and pellet the beads until the supernatant is clear.
6. Carefully remove the supernatant containing your library that is now free of any streptavidin-binding molecules.
7. Purify the supernatant using silica columns, sbeadex SAB, AMPure XP beads or other clean-up method.

The final Nextera cleaned-up/purified library is now compatible with the HybCap Oligo Probes and protocol and can be used an input into the enrichment process like a normal sequencing library.

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