KIT MANUAL

TwinStrand Duplex Sequencing™ Kit Manual

Unparalleled Accuracy | Unprecedented Sensitivity

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

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3131 Elliott Ave, Suite 750 | Seattle, WA 98121 | +1(877) 202-TWIN | support@twinstrandbio.com
twinstrandbio.com

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

PATENT MARKING

Patent: www/twinstrandbio.com/legal

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Overview

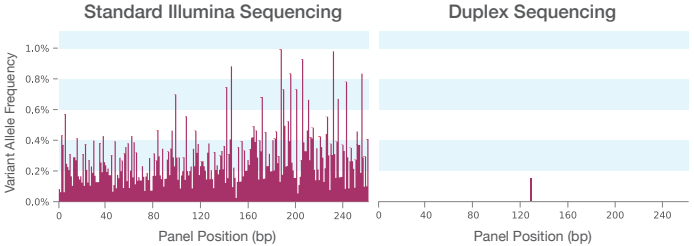
Duplex Sequencing Technology

TwinStrand Duplex Sequencing™ Technology is a biochemical and informatics-based error correction approach that provides unprecedented sequencing accuracy and enables highly sensitive detection of ultra-low frequency genetic variants.

Duplex Sequencing Kits contain all the reagents necessary (besides user-supplied water and ethanol) to convert fragmented DNA, across a broad range of input amounts, into Duplex Sequencing Libraries for next-generation sequencing on any Illumina sequencing platform.

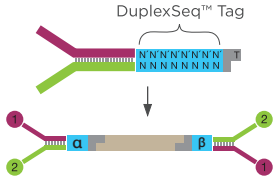
This manual provides a step-by-step protocol for Duplex Sequencing Library preparation and general guidelines regarding the simple adjustments needed to accommodate different hybrid capture panels and amounts of input DNA. For each application-specific kit, please refer to the separate Panel Information Sheets for exact recommendations, where noted in the protocol.

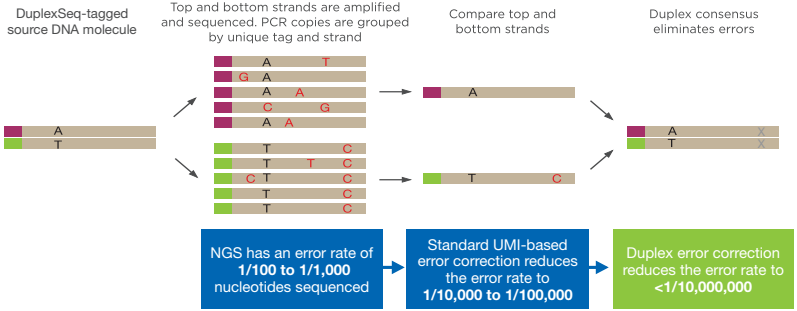
Raw sequencing data produced from the Duplex Sequencing Libraries generated by this kit is intended to be processed into fully error-corrected Duplex Consensus Sequences using the TwinStrand DuplexSeq™ software, which is available through TwinStrand's portal on the DNAnexus platform. Access information and software instructions are included with each kit.



The same gene sequenced by standard Illumina sequencing (left) and with Duplex Sequencing (right). With conventional methods, every position in the gene appears mutated in 0.1 – 1% of molecules sequenced. Duplex Sequencing eliminates the background noise, revealing the previously hidden single true mutation.

- Duplex Sequencing capitalizes on the naturally-occurring complementarity between strands of the DNA double-helix
- Each strand of each original molecule is uniquely labelled such that both can be tracked throughout amplification and sequencing for subsequent error correction





Using This Manual

Icons:

The following icons are used throughout the manual with the following meanings:

	Reagents stored (or steps executed) in an amplicon free, Pre-PCR area
	Reagents stored (or steps executed) in a Post-PCR work area
	To be stored frozen at -20°C
	To be stored refrigerated at 4°C
	Hazardous material warning
	Safe stopping point during the protocol
	General tips and tricks
	Important information relating to a specific step or reagent

The color of the tops of the kit boxes guide which work area each box should be in: "Green is Clean" (i.e., amplicon free pre-PCR area). Throughout the manual the above green and blue work area logos indicate the correct location for each protocol step.

Information Sheets:

For application-specific kits, this manual is supplemented with a Panel Information Sheet for guidelines on DNA input, required number of sequencing clusters and anticipated Duplex Depth. If you are using the Universal Kit and supplying your own probe panel, refer to Appendix III for library input and sequencing guidelines.

Configuration Reagents:

There are five classes of reagents that vary between kits based on their intended application and species: Cot-1 DNA (**COT**), Control Panels (**CTP**), application-specific Panels (**PAN**), DNA Technical Controls (**DTC**) and DNA Standards (**DNS**). All reagents are labeled with their three-letter code on the tube cap and the specific product name on the side. Every CTP, PAN, DTC and DNS reagent is accompanied by an Information Sheet with detailed specifications and use guidelines.

Before using the supplied kit:

- Carefully review all safety information, guidelines, protocol steps and notes
- Thoroughly read all relevant Appendixes and Information Sheets
- Ensure all necessary equipment is available, programed, calibrated and in working order
- Confirm consumables are compatible with equipment and an adequate supply is on hand
- Note the point at which the protocol instructs to move work from the pre-PCR to the post-PCR area
- Ensure work space and equipment is clean and free of amplicons in the pre-PCR area
- Keep a copy of all necessary documentation (Kit Manual, Panel Information Sheets, DNA Information Sheets) accessible in each work area
- If you are a first-time user, we *strongly* recommend carrying out the protocol with the included DNA Technical Control (DTC) and a TwinStrand-supplied panel (PAN or CTP) before processing valuable samples

Support

For additional assistance with steps in this manual contact us at:

support@twinstrandbio.com

+1 (877) 202-TWIN

Safety

When performing the Duplex Sequencing Kit workflow, please follow standard laboratory safety precautions including:

- Use of appropriate personal protective equipment (eye protection, lab coat, closed shoes, gloves, etc.)
- Ensuring your work space is equipped with appropriate safety equipment (eye wash station, fire extinguishers, etc.)
- Proper handling of potentially harmful chemical material. Look for the Hazard icon throughout this manual. See MSDSs at www.twinstrandbio.com/safety.
- Proper pathogen precautions when working with biological materials (tissues, body fluids, cell culture, etc.) prior to DNA extraction.

HAZARD: This set of reagents contains formamide, an aliphatic amide that may be toxic to reproduction. Injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat. Disposal as chemical waste is subject to user compliance in accordance with the governmental safety standards for the region and product characteristics at the time of disposal.

KIT STORAGE

Kits are shipped with boxes A-D at +4˚C. Upon arrival, boxes A & C should be stored frozen at -20˚C and boxes B & D should be stored refrigerated at +4˚C. We strongly recommend following best practices for DNA-amplification-based protocols, which entails maintaining separate workspaces and cold storage units for pre- and post-PCR reagents to avoid potential amplicon cross-contamination. The storage temperature and work area for each box are indicated by icons on the label. TwinStrand™ Buffer may be removed from boxes and stored at room temperature. Reagents stored at -20˚C are stable up to 10 freeze-thaw cycles.

Kit Documentation:

Two sets of instructions are provided with every kit to maintain one set in each distinct work area (pre-PCR and post-PCR). The kit documentation includes:

Kit Manual	Panel Information Sheet(s)
Bioinformatics Guide	DNA Information Sheet(s)

KIT CONTENTS

BOX A: Pre-PCR Components, stored at -20˚C

REAGENT		24 Sample Kit Volume	48 Sample Kit Volume
ERAT	End Repair / A-Tail Mix	540 µL	1.08 mL
LIG	Ligation Reaction Mix	790 µL	1.58 mL
ADAP	DuplexSeq™ Adapters	150 µL	300 µL
LCM	Library Conditioning Mix	100 µL	200 µL
cPCR	PCR Master Mix (Pre-PCR)	525 µL	1.05 mL
IND*	Indexing Primers (in 96-well plate)	7 µL/well	7 µL/well
DTC**	DNA Technical Control	100 µL	100 µL
DNS***	DNA Standard(s)	≥100 µL	≥100 µL

Each kit is shipped with a plate of 48 unique dual indexes comprising either set A (IND-A), Set B (IND-B) or a 96 index set of both together (IND-A+B). See Appendix V for the specific arrangement of indexes.

***Each kit contains a species-specific DNA Technical Control at concentration of 25 ng/µL (2.5 µg total). The species is listed on the side of the tube. See the corresponding DNA Information Sheet accompanying your kit.*

****For application-specific products, one or more DNA Standards may be included. The concentration, volume, species and nature of the standard is listed on the side of the tube. See accompanying DNA Information Sheet.*

BOX B: Pre-PCR Components, stored at +4˚C

REAGENT		24 Sample Kit Volume	48 Sample Kit Volume
cSPRI	SPRI Beads (Pre-PCR)	1.80 mL	3.60 mL
cTSB	TwinStrand™ Buffer (Pre-PCR)	775 µL	1.55 mL

TIP *For reagents that are present in both pre- and post-PCR kit boxes, a "c" or a "d" precedes the reagent name as a quick reminder of "clean" (pre-PCR) and "dirty" (post-PCR).*

BOX C: Post-PCR Components stored at -20˚C

REAGENT		24 Sample Kit Volume	48 Sample Kit Volume
HYB	Hybridization Buffer	1.30 mL	2.60 mL
CAP	Capture Mix	100 µL	200 µL
BWB	Bead Wash Buffer (2x)	7.00 mL	14.0 mL
EQB	Equilibration Buffer (10x)	1.70 mL	3.40 mL
SWB	Stringent Wash Buffer (10x)	1.10 mL	2.20 mL
dPCR	PCR Master Mix (Post PCR)	1.00 mL	2.00 mL
PRM	P5/P7 Primers	260 µL	520 µL
COT*	Species-Specific Cot-1 DNA	264 µL	528 µL
CTP**	Species-Specific Control Panel	11 µL	11 µL
PAN***	Application-Specific Hybrid Capture Panel	53 µL	106 µL

**Each kit is supplied with Cot-1 DNA, the species of which is listed on the side of the tube.*

***Every kit contains a small control panel, which entails a densely tiled 2.4 kb set of probes directed at a portion of the species-specific genome that is listed on the side of the tube. The control panel is supplied at an optimized concentration and with enough material for 5 libraries. See the Panel Information Sheet accompanying your kit.*

****For application-specific products, one or more application-targeted panel is included in your kit. The specific panel identity is listed on the side of the tube. Each is supplied at an optimized concentration and should be used undiluted. See the Panel Information Sheet(s) accompanying your kit. Not included with Universal Kits where panel is user-supplied.*

BOX D: Post-PCR Components, stored at +4˚C

REAGENT		24 Sample Kit Volume	48 Sample Kit Volume
dSPRI	SPRI Beads (Post-PCR)	3.96 mL	7.92 mL
STREP	Streptavidin Beads	1.25 mL	2.50 mL
FORM	Formamide	10.0 mL	10.0 mL
dTSB	TwinStrand™ Buffer (Post-PCR)	4.00 mL	8.00 mL

GUIDELINES FOR SAMPLE PROCESSING

1. *Preventing Cross-Contamination*

Duplex Sequencing is extremely sensitive and even slight cross-contamination may impact how results are interpreted. Great care should be taken to prevent contamination at all stages of sample processing, including collection and handling of biological materials, extraction of DNA and preparation of Duplex Sequencing Libraries.

When working with tissues or cells, use new sterile instruments and disposable dishes with every sample. When extracting DNA and preparing libraries, always use filtered pipette tips and change these between samples. Use appropriate length tips such that the shaft of the pipette is not at risk of coming into contact with the inside of tubes. When applying adhesive seal to PCR plates, use even pressure across the entire plate to carefully seal each well. Pay particular attention to edge and corner wells. Always check the adhesive seal for condensation before removing the seal from the plate. If condensation is present, briefly centrifuge the plate to remove condensation. When unsealing a plate, do so slowly and with a steady, even tension to prevent splashing. Never re-use plate seals; apply a fresh seal every time a plate is opened.

2. *Pre- and Post-PCR Work Areas*

DNA samples and pre-PCR reagents should be handled in an area isolated from PCR products to reduce the risk of amplicon contamination. Perform Library Preparation at an amplicon-free bench, freshly wiped down with 0.5% sodium hypochlorite (10% commercial bleach) and with a new disposable bench cover. Post-PCR steps should be performed at a different bench than used for the Library Preparation and one that is also bleached regularly. Wipe pipettes with above bleach solution and then water and allow to dry. Use only filtered pipette tips. Wear a clean lab coat and gloves and change both when moving between pre- and post-PCR areas. It is best practice to maintain a separate set of pipettes, tube racks and other laboratory equipment in each work area which are, ideally, in different rooms.

3. *DNA Extraction*

We recommend Qiagen silica column-based kits or “salting out” methods for DNA extraction. Please refer to the Qiagen website for the appropriate kit for your sample type if using the former. Do not use phenol-chloroform extraction or temperatures greater than 56℃. Modify incubation temperatures in protocols such that none are higher than 56℃ and digestion durations are the minimum necessary for your sample type. Use of high temperature to “reverse cross-linking”, as suggested by many FFPE extraction protocols is not recommended. Do not elute into water; use a buffered solution such as 10 mM Tris-HCl with 0.1 mM EDTA (TE-low), 10 mM Tris-HCl (Qiagen Buffer EB) or a similar solution supplied in your DNA extraction kit. EDTA concentrations up to 2 mM are compatible with downstream processing.

4. ***Reaction Preparation***

   Duplex Sequencing Kits have been specifically formulated for a high degree of working stability. Prepare reactions at room temperature. Do not put reagents on ice or in a benchtop cooler. We recommend gathering the reagents necessary for a single step, using the reagents, and then returning them back into their proper storage location. Ensure all reagents are fully thawed (if applicable) at room temperature and vortexed before use. Vortexing reagents for 5-10 seconds will not adversely affect them.

5. ***Bead Handling***

   Resuspend SPRI and STREP beads thoroughly prior to use. Warming the beads up to room temperature before use is not necessary. During bead incubations, do not agitate the PCR plate when the plate is on the magnetic rack; be careful not to disturb the bead pellet. If beads are aspirated into the pipette tip when removing supernatant, dispense beads back into the PCR plate on the magnet and allow liquid to clear again (~2 minutes) before proceeding. During bead washes and cleanups, be careful not to over-dry the beads. If beads in the pellet are over-dried, the pellet will begin to crack apart. Over-dried beads will take longer to resuspend, and DNA loss may occur. Bead drying times may need to be adjusted based on the ambient humidity and temperature of your workspace.

   DO NOT FREEZE SPRI OR STREPTAVADIN BEADS. If the beads are frozen by mistake, please discard and contact us.

6. ***Plate Centrifugation***

   Centrifugation is necessary to remove any liquid from the plate seal, whether it be post-incubation condensation or post-vortexing splashes. Apply up to 150 x g (relative centrifugal force) for 30 seconds for this purpose. If liquid remains visible on the seal after centrifugation, use higher speed. If plate contains SPRI or STREP beads, make sure the beads remain visibly suspended after centrifugation. If beads pellet after centrifugation, shake or vortex the plate to resuspend the beads and then apply lower speed for centrifugation.

7. ***Requirements for DNA Fragmentation***

   This protocol is designed for use on randomly fragmented double-stranded DNA. DNA can be ultrasonically sheared or provided as a pre-fragmented product of caspase degradation, such as occurs with cell-free DNA from plasma or other body fluids. At this time, we do not recommend random enzymatic fragmentation methods, as some methods introduce large numbers of nicks and/or stretches of single-stranded DNA which disrupt the native DNA duplex structure. Non-random fragmentation approaches, such as the use of restriction endonucleases or CAS-family nucleases, should not be used with this kit due to the invariant nature of the resulting cleavage sites (contact us for further information about alternate solutions).

For high molecular weight genomic DNA, empirically testing shearing conditions to ensure a 300 bp average fragment size is recommended. Shearing DNA to average fragment size other than 300 bp may impact library construction efficiency and quality of sequencing results. When using an ultrasonicator, DNA should be sheared in TE-low (10mM Tris-HCl with 0.1 mM EDTA) or similar, *NOT WATER*. If in doubt about the storage buffer composition in which a sample is received, we recommend first adding a 1/10th volume of 10x TE-low (100 mM Tris HCl, 1 mM EDTA). Final EDTA concentrations up to 2 mM are compatible with downstream processing. Cell-free DNA does not need to be sheared.

The maximum volume of DNA input into Library Preparation is 50 µL but 29.5 µL has been established as optimal. If DNA input volume is larger than 29.5 µL, refer to Appendix IV – Acceptable Protocol Adjustments.

8. DNA Quantification

We recommend quantifying DNA prior to fragmentation using a double-stranded DNA-binding fluorescent dye (e.g. Qubit, Thermo Fisher Scientific) rather than UV spectrometry (e.g. Nanodrop, Thermo Fisher Scientific). The latter may sometimes overestimate concentrations due to RNA or other impurities. All DNA input mass references in this manual and the accompanying Panel Information Sheets refer to the *PRE-SHEARING* DNA mass.

9. DNA Inputs

When starting with unsheared genomic DNA, we recommend an input mass (pre-shearing) of 50 ng – 2 µg. The expected Duplex Depth to be achieved for a given input mass of DNA is discussed further in Appendix III and your specific Panel Information Sheets. To achieve higher Duplex Depths per sample, prep replicate libraries and merge Duplex Data. With pre-fragmented cell-free DNA, lower inputs can be used and the Duplex Depths that can be achieved on a per-nanogram basis will be higher than for mechanically sheared DNA. DNA obtained from formalin fixed paraffin embedded tissue (FFPE) will generally give a lower Duplex Depth than an equivalent mass of non-fixed tissue, to an extent that can vary significantly depending on exact fixation process and sample age. For DNA inputs outside the recommended range, we strongly suggest careful empiric testing with the specific type of DNA you are using. With very low inputs, excessive loss with shearing can occur. With higher inputs, beyond a point, Duplex Depths will decrease rather than increase, due to reaction saturation.

TIP *DNA integrity number (DIN) > 7 on a TapeStation generally indicates high quality DNA, although oxidative or other forms of non-fragmentation damage may still be present and not appreciable.*

TIP *The Duplex Depth that can be achieved for a given input mass of DNA depends on the size of the genome being sequenced from. If using a Universal Kit and designing your own panel for a non-mammalian genome, for a multi-copy gene or transgene or for plasmid or organelle genomes (mitochondria, chloroplasts etc.) contact us for DNA input and sequencing cluster recommendations.*

10. DNA Technical Controls and Control Probe Panels

Every Duplex Sequencing Kit contains at least one DNA Technical Control (**DTC**)–a species-specific high molecular weight DNA–and one species-specific Control Panel (**CTP**)–an arbitrarily-selected, contiguous 2.4 kb region of the target genome. Performance of these control reagents will provide a valuable benchmark for interpreting results if any troubleshooting is necessary. Particularly as a first-time user, it is highly recommended to include a library generated with the provided DNA Technical Control (250-500 ng input for mammalian samples) and the application-specific Panel (or control panel when using a Universal kit). When processing new sources of DNA or testing a new self-supplied probe panel, the Control Panel can be used in combination with the new DNA and/or the DNA Technical Control can be used in combination with the new panel to efficiently isolate variables.

The Control Panel volume included in each kit is sufficient for 5 control libraries. Refer to the accompanying DNA Information Sheets and Panel Information Sheets for more details. DNA Technical Controls (supplied at 25 ng/µL) can also be used to test your DNA quantification system, however, this will reduce the final volume available for control reactions.

11. DNA Standards

Some application-specific Duplex Sequencing Kits are supplied with DNA standards (**DNS**) with known variant frequencies. When included, these standards typically comprise a low mutation burden sample and one or more samples where either DNAs bearing known variants have been spiked in at specific low frequencies or where the biological source of the DNA was exposed to a mutagen to elevate the overall abundance of random mutations across the genome. See details of each included DNA Standard in the accompanying DNA Information Sheets.

12. Preparation of Capture Probes

All TwinStrand-supplied panels are provided at an optimized probe concentration, designed for maximum capture uniformity and do not require dilution prior to use. User-supplied capture probes to be used with Universal Kits should be 5'-biotinylated 120mer DNA oligonucleotides. When purchasing individual oligonucleotides, we recommend ordering as high coupling efficiency chemistry (e.g. branded as Ultramers by Integrated DNA Technologies (IDT), EXTREmers by Eurofins, Longmers by others) with standard desalting. As a starting point for testing, probes should be mixed one-to-one and the final concentration adjusted to 0.75 pmol/µL*. Custom or stocked pre-mixed probe panels from Twist Bioscience or IDT (xGen Lockdown probe pools) are also compatible with the kit; optimal final concentration should be established empirically. If using a higher volume of probes, refer to Appendix IV – Acceptable Protocol Adjustments.

**This is the total probe mix concentration. The equimolar concentration of individual probes will vary depending on the number of probes per panel.*

We *do not* recommend pooling multiple samples per capture. Pooled captures introduce a risk of generating very low frequency PCR crossover events between samples. Additionally, Duplex data yield may be reduced if libraries are pooled for capture. All TwinStrand kits contain sufficient capture reagents for two sequential captures of every sample individually.

13. Master Mix for Multiple Samples

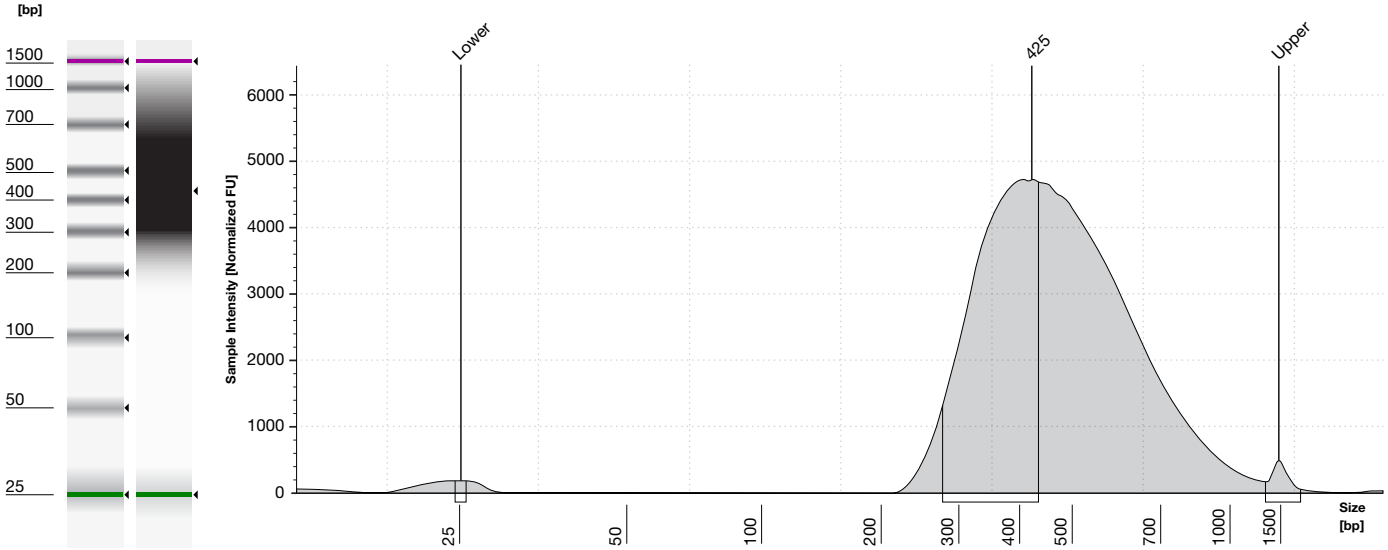
To reduce sample-to-sample variability we recommend making master mixes for all steps when preparing multiple samples (except for the PCR-1 (Indexing) Reaction). To ensure optimal use of reagents, yet still accommodate for slight pipetting errors, calculate 5-10% overage when preparing master mixes. The kits are provided with extra reagent volume for this calculation.

14. Checking Final Libraries Pre-Cleanup

The number of cycles needed for the final PCR (PCR-3 when doing sequential captures or PCR-2 if only using one capture) varies with DNA input, panel design, thermocycler calibration and lab practice. For TwinStrand-supplied panels, the approximate number of final PCR cycles required is listed on the corresponding Panel Information Sheet. However, one should always check final libraries on an electrophoresis system (e.g. fragment analyzer or agarose gel), *PRIOR TO FINAL CLEANUP*. To do so, simply remove the PCR plate from thermocycler, centrifuge briefly and open at room temperature on bench to take an aliquot.

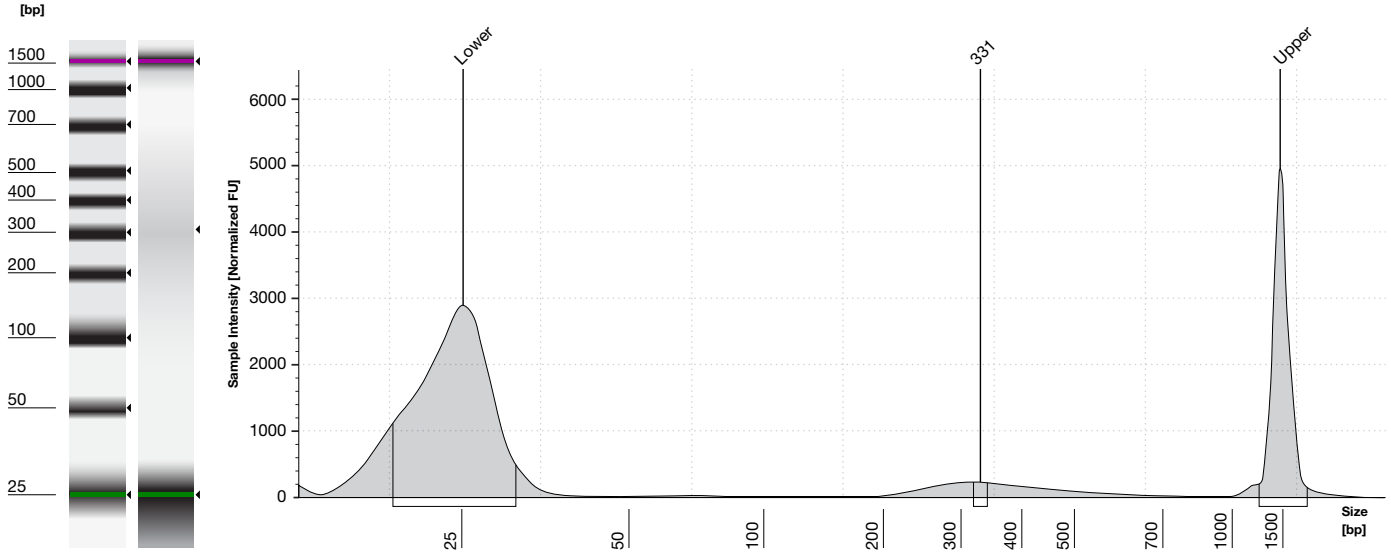
The acceptable range of library is approximately 1-80 ng/µL. If the library quantity appears low before the final cleanup, run the remaining PCR reaction for an additional 3-5 extra cycles to increase the PCR product mass, which should roughly double per cycle. If the library is not detectable, run the remaining reaction for an additional 8-10 extra cycles. If the library is not detectable after 30 total cycles (final PCR), the sample preparation has failed. Refer to Appendix I – Troubleshooting for possible cause of failure. The PCR reactions can be left at room temperature for 1 hour during this analysis. For extra PCR cycles, the initial 45 second hot-start incubation at 98°C can be omitted.

The following electropherogram and gel-like image are examples of a well-amplified library (2 µl) that was run on an Agilent High Sensitivity D1000 ScreenTape System and is ready for final cleanup. The final trace pre- and post- purification will be the same in terms of fragment size distribution.



The gaussian electropherogram profile should peak at 300-500 bp (depending on the average DNA fragment size; adapters + indexes add 153 bp to DNA inserts). The distribution of library fragments should range in size from ~200 bp to ~1000 bp and should be easily detectable by gel electrophoresis or fragment analyzer output. With cell-free DNA libraries, the peak will be on the lower end of this size range and the distribution profile will be narrower.

The following electropherogram and gel-like image are examples of a library (2 µL) needing 3-5 extra cycles of final PCR that was run on an Agilent High Sensitivity D1000 ScreenTape System.



TYPICAL PROCESSING WORKFLOW

Day 1: Pre-PCR

DNA Shearing ▸ Library Preparation ▸ ✋ ▸ Library Conditioning ▸ PCR-1 (Indexing) Reaction Setup

Post-PCR

PCR-1 (Indexing) Reaction Thermocycling ▸ ✋ ▸ Overnight Capture-1 Hybridization

Day 2: Post-PCR

DNA Bind and Wash-1 ▸ PCR-2 ▸ ✋ ▸ Overnight Capture-2 Hybridization

Day 3: Post-PCR

DNA Bind and Wash-2 ▸ PCR-3 ▸ QC ▸ Final Cleanup ▸ ✋ ▸ Library Quantification and Pooling

TIP *The three-day timeline is comfortable for most users and is what we recommend starting with. When a faster turn-around time is required, the workflow can be reduced to two days by using an overnight first capture on day 1 followed by a 4 hour second capture on day 2.*

SAFE STOPPING POINTS

As optional safe stopping points, you may store samples at -20°C after elution from any SPRI (cSPRI/dSPRI) clean up. If a safe stopping point is not specified in the protocol, proceed immediately to the next step. You may incubate capture hybridizations anywhere from 4 hours to overnight (up to 20 hours total).

REQUIRED REAGENTS

Freshly made 70% ethanol and molecular biology grade H_2O must be user-supplied. While a species-specific Control Panel and DNA Technical Control are provided in every kit, larger hybrid capture panels and DNA Standards are only included in application-specific kits.

REQUIRED CONSUMABLES

PCR plates
(Bio-Rad Laboratories, Catalog # HSP9601 or equivalent)

Plate seals
(Thermo-Fisher Scientific, Catalog # 4306311 or equivalent)

Filtered pipette tips

1.5 mL microfuge tubes

Conical 5 mL and/or 15 mL tubes

Reagent reservoirs

REQUIRED EQUIPMENT

Thermocycler with heated lid, compatible with skirted PCR plates

Magnetic PCR plate rack (e.g. Invitrogen Catalog # 12027)

Magnetic tube rack for 1.5 mL (e.g. EMD Millipore, Catalog # LSKMAGS08) and/or conical size (e.g. Invitrogen Catalog # 12301D) Microfuge and plate centrifuge

Vortexer

Fluorometer compatible with DNA-quantifying dyes (e.g. Qubit, Thermo Scientific)

Method of shearing DNA (e.g. Covaris ultrasonicator)

Calibrated pipettes: single channel 2 µL to 1000 µL; multichannel 2 µL to 200 µL

Electrophoresis system
(e.g. Agilent TapeStation or agarose gel system)

RECOMMENDED EQUIPMENT

High speed plate shaker (e.g. Bulldog Bio, Catalog # 1808-0505)

SEQUENCERS

Samples prepared with our DuplexSeq™ Adapters can be run on any Illumina sequencing platform. The number of raw sequencing reads/clusters required per sample for a given panel and input amount of DNA should be noted on your Panel Information sheet and/or Appendix III to confirm that your sequencer capacity is adequate for your application. Our unique dual index sets require 8 bp indexing reads.

Please note, the index2/i5 sequence orientation is dependent on the sequencer used. Use the indices (Appendix V) as listed for NextSeq 500/550 and HiSeq 3000/4000 sequencers. Use the reverse complement of the i5 index for MiSeq, HiSeq 2000/2500 or NovaSeq 6000 instruments.

PROTOCOL

Read all previous sections before commencing with library construction. This protocol is designed for use with randomly fragmented DNA inputs ranging from 50 ng – 2 µg *(PRE-SHEARING MEASUREMENT)*. Lower amounts (<50 ng) of cell-free DNA can be used. It is highly recommended to include a library generated with the provided species-specific DNA Technical Control (250-500 ng) and the application-specific Panel (or control panel if using a Universal kit), particularly during the first use of the kit. Please read Guidelines for Sample Processing – DNA Technical Controls and Control Probe Panels for more information, as well as accompanying Panel and DNA Information Sheets.

Thaw the reagents needed for each step at room temperature and vortex before use. Do not put reagents on ice.

TIP *Indexing primer pairs (IND plate used in the 2nd section of the protocol) are provided in columns of 8. We suggest considering the index layout (Appendix V) when deciding on how to arrange your DNA samples for library processing.*

Library Preparation

1. In the Pre-PCR area prepare End Repair / A-Tail (ERAT) reaction in a 96-well PCR plate using the following recipe. Final reaction volume should be 50 µL total.

See Appendix IV for acceptable protocol adjustment for higher DNA shearing volumes

1x ERAT Reaction
20.5 µL End Repair / A-tail Mix (ERAT)
29.5 µL Fragmented DNA Sample

2. Seal plate, vortex to mix, and briefly spin down to collect volume into the wells. Place plate in thermocycler in Pre-PCR area and run the following program with **lid heated to 75°C.**

Step	Temperature	Time
1	20°C	30 minutes
2	65°C	30 minutes
3	4°C	∞

Once the thermocycler reaches 4°C, you may remove the plate.

3. Prepare the following Ligation Reaction by adding ligation reagents directly to the completed ERAT reaction.

1x Ligation Reaction
50 µL ERAT reaction
30 µL Ligation Reaction Mix (LIG)
6 µL DuplexSeq™ Adapters (ADAP)

4. Seal plate, vortex to mix, and briefly spin down to collect volume into the wells. Place plate in thermocycler and run the following program **without a heated lid.**

Step	Temperature	Time
1	20°C	60 minutes
2	4°C	∞

Once the thermocycler reaches 4°C, you may remove the plate.

5. Clean up the reaction using the provided SPRI Beads (cSPRI).

- Add 68.8 µL (0.8x ratio) of resuspended cSPRI to each well. Pipette up and down until cSPRI beads are thoroughly mixed and incubate at room temperature for 5 minutes. (Note: If input DNA volume is greater than the recommended 29.5 µL in step 1, increase SPRI beads volume accordingly after Ligation Reaction to maintain the 0.8x volume ratio.)
- Place on magnet for 5 minutes, then remove and discard supernatant.
- With plate on magnet, add 150 µL of 70% ethanol. Do not resuspend bead pellet. Wait 30 seconds, then remove and discard supernatant.
- Repeat ethanol wash: with plate on magnet, add 150 µL of 70% ethanol. Do not resuspend beads. Wait 30 seconds, then remove and discard supernatant.
- After 2nd wash, use a 10 µL pipette to remove any residual ethanol. Remove plate from magnet and allow to dry at room temperature for 5 minutes. Do not over-dry.
- Add 23 µL of TwinStrand Buffer (cTSB) to washed cSPRI beads and pipette up and down to resuspend and elute the library DNA from the beads.
- Incubate at room temperature for 5 minutes.
- Place on magnet and incubate for 2 minutes.
- Transfer 21 µL of the library-containing supernatant to a fresh PCR plate and discard cSPRI beads.

SAFE STOPPING POINT: You may seal the plate and store samples at -20°C or proceed with the Library Conditioning Reaction.

TIP *70% ethanol should be freshly prepared for each library prep from a 100% stock. If not freshly prepared and stored in tightly sealed container when not in use, the preferential evaporation of the alcohol can lead to an incorrect concentration which can cause DNA loss during library processing.*

6. Prepare the following Library Conditioning Reaction.

1x Library Conditioning Reaction

21 µL Ligated DNA in cTSB
4 µL Library Conditioning Mix (LCM)

7. Seal plate, vortex to mix, and briefly spin down to collect volume. Place plate in thermocycler and run the following program with **lid heated to 45°C.**

Step	Temperature	Time
1	37°C	60 minutes
2	4°C	∞

Once the thermocycler reaches 4°C, you may remove the plate.

PCR-1 (Indexing) Reaction

1. Prepare the following Indexing Reaction using provided indexing primers (IND plate). Each well of the plate contains one sample worth of indexing primers. Make careful note of which indexing primer pair is used for each sample. Seal plate, vortex to mix, briefly spin down and then *TRANSFER TO POST-PCR AREA*.

1x Indexing Reaction

25 µL Ligated Conditioned DNA

20 µL PCR Master Mix (cPCR)

5 µL Indexing Primers (IND)

2. Place plate in thermocycler in *POST-PCR AREA* and run the following program with **lid heated to 105°C.**

Step	Temperature	Time	Cycles
1	98°C	45 seconds	1
2	98°C	15 seconds	10 Cycles
3	60°C	90 seconds	
4	72°C	45 seconds	
5	72°C	60 seconds	1
6	20°C	∞	1

Once the thermocycler reaches 20°C, you may remove the plate

3. Clean up the reaction using the provided SPRI Beads (Post-PCR box, dSPRI).

- Add 50 µL (1.0x ratio) of resuspended dSPRI to each well. Pipette up and down to mix dSPRI beads thoroughly. Incubate at room temperature for 5 minutes.
- Place on magnet for 5 minutes then remove and discard supernatant.
- With plate on magnet, add 150 µL of 70% ethanol. Do not resuspend bead pellet. Wait 30 seconds, then remove and discard supernatant.
- Repeat ethanol wash: with plate on magnet, add 150 µL of 70% ethanol. Do not resuspend beads. Wait 30 seconds, then remove and discard supernatant.
- After 2nd wash, use 10 µL pipette to remove any residual ethanol. Remove plate from magnet and allow to dry at room temperature for 5 minutes. Do not over-dry.
- Add 22 µL of TwinStrand Buffer (dTSB) to washed dSPRI beads and pipette up and down to resuspend and elute the library DNA from the beads.
- Incubate at room temperature for 5 minutes.
- Place on magnet and incubate for 2 minutes.
- Transfer 20 µL of library-containing supernatant to a fresh PCR plate and discard dSPRI beads.

SAFE STOPPING POINT: You may seal the plate and store samples at -20°C or proceed to Capture 1.

Capture-1

1. Prepare the Capture Reaction Master Mix. Refer to Guidelines for Sample Processing for directions on dilution of biotinylated capture probes not supplied by TwinStrand.

The following preparation volume makes enough Capture Reaction Master Mix for Capture-1 and Capture-2 for a single sample. The remaining Capture Reaction Master Mix left after Capture-1 can be stored at -20°C for up to 7 days and thawed for Capture-2. After thawing, vortex reagents to mix and dissolve any precipitates. If foam forms after vortexing the Hybridization Buffer, let the buffer sit at room temperature five minutes for the foam to dissipate.

Capture Reaction Master Mix

4 µL Capture Mix

10 µL Cot-1 DNA (COT)

48 µL Hybridization Buffer (HYB)

26 µL Formamide (FORM)

2 µL Probe Panel (PAN)

If using different panels for some samples during a library preparation, such as a control panel (CTP), make two or more submaster mixes It is important to remember to use the correct saved submaster mixes for each sample in the the 2nd capture.

2. Prepare the following Capture Reaction by adding Capture Reaction Master Mix directly to the cleaned Indexing PCR products.

1x Capture Reaction

20 µL Cleaned DNA from Indexing PCR product

45 µL Prepared Capture Reaction Master Mix

3. Seal plate, vortex to mix, and briefly spin down to collect volume. Place plate in thermocycler and run the following program with **lid heated to 105°C.** Make sure your plate is sealed properly across all wells and around the plate edges and corners.

Step	Temperature	Time
1	95°C	10 minutes
2	65°C	4–20 hours*

** Minimum time at 65°C is 4 hours, but incubation may proceed up to 20 hours.*

DNA Bind and Wash-1

1. While the Capture Reaction is incubating, prepare the Streptavidin (STREP) beads. The following preparation makes enough washed STREP beads for Capture-1 and Capture-2 for a single sample. Prepare 400 µL of 1x BWB *PER SAMPLE* by diluting Bead Wash Buffer (2x) with an equal volume of water. Washed STREP can be stored at 4°C for up to 7 days.

1x Bead Wash Buffer (1x BWB)
200 µL Bead Wash Buffer (2x) (BWB)
200 µL H_2O

2. Thoroughly vortex STREP beads and aliquot 50 µL *PER SAMPLE* into a PCR plate.

- Place plate/tube on magnet for 3 minutes until STREP beads are separated. Discard supernatant and remove plate from magnet.
- Add 150 µL of 1x BWB *PER SAMPLE*, seal plate, and vortex until STREP beads are resuspended. Briefly spin down the plate.
- Place on magnet 3 minutes and discard supernatant.
- Add 150 µL of 1x BWB *PER SAMPLE*, seal plate, and vortex until STREP beads are resuspended. Briefly spin down the plate.
- Place on magnet 3 minutes and discard supernatant.
- Resuspend washed STREP in 100 µL of 1x BWB *PER SAMPLE*.

TIP *For preparing large bead volumes use a single conical tube and magnetic tube rack.*

3. After the Capture Reaction is finished incubating:

- Aliquot 50 µL of washed and resuspended STREP beads *PER SAMPLE* into clean wells of a fresh PCR plate.
- Place the plate on magnet for 1 minute.
- While the STREP beads are on the magnet, retrieve the Capture Reaction from the thermocycler and spin down briefly.
- Remove and discard the supernatant from the STREP beads and remove the plate from the magnet.
- Transfer the entire Capture Reaction (65 µL) into the corresponding wells in the plate with STREP beads and thoroughly resuspend the beads.

STREP BEADS SHOULD NOT BE PELLETED PRIOR TO INCUBATION. STREP beads will settle during incubation, but this does not affect downstream processing

- Seal and place plate in thermocycler and run the following program with **lid heated to 105°C.**

Temperature	Time
65°C	45 minutes

Proceed *IMMEDIATELY* to next step.

4. While DNA is incubating/binding with STREP beads (Step 3, above):

- Thaw Equilibration Buffer (10x) (EQB) and Stringent Wash Buffer (10x) (SWB).
- Prepare the following 1x dilutions per sample. The following preparations make enough buffer for Captures 1 and 2 for a single sample.

1x Equilibration Buffer (1x EQB)
450 µL H_2O
50 µL Equilibration Buffer (10x) (EQB)

1x Stringent Wash Buffer (1x SWB)
125 µL H_2O
100 µL Formamide (FORM)
25 µL Stringent Wash Buffer (10x) (SWB)

Prior to washes, allow wash buffers to come up to room temperature. Wash buffers do not need to be heated and all washes are performed at room temperature. Diluted buffers can be stored at 4°C or room temperature for up to 7 days.

5. After DNA is finished incubating/binding with STREP beads, remove plate from thermocycler and wash the beads at room temperature.

- Transfer plate to magnet for 1 minute for STREP beads to clear solution. Discard supernatant and remove from magnet.
- Add 125 µL of 1x EQB to each well. Pipette up and down to resuspend STREP beads and incubate at room temperature for 2 minutes.
- Place on magnet for 2 minutes, then discard supernatant and remove from magnet.
- Add 125 µL of 1x SWB to each well. Pipette up and down to resuspend STREP beads and incubate at room temperature for 5 minutes.
- Place on magnet for 2 minutes, then discard supernatant and remove from magnet.
- Next, add 125 µL of 1x EQB to each well. Pipette up and down to resuspend STREP beads and incubate at room temperature for 2 minutes.
- Place on magnet for 2 minutes, then discard supernatant and remove from magnet.
- Add 25 µL of TwinStrand Buffer (dTSB) to each well and pipette up and down to resuspend STREP beads with the bound, target-enriched library.

PCR-2

1. Thaw PCR Master Mix (dPCR) at room temperature and vortex before use. Prepare the following reaction. (Note: PCR-2 and PCR-3 are performed “on bead.”)

1x PCR-2 Mix

25 µL STREP bead solution with bound DNA

20 µL dPCR

5 µL P5/P7 Primers (PRM)

Seal plate, vortex to mix, and spin down such that STREP beads are still resuspended but not on underside of seal. STREP beads will settle during PCR, but this does not affect downstream processing. Put in thermocycler and run the following program with **lid heated to 105°C.**

Step	Temperature	Time	Cycles
1	98°C	45 seconds	1
2	98°C	15 seconds	16 Cycles
3	60°C	45 seconds	
4	72°C	45 seconds	
5	72°C	60 seconds	1
6	20°C	∞	1

Once the thermocycler reaches 20°C, you may remove the plate.

2. Clean up the reaction using the provided SPRI Beads (dSPRI).

- Add 50 µL (1.0x ratio) of resuspended dSPRI beads to each well. Pipette up and down to mix dSPRI beads thoroughly and incubate at room temperature for 5 minutes.
- Place on magnet for 5 minutes, then remove supernatant and discard.
- With plate on magnet, add 150 µL of 70% ethanol. Do not resuspend. Wait 30 seconds, then remove and discard supernatant.
- Repeat ethanol wash: with plate on magnet, add 150 µL of 70% ethanol. Do not resuspend. Wait 30 seconds, then remove and discard supernatant.
- After 2nd wash, use 10 µL pipette to remove residual ethanol. Remove plate from magnet and allow sample to dry at room temperature for 5 minutes. Do not over-dry.
- Add 22 µL TwinStrand Buffer (dTSB) to dSPRI beads and resuspend to elute the library. Incubate at room temperature for 5 minutes.
- Place on magnet and incubate for 2 minutes.
- Transfer 20 µL of library-containing supernatant to a fresh PCR plate and discard dSPRI beads.

SAFE STOPPING POINT: You may seal the plate and store samples at -20°C or proceed to Capture-2.

Capture-2

1. Prepare the Capture Reaction by thawing the pre-made Capture Reaction Master Mix at room temperature and vortex before use. Add to the cleaned PCR-2 products as stated below:

Capture Reaction

20 µL Clean DNA from PCR-2

45 µL Prepared Capture Reaction Master Mix

Remember, as with Capture-1, if using different panels for some samples, one should have made separate submaster "Capture Reaction Mixes" previously. See Capture-1, Step-1.

2. Seal plate, vortex to mix, and spin down. Place plate in thermocycler and run the following program with **lid heated to 105°C.**

Step	Temperature	Time
1	95°C	10 minutes
2	62°C *	4–20 hours**

**Note the temperature difference between Capture-1 (65°C) and Capture-2 (62°C).*

***Minimum time at 62°C is 4 hours, but incubation may proceed up to 20 hours.*

DNA Bind and Wash-2

1. After the Capture Reaction is finished incubating:

- Aliquot 50 µL of washed and resuspended Streptavidin (STREP) beads per sample into clean wells of a fresh PCR plate.
- Place the plate on magnet for 1 minute, then remove and discard supernatant. Remove from magnet. *DO NOT ALLOW STREP BEADS TO DRY OUT*.
- Remove the Capture Reaction plate from thermocycler, unseal and transfer entire Capture Reaction (65 µL) into corresponding wells in the plate with STREP beads and thoroughly resuspend the beads.

STREP BEADS SHOULD NOT BE PELLETED PRIOR TO INCUBATION. STREP beads will settle during incubation, but this does not affect downstream processing

- Seal and place plate in thermocycler and run the following program with **lid heated to 105°C.**

Temperature	Time
62°C*	45 minutes

Note the temperature difference between Capture-1 (65°C) and Capture-2 (62°C).

Proceed **immediately** to next step.

2. After DNA is finished incubating/binding with STREP beads, remove plate from thermocycler and begin bead washes with previously made 1x Equilibration Buffer (1x EQB) and 1x Stringent Wash Buffer (1x SWB):
   - Transfer plate to magnet for 1 minute for STREP beads to clear solution. Discard supernatant and remove from magnet.
   - Add 125 µL of 1x EQB to each well. Pipette up and down to resuspend STREP beads and incubate at room temperature for 2 minutes.
   - Place on magnet for 2 minutes, then discard supernatant and remove from magnet.
   - Add 125 µL of 1x SWB to each well. Pipette up and down to resuspend STREP beads and incubate at room temperature for 5 minutes.
   - Place on magnet for 2 minutes, then discard supernatant and remove from magnet.
   - Next, add 125 µL of 1x EQB to each well. Pipette up and down to resuspend STREP beads and incubate at room temperature for 2 minutes.
   - Place on magnet for 2 minutes, then discard supernatant and remove from magnet.
   - Add 25 µL of TwinStrand Buffer (dTSB) to each well and pipette up and down to resuspend STREP beads with the bound, target-enriched library.

PCR-3

1. Thaw PCR Master Mix (dPCR) at room temperature and vortex before use. Prepare the following reaction (Note: PCR-2 and PCR-3 are performed "on bead"):

   1x PCR-3 Mix
   25 µL STREP bead solution with bound DNA
   20 µL dPCR
   5 µL P5/P7 Primers (PRM)

   Seal plate, vortex to mix, and spin down briefly such that STREP beads are still resuspended but not on the underside of the seal. Put in thermocycler and run the following program with **lid heated to 105°C.**

**Note that the optimal final PCR cycle number varies with DNA input, panel design and lab practices. If using a TwinStrand supplied panel, please refer to the accompanying Panel Information Sheet for the recommended starting cycle number. For extra PCR cycles, due to low or no visible signal, the initial 45 second hot-start incubation at 98°C can be omitted.*

Step	Temperature	Time	Cycles
1	98°C	45 seconds	1
2	98°C	15 seconds	N* Cycles
3	60°C	45 seconds	
4	72°C	45 seconds	
5	72°C	60 seconds	1
6	20°C	∞	1

For general guidance on the number of cycles for PCR-3, please refer to the table below:

Panel Size (kb)	DNA Input (ng)	Number of PCR-3 Cycles
1	100	11
	500	10
5	100	10
	500	9
50	100	9
	500	8
100	100	8
	500	7

Additional PCR-3 cycles may be required if library quantity appears low or no visible signal; please see Guidelines for Sample Processing – Checking Final Libraries Pre-Cleanup.

- After amplification, check libraries on an electrophoresis system (e.g. fragment analyzer or agarose gel) *BEFORE CLEANUP*. Remove plate from thermocycler, centrifuge briefly and take an aliquot to analyze libraries. The PCR reactions can be left at room temperature for 1 hour during this analysis.

2. Clean up the reaction using the provided SPRI Beads (dSPRI).

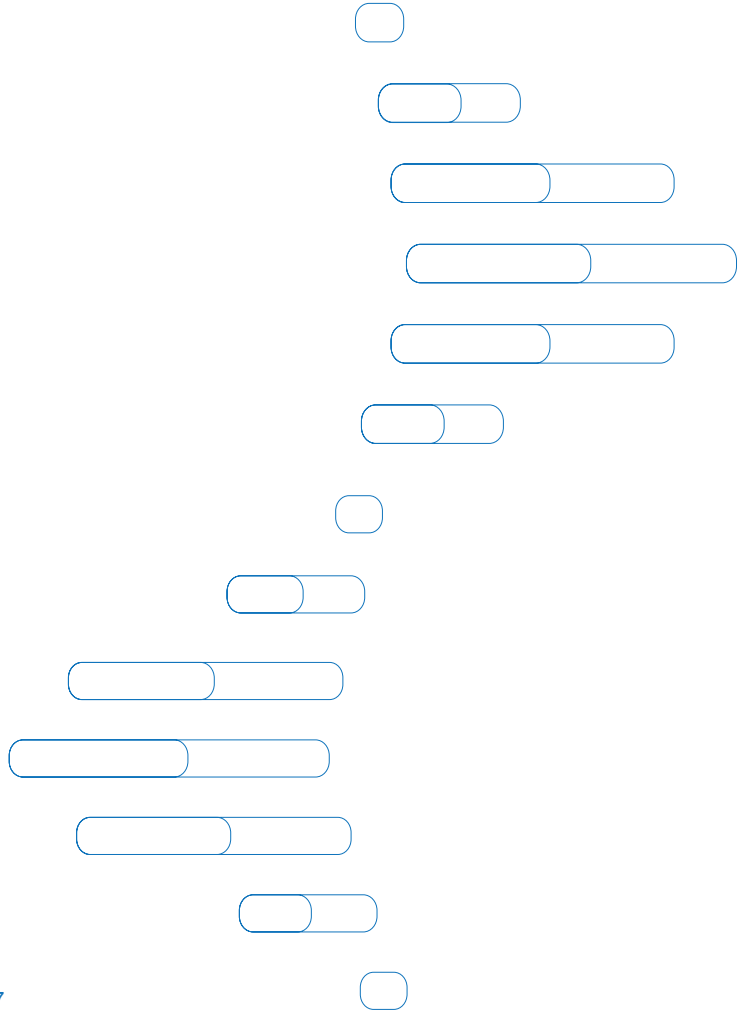
- Add 50 µL of resuspended dSPRI beads (or adjust to maintain 1.0x ratio volume if aliquot taken for fragment analyzer/gel) to each well. Pipette up and down to mix dSPRI beads thoroughly and incubate at room temperature for 5 minutes.
- Place on magnet for 5 minutes, then remove and discard supernatant.
- With plate on magnet, add 150 µL of 70% ethanol. Do not resuspend. Wait 30 seconds, then remove and discard supernatant.
- Repeat ethanol wash: with plate on magnet, add 150 µL of 70% ethanol. Do not resuspend. Wait 30 seconds, then remove and discard supernatant.
- After 2nd wash, use 10 µL pipette to remove any residual ethanol, remove from magnet and allow to dry at room temperature for 5 minutes. Do not over-dry.
- Add 22 µL TwinStrand Buffer (dTSB) to dSPRI beads and resuspend to elute library. Incubate at room temperature for 5 minutes.
- Place on magnet and incubate for 2 minutes.
- Transfer 20 µL of library-containing supernatant to a fresh PCR plate and discard dSPRI beads.

SAFE STOPPING POINT: You may seal the plate and store samples at -20°C or proceed to quantify and pool libraries for sequencing.

3. Quantify the individual cleaned-up final libraries using a fluorometer. If the libraries were of a relatively uniform size distribution prior to cleanup, it is acceptable, and generally simpler, to pool by mass rather than molarity. The relative mass of each library going into the multiplexed pool should be proportional to the fraction of clusters on a flow cell (or lane) that is intended to be dedicated to each sample. See Panel Information Sheets and/or Appendix III for guidelines.

Carry out final analysis of the pooled libraries by both fluorometry (e.g. Qubit) for final quantification and electrophoresis (e.g. fragment analyzer or agarose gel) to confirm the fragment size distribution. Expect a distribution of DNA fragments ranging in size from ~200 bp to ~1000 bp with the sample peak between 300-500 bp. With cell-free DNA libraries, the peak will be on the lower end of this size range and the profile will be narrower. The distribution for the pooled libraries will be consistent with the individual libraries prior to cleanup (refer to Guidelines for Sample Processing – Checking Final Libraries Pre-Clean Up).

Proceed with processing of library pool and sequence per Illumina's or your institution's specifications.



Appendix I – TROUBLESHOOTING

PROBLEM	SOLUTION
Libraries are not visible on the electrophoresis system and/or measurable by fluorometer before cleanup.	If library quantity appears low (i.e. below about 1-3 ng/µL) before the final cleanup, run remaining PCR reaction for an additional 3-5 extra cycles. The mass should roughly double per cycle. If the library is not detectable, run the remaining reaction for an additional 8-10 extra cycles to increase the PCR product mass until the fragment analyzer trace or gel indicates enough library is obtained. Refer to Guidelines for Sample Processing – Checking Final Libraries Pre-Clean Up. When libraries pass QC, proceed to cleanup step with remaining reaction volume. Adjust bead volume accordingly to maintain 1.0x volume ratio. If libraries are not quantifiable after 30 total cycles of the final PCR, the sample preparation has failed, and the libraries need to be generated again.
Libraries not observed after 30 cycles of final PCR.	Good DNA quality and accurate quantification are important for consistent library construction. It is highly recommended to control for DNA quality by running a control reaction (using a TwinStrand-supplied DNA Technical Control and a TwinStrand supplied panel–either an application-specific Panel or a species-specific Control Panel) in subsequent sample batches if failures are seen. Other common causes of library failure include: 1) not using freshly made ethanol, 2) over-drying beads, 3) freezing beads, 4) bead loss when working with magnetic plates, 5) evaporation during hybridization or PCR due to incompletely sealed wells or incorrect lid temperature, and 6) non-recommended modifications to the protocol. We recommend processing a small number of samples until comfortable with the protocol.
Libraries are not generating acceptable Duplex Depth.	Confirm sample preparation is working well, as indicated by electrophoresis trace and library quantification. If the trace and quantification look correct, this may be an indication that your library is under-sequenced (i.e. an inadequate number of clusters/reads generated per sample). Check the peak tag family size (PTFS) in the DuplexSeq Metrics Report; ideal size for PTFS is 10-15. Sub-optimal sequencing results can be caused by inputting less than intended DNA. Overestimation of DNA concentration by using UV spec is a common cause. Use a double-stranded DNA binding dye method instead. Poor results can also result from putting too much sheared DNA into the library construction (inputs above 2-2.5 µg will saturate the reaction and decrease, rather than increase Duplex Depth). Damaged or degraded DNA will not generate as much Duplex Data as high quality DNA with the same input mass. Assess genomic DNA quality by agarose gel or running a genomic tape on a fragment analyzer. Lastly, your capture efficiency may be suboptimal. Check the percent on-target. For a well-designed probe panel, greater than 90% of sequences will be on-target after 2 captures.

Appendix I – TROUBLESHOOTING CONTINUED

PROBLEM	SOLUTION
Failure to identify samples during sequence demultiplexing process	The index2 (i5) sequence orientation is dependent on the sequencer used. Use the indexes as listed for iSeq 100, MiniSeq, NextSeq 500/550, and HiSeq3000/4000 sequencers. Use the reverse complement of the i5 index for MiSeq, HiSeq2000/2500, and NovaSeq 6000.
Problem not listed	Email support@twinstrandbio.com.

Appendix II – DEFINITIONS

Duplex Depth: Duplex Depth at a particular genomic position represents the true molecular depth based on Duplex Consensus Sequence (DCS) coverage. Each DCS represents an original, unique double-stranded DNA (dsDNA) molecule from which information from both strands could be assessed. Thus, Duplex Depth at a site refers to the number of original dsDNA molecules sequenced across this nucleotide position where the sequences of both original strands are found to be in agreement.

Informative Duplex Bases: The total number of non-N bases present among all Duplex Consensus Sequences (DCS) for a sample. This number serves as the denominator for calculating overall mutation frequency, such as is used with mutagenesis assays.

Maximum Duplex Depth: The maximum Duplex Depth recovered across the the entire panel region of a reference genome. This metric can vary due to stochastic factors and focal copy number variation between samples so should be used with caution as an overall library preparation metric.

Minimum Duplex Depth: The minimum Duplex Depth recovered across the entire panel within the specified target region of a reference genome. This metric can vary due to stochastic factors and focal copy number variation between samples so should be used with caution as an overall library preparation metric.

Mean Duplex Depth: The average Duplex Depth recovered across a the entire panel region of a reference genome. This is a useful measure of how well the sample preparation worked overall.

Tag family size: The number of times a given strand of an original library molecule's amplicon duplicates were sequenced. For example, if the tag family size is 7, this means 7 PCR copies of a single strand of the same tagged molecule were sequenced.

Peak tag family size (PTFS): An aggregate measure of how many copies of an average library molecule was sampled. This translates to how well a library was sequenced and correlates with the number of unique ligated molecules and raw reads allocated per library. For most applications, a peak tag family size of 10-15 is ideal.

Percent (%) on-target: The percentage of raw bases sequenced that correspond to the specified target regions in a reference genome. This is an indication of how well hybrid capture worked. A well performing panel targeting a unique portion of a genome with double capture can achieve a 99% on-target rate. Small panels, panels containing repetitive elements or loci with pseudogenes elsewhere in the genome or use of a single capture will generally reduce the on-target rate.

Appendix III - LIBRARY INPUT AND SEQUENCING GUIDELINES

PANEL SIZE (kb)	DNA INPUT* (ng)	ESTIMATED NUMBER OF CLUSTERS (Million)	NUMBER OF PAIRED-END READS (Million)	EXPECTED MAX DUPLEX DEPTH (Thousand)
1	100	1 – 1.5	2 – 3	2 – 4
	250	2 – 3	4 – 6	5 – 10
	500	4 – 6	8 – 12	10 – 20
5	100	3 – 8	6 – 16	2 – 4
	250	7.5 – 15	15 – 30	5 – 10
	500	15 – 30	30 – 60	10 – 20
50	100	20 – 50	40 – 100	2 – 4
	250	50 – 100	100 – 200	5 – 10
	500	100 – 200	200 – 400	10 – 20
100	100	35 – 60	70 – 120	2 – 4
	250	85 – 150	170 – 300	5 – 10
	500	160 – 300	320 - 600	10 – 20

These guidelines assume use of 2x150 bp paired-end sequencing, that reads are >95% on target, that DNA is fragmented by sonication to a median fragment size of 300 bp and DNA is relatively undamaged from storage, extraction, or other handling conditions (e.g. not FFPE, no high temperature or phenol-chloroform exposure). Variations in these factors may lead to significant deviations from estimates. Many factors, especially the specific panel design, can affect sequencing efficiency and new panels should be tested empirically. Please refer to the accompanying Panel Information Sheets for TwinStrand-supplied panels for more specific guidelines.

**High molecular weight DNA quantified prior to mechanical shearing.*

Appendix IV - ACCEPTABLE PROTOCOL ADJUSTMENTS

1. **Concentrate DNA Prior to Mechanical Shearing**

   In cases where a DNA sample is at a low concentration, it may be necessary to concentrate it before shearing. DNA can be concentrated with a 1:1 ratio of SPRI beads:DNA and then eluted into an appropriate volume. Expect ~10% DNA loss from this extra cleanup. Our kits do not provide SPRI beads for sample concentration. We recommend sourcing extra SPRI beads from Bulldog Bio or Beckman Coulter. Do not combine SPRI beads from different sources. Do not mix cSPRI with dSPRI.

2. **Larger DNA Shearing Volume for Library**

   Up to 50 µL of sheared DNA can be added to the End Repair / A-Tail (ERAT) Reaction without changing the volume of ERAT Mix. Put the entire ERAT product volume into the Ligation Reaction, without changing the volume of Ligation Reaction Mix or DuplexSeq Adapters. Increase SPRI bead volume accordingly after Ligation Reaction to maintain a 0.8x volume ratio. For example, if 50 µL is the input volume for sheared DNA into ERAT, the Ligation Reaction volume will be 106.5 µL (50 µL input DNA + 20.5 µL ERAT + 30 µL LIG + 6 µL DuplexSeq Adapters). To maintain the 0.8x volume ratio, the SPRI bead volume should be 85.2 µL. Our kits do not provide extra cSPRI beads for this volume of post-ligation clean up. We recommend sourcing extra SPRI beads from Bulldog Bio or Beckman Coulter. Do not combine SPRI beads from different sources. Do not mix cSPRI with dSPRI.

3. **Temporary Storage of Sheared DNA**

   After shearing, we recommend beginning the End Repair / A-tail (ERAT) Reaction immediately. Alternatively, sheared DNA may be stored at 4°C for up to 16 hours before Library Preparation. If you need to store sheared DNA longer than 16 hours, store at -20°C. Freezing at this step may result in a slight loss in Duplex Depth.

4. **Skipping the Library Conditioning Reaction**

   Library Conditioning reduces the risk of very low level artifacts from different forms of DNA damage in short, single-stranded portions of input DNA fragments. The Library Conditioning Reaction is generally recommended, particularly for extremely sensitivity applications, such as mutagenesis evaluation, and/or where DNA is known to be heavily damaged (e.g. FFPE). However, this step may reduce Duplex Depths to an extent that is proportional to the amount of damage. On occasion we have observed more than 50% loss of Duplex Depth for exceptionally damaged DNA. Skipping Library Conditioning does not prevent the construction of Duplex Sequencing Libraries but will require a more thorough examination of putative variants. To bypass the Library Conditioning Reaction, increase the elution volume of cTSB in the post-Ligation cleanup from 23 µL to 27 µL, and transfer 25 µL of library-containing supernatant to the PCR-1 (Indexing) reaction.

5. **Higher Probe Volume for Capture**

   If using probes not provided by TwinStrand, up to 5 µL of probes per capture reaction can be added to Capture Reaction Master Mix without making any other changes to the protocol.

6. Skipping the Second Capture

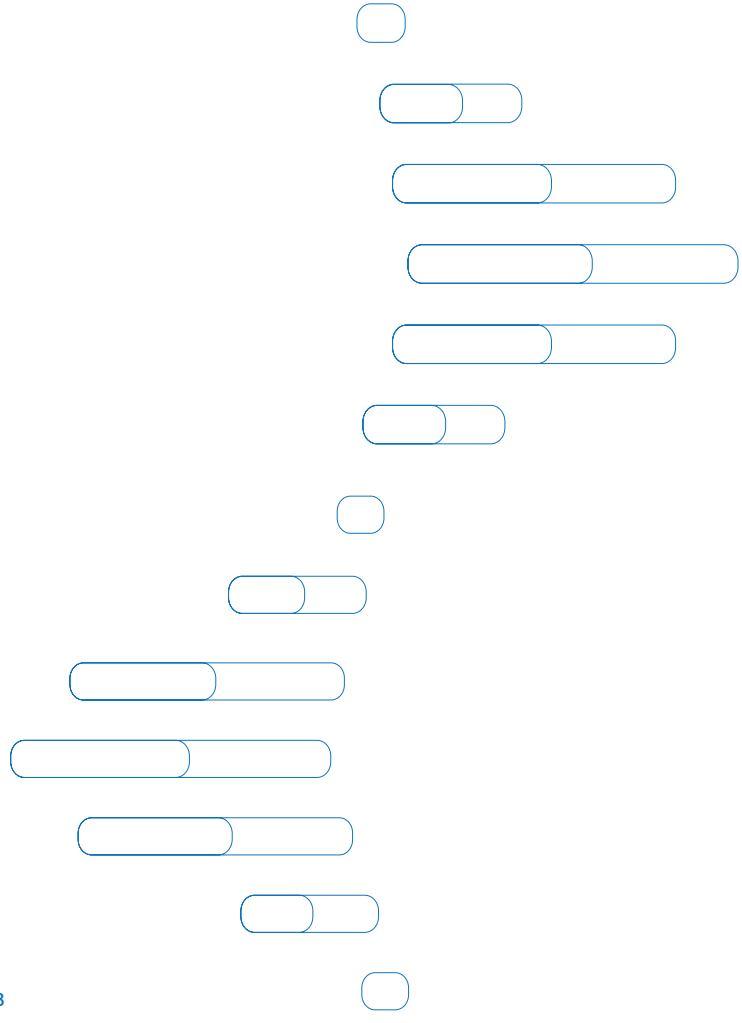
Each kit contains enough reagents for two sequential captures per sample. Sequential captures generally increase the percent on-target sequence generated compared with a single capture. However, this will vary by panel. Smaller probe panels (<10-20 kb), particularly those comprised of loosely tiled probes or probes with substantial homology to non-targeted regions elsewhere in the genome, most benefit from two captures. Larger panels (>40-50 kb), those with densely tiled probes (i.e. long stretches of adjacent probes without many intervening non-probed regions) and probes that are highly unique to the targeted regions, least benefit from two captures.

The trade-off between one and two captures weighs the potential for higher sequencing costs due to more off-target reads with one capture versus spending 3 instead of 2 days on library preparation for two captures. We recommend beginning with two captures when working with a new panel and then empirically testing the cost-benefit of one capture for your application. A well performing larger panel can achieve >95% on target with a single capture, whereas a challenging small panel may increase from <20% on-target to >95% on-target with the inclusion of a second capture.

To bypass the second capture, simply perform PCR-2 as the final PCR. For TwinStrand supplied panels, refer to the accompanying Panel Information Sheet for single capture PCR-2 cycle number recommendations. Check the PCR product on a gel or TapeStation prior to cleanup as described in: Guidelines for Sample Processing – Checking Final Libraries Pre-Cleanup. Add additional cycles as necessary then clean up reaction.

7. Using a Plate Shaker or Vortexer

In this protocol, any step that reads “pipette up and down to mix” can be replaced by use of a plate shaker or vortexer, if available. Mixing on a high-speed orbital plate shaker reduces well-to-well variability and is highly recommended when processing large numbers of samples. Seal the plate well before shaking and follow specifications for your model; make sure reagents are uniformly mixed or resuspended. After shaking/vortexing, spin down plate in a plate centrifuge such that all liquid is in the bottom of the wells, with none remaining on the seal. If spinning down with either SPRI or STREP beads, do so such that beads remain suspended in solution but not on the underside of the seal.



Appendix V - Plate Maps for Index Primers

Plate Map for unique dual-index pairs for following sequencers: **iSeq 100, MiniSeq, NextSeq 500/550, HiSeq 3000/4000**

Index Set A	1			2			3			4			5			6		
	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read
A	1	AATCCGAG	TTAGACGC	9	CAGAGACT	GAGTAGTC	17	CAGGTCTA	CGCTTCAT	25	GCTTACGA	GTCAATGC	33	CTACTCCT	GAGTGCAA	41	AGGCGTAA	TAAGGCCA
B	2	CAGCGTTA	TAACGCTG	10	TACCAGTC	GCCATAGA	18	GTCTGTCA	TGACAGAC	26	CGGATTGC	GCAATCCG	34	TAGCACGA	CTGCGTTA	42	AGCATGGT	TGGCCATA
C	3	GATTCTCG	CAATACCG	11	GTCGAGAA	ATCCGGTT	19	CGACATAC	ATATCGCG	27	CGGATAAG	GATGCCTA	35	CGCAGATA	TGCTTCTG	43	GCTGTAGT	GCAAGAAG
D	4	ACACCACA	GCTTAGCT	12	TCTCTGGT	CAACCACT	20	TAGCGCTT	CTCAACCT	28	GCGTTACA	TTGATGCG	36	GCCTTGTT	TATGCACG	44	ACTCACCA	AGATGCAG
E	5	AATGAGGC	GATACGCT	13	AGAATCCG	CACTCACA	21	ATGAGCAG	AGCCTGTA	29	AGTACAAG	CTTGTACT	37	ATTGGCGT	CTGTTGTC	45	ACGAAGAC	TGTTGCGA
F	6	TTGAGAGC	GCTAGGAA	14	AGCAGCTT	CGCGATAA	22	TGTGGATC	CGTCCAAT	30	AGGTAACG	AACGGTTC	38	AGATTGGC	CTTAGCGA	46	CGTAACTC	GACAGTCA
G	7	TTGCAGTG	TCAGGACT	15	ATACCGAC	CGAACCAA	23	AGATGTAC	GTACATCT	31	ACCAGAAC	ACTCGTTC	39	AGCTGACA	CTCAGAAG	47	CTATGGCA	AGTGACAG
H	8	ACGGTGTA	CAGAAGGT	16	GCAGTTGA	ACCTGTAG	24	GTAGCTCA	ACCGTCAT	32	CGTACTGT	TCTGGTAG	40	GAGGCAAT	CGATAGGA	48	CTGTGCTT	GTTGAGGT

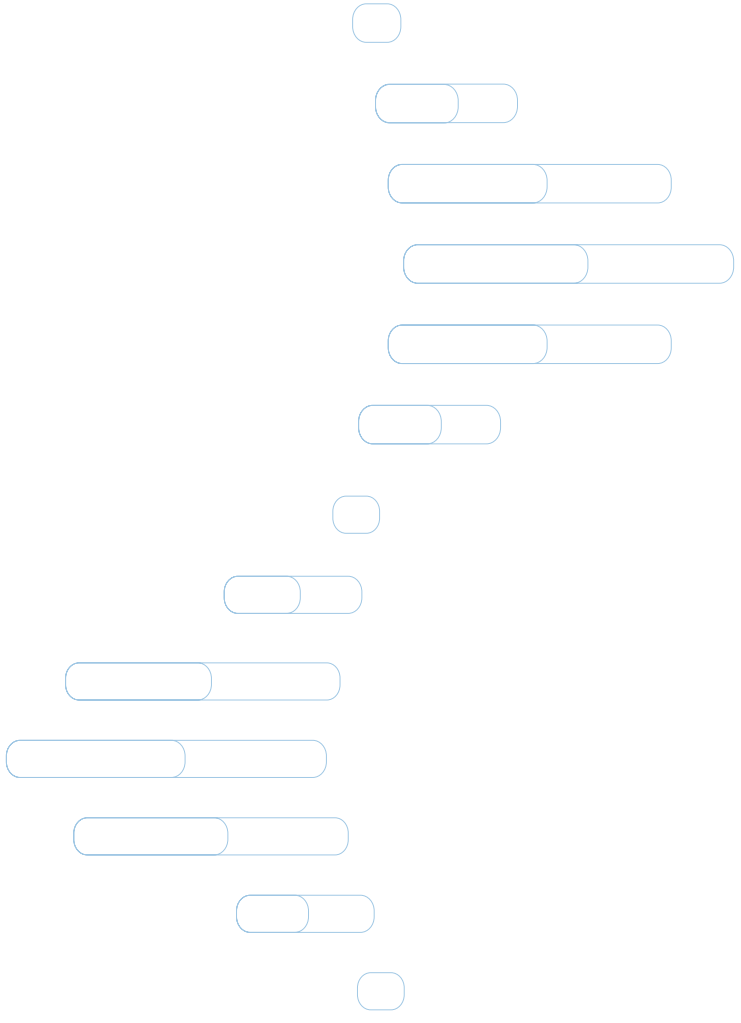
Index Set B	7			8			9			10			11			12		
	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read
A	49	TACTGTCG	GCATAGTG	57	GTTCATCG	GTCCTAAC	65	TGGTGGAA	AGGAGTCT	73	ACGTTCGT	CACATACG	81	ATGCCGTA	TACGAGCA	89	TCTCAAGC	AAGCTCTC
B	50	AGGATCGA	TCGAACCA	58	ACAACGGA	TCCAGTTG	66	CAGTCTTG	AGAGAGTC	74	TAGCCTAG	ACTTCCTG	82	TCGCTATG	ATCGCCAA	90	CTCCAAGT	AAGCCTCT
C	51	ACGTCTTC	CAGTAAGC	59	GATGGCAT	ACTGCTCT	67	CGTCATGA	TCTGCGAA	75	TCCGAATG	CAATGGAG	83	CTAGTGGA	AACTGGCT	91	GAGACGAA	TAACTGCG
D	52	ACGGCATT	TGTCTACG	60	GCTCCATT	ATTACCGC	68	ACGTCAAG	CTTCCTTG	76	TACCGATG	GATCTCCA	84	TAAGCCGT	GCACTTAG	92	TTAGGCAG	TTCCGTAC
E	53	CATTCGGT	GCATAAGC	61	AGAACAGC	TCCGATGT	69	CGACTATG	CATTCCAC	77	AGTGGCTA	AGCCATTG	85	GCATCCAA	TGGTACTC	93	TGACCGTT	GAAGTGAC
F	54	TGCCATCA	CTTGTCTC	62	GAGTCCTT	TTGCCTGT	70	CAAGCTTC	GTGGTATG	78	ACGCAACT	CTCGAATC	86	TGTGCTAC	AGAGCACT	94	AGTTCGCA	GAATGCCT
G	55	CATCGGTT	ACACGGAA	63	GTCGGATT	GACCACTT	71	GTCACCTA	CAACGTCA	79	CACCTTAC	GTAAGGTG	87	TTCAGCCA	GACACTGT	95	TCGCATAC	GACAGATC
H	56	TCTACCTC	TGCACTCT	64	AGGCTGAT	GCCTGTTA	72	TCTCCTCA	TGTTCGAG	80	GAAGCATG	CTTCGACA	88	TGAAGACG	CTGGATGA	96	ACCGCTTA	TGCCACAT

Plate Map for unique dual-index pairs for following sequencers: **NovaSeq 6000, MiSeq, HiSeq 2000/2500**

Index Set A	1			2			3			4			5			6		
	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read
A	1	AATCCGAG	GCGTCTAA	9	CAGAGACT	GACTACTC	17	CAGGTCTA	ATGAAGCG	25	GCTTACGA	GCATTGAC	33	CTACTCCT	TTGCACTC	41	AGGCGTAA	TGGCCTTA
B	2	CAGCGTTA	CAGCGTTA	10	TACCAGTC	TCTATGGC	18	GTCTGTCA	GTCTGTCA	26	CGGATTGC	CGGATTGC	34	TAGCACGA	TAACGCAG	42	AGCATGGT	TATGGCCA
C	3	GATTCTCG	CGGTATTG	11	GTCGAGAA	AACCGGAT	19	CGACATAC	CGCGATAT	27	CGGATAAG	TAGGCATC	35	CGCAGATA	CAGAAGCA	43	GCTGTAGT	CTTCTTGC
D	4	ACACCACA	AGCTAAGC	12	TCTCTGGT	AGTGGTTG	20	TAGCGCTT	AGGTTGAG	28	GCGTTACA	CGCATCAA	36	GCCTTGTT	CGTGCATA	44	ACTCACCA	CTGCATCT
E	5	AATGAGGC	AGCGTATC	13	AGAATCCG	TGTGAGTG	21	ATGAGCAG	TACAGGCT	29	AGTACAAG	AGTACAAG	37	ATTGGCGT	GACAACAG	45	ACGAAGAC	TCGCAACA
F	6	TTGAGAGC	TTCCTAGC	14	AGCAGCTT	TTATCGCG	22	TGTGGATC	ATTGGACG	30	AGGTAACG	GAACCGTT	38	AGATTGGC	TCGCTAAG	46	CGTAACTC	TGACTGTC
G	7	TTGCAGTG	AGTCCTGA	15	ATACCGAC	TTGGTTCG	23	AGATGTAC	AGATGTAC	31	ACCAGAAC	GAACGAGT	39	AGCTGACA	CTTCTGAG	47	CTATGGCA	CTGTCACT
H	8	ACGGTGTA	ACCTTCTG	16	GCAGTTGA	CTACAGGT	24	GTAGCTCA	ATGACGGT	32	CGTACTGT	CTACCAGA	40	GAGGCAAT	TCCTATCG	48	CTGTGCTT	ACCTCAAC

Index Set B	7			8			9			10			11			12		
	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read	Index Primer Pair	i7 read	i5 read
A	49	TACTGTCG	CACTATGC	57	GTTCATCG	GTTAGGAC	65	TGGTGGAA	AGACTCCT	73	ACGTTCGT	CGTATGTG	81	ATGCCGTA	TGCTCGTA	89	TCTCAAGC	GAGAGCTT
B	50	AGGATCGA	TGGTTCGA	58	ACAACGGA	CAACTGGA	66	CAGTCTTG	GACTCTCT	74	TAGCCTAG	CAGGAAGT	82	TCGCTATG	TTGGCGAT	90	CTCCAAGT	AGAGGCTT
C	51	ACGTCTTC	GCTTACTG	59	GATGGCAT	AGAGCAGT	67	CGTCATGA	TTCGCAGA	75	TCCGAATG	CTCCATTG	83	CTAGTGGA	AGCCAGTT	91	GAGACGAA	CGCAGTTA
D	52	ACGGCATT	CGTAGACA	60	GCTCCATT	GCGGTAAT	68	ACGTCAAG	CAAGGAAG	76	TACCGATG	TGGAGATC	84	TAAGCCGT	CTAAGTGC	92	TTAGGCAG	GTACGGAA
E	53	CATTCGGT	GCTTATGC	61	AGAACAGC	ACATCGGA	69	CGACTATG	GTGGAATG	77	AGTGGCTA	CAATGGCT	85	GCATCCAA	GAGTACCA	93	TGACCGTT	GTCACTTC
F	54	TGCCATCA	GAGACAAG	62	GAGTCCTT	ACAGGCAA	70	CAAGCTTC	CATACCAC	78	ACGCAACT	GATTCGAG	86	TGTGCTAC	AGTGCTCT	94	AGTTCGCA	AGGCATTC
G	55	CATCGGTT	TTCCGTGT	63	GTCGGATT	AAGTGGTC	71	GTCACCTA	TGACGTTG	79	CACCTTAC	CACCTTAC	87	TTCAGCCA	ACAGTGTC	95	TCGCATAC	GATCTGTC
H	56	TCTACCTC	AGAGTGCA	64	AGGCTGAT	TAACAGGC	72	TCTCCTCA	CTCGAACA	80	GAAGCATG	TGTCGAAG	88	TGAAGACG	TCATCCAG	96	ACCGCTTA	ATGTGGCA

Notes



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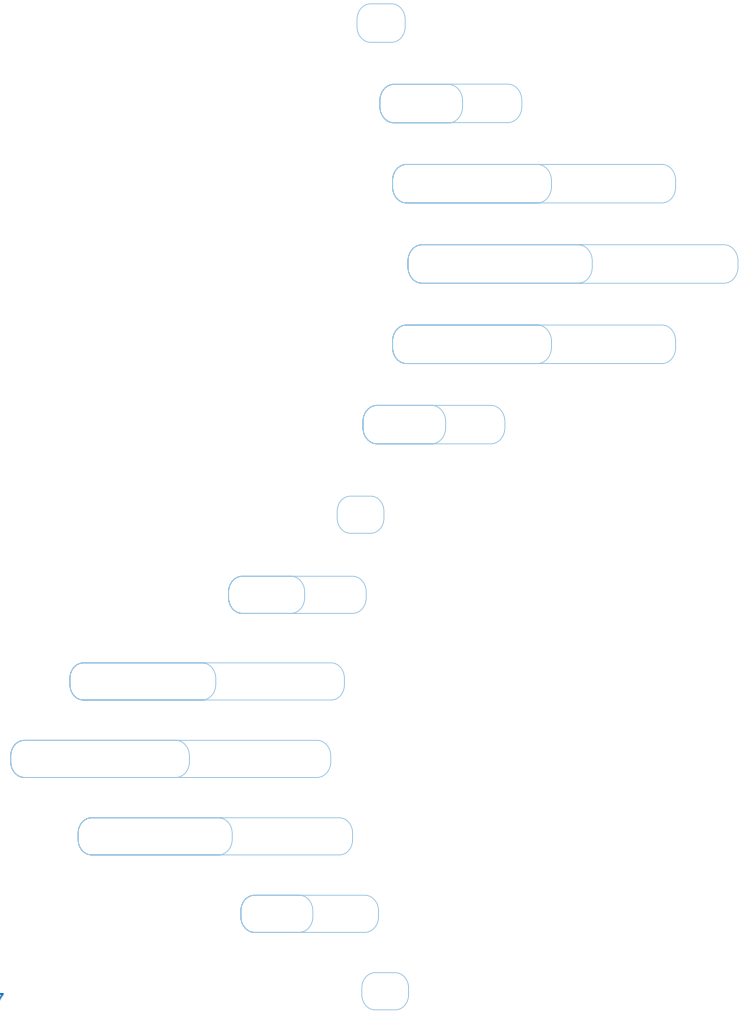

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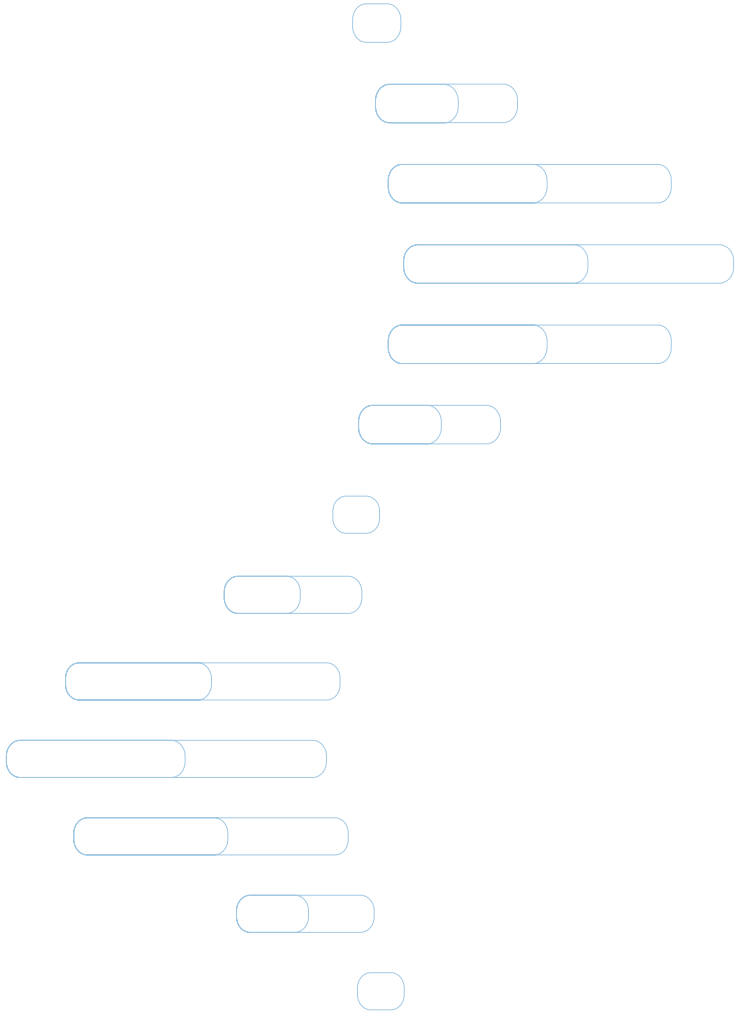

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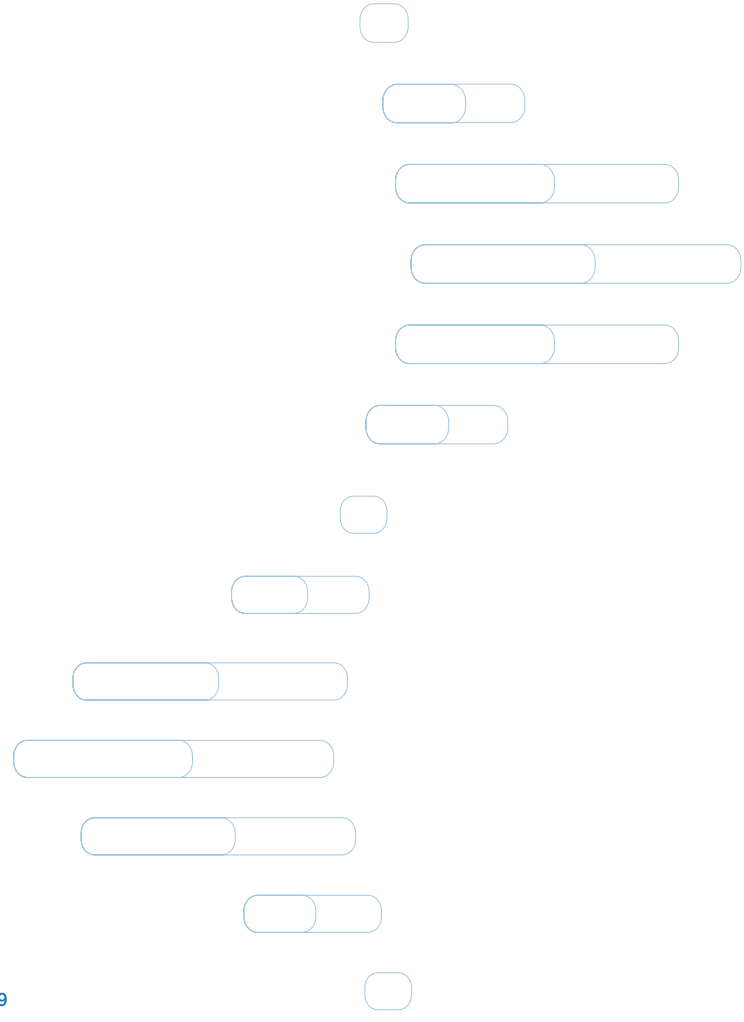

3131 Elliott Ave, Suite 750 | Seattle, WA 98121 | +1(877) 202-TWIN | support@twinstrandbio.com
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