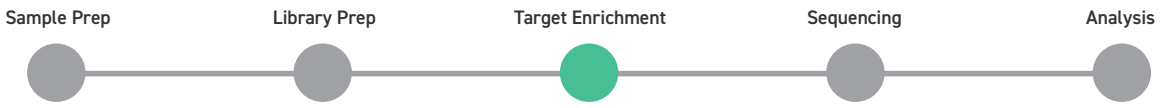
Twist FlexPrep Target Enrichment Protocol

For use with the Twist NGS Workflow

The Twist FlexPrep Target Enrichment Protocol generates target-enriched DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems. As a component of the Twist FlexPrep System, this protocol has been optimized for use with the Twist FlexPrep UHT Library Preparation Kit, using the Twist FlexPrep Normalization Adapters and the Twist UDI Primers. This manual details the steps for a cost-effective protocol, allowing for library input up to a 96-plex using a 16-hour hybridization step. This protocol should only be performed with the reagents specified or their equivalents.



Twist NGS Workflow. *The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist FlexPrep Target Enrichment Protocol works in conjunction with the other component protocols.*

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

PROTOCOL COMPONENTS

Please read the product packaging and storage recommendations carefully for each kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
TWIST FLEXPREP HYBRIDIZATION AND WASH KIT (FOR TARGET ENRICHMENT WITH TWIST FLEXPREP HYBRIDIZATION)			
109156 (192 samples) 109163 (1152 samples)	Twist FlexPrep Hybridization Reagents	· Hybridization Mix · Hybridization Enhancer · Amplification Primers	–20°C
109157 (192 samples) 109164 (1152 samples)	Twist FlexPrep Wash Buffers	· Binding Buffer · Standard Wash Buffer 1 · Wash Buffer 2	2-8°C
109154 (192 samples) 109161 (1152 samples)	Twist FlexPrep Universal Blockers	For the prevention of nonspecific capture: · Universal Blockers · Blocking Solution	–20°C
109155 (192 samples) 109162 (1152 samples)	Twist FlexPrep Binding Beads	For target enrichment and purification: · Streptavidin Binding Beads	2-8°C
TWIST PROBE PANELS (ORDERED SEPARATELY)			
Choice of panel type and reaction size	Twist Fixed Panel	Fixed content enrichment panel for hybridization reactions	–20°C
	Twist Custom Panel	Custom enrichment panel for hybridization reactions	–20°C
	(Optional) Secondary Twist Probe Panel	Secondary enrichment panel for adding content to a fixed or custom panel	–20°C

For full kits that come with the Twist FlexPrep UHT Library Preparation components, use the following catalog numbers:

109223: Twist FlexPrep UHT Library Prep and Enrichment Kit, 192 Samples

109226: Twist FlexPrep UHT Library Prep and Enrichment Kit, 1152 Samples

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INTENDED USE

This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding use of the product for applications in which it is not intended.

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate enriched libraries using the Twist FlexPrep Target Enrichment workflow.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
1.5-ml compatible magnetic stand	Beckman Coulter, Thermo Fisher Scientific
96-well compatible magnetic plate	Alpaqua, Permagen Labware
96-well deep well block	Eppendorf
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Qubit dsDNA Broad Range Quantification Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Thermomixer (preferred) or heat block for 1.5-ml tubes x2	Eppendorf
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol, and follow the provided instructions. Twist cannot guarantee the performance of the Twist FlexPrep Hybridization Target Enrichment Workflow if modifications are made to the protocol.

Test the compatibility of your thermal cycler and PCR tubes by incubating them at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.

The FlexPrep Hybridization Mix is a viscous reagent. Pipette slowly to ensure accuracy.

FOR TECHNICAL SUPPORT, CONTACT CUSTOMERSUPPORT@TWISTBIOSCIENCE.COM

PROTOCOL OVERVIEW

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

	FLEXPREP HYBRIDIZATION TARGET ENRICHMENT WORKFLOW (AMPLIFIED, INDEXED LIBRARIES)	TIME
STEP 1	**Prepare libraries for hybridization** Indexed library pool STOPPING POINT	**1 hour**
STEP 2	**Hybridize capture probes with pools** Hybridized targets in solution	**16 hours**
STEP 3	**Bind hybridized targets to streptavidin beads** Captured targets on beads	**1.5 hours**
STEP 4	**Post-capture PCR amplify, purify, and perform QC** Enriched libraries STOPPING POINT	**1 hour**
STEP 5	**Sequence on an Illumina platform** Libraries ready for sequencing on Illumina platform	**—**

STEP 1 PREPARE LIBRARIES FOR HYBRIDIZATION

This step involves aliquoting the appropriate amount of amplified, indexed FlexPrep libraries (generated previously using the Twist FlexPrep UHT Library Preparation Kit) and preparing the hybridization reaction solution.

This protocol supports a multiplex (96-plex) hybridization capture.

Reagents Required

- Amplified, indexed FlexPrep libraries
- Ethanol
- Molecular biology grade water
- From Twist FlexPrep Universal Blockers:
  - Universal Blockers
  - Blocker Solution (If using a non-human capture panel, replace with species-specific blocking solution, not provided)
- DNA Purification Beads found in the Twist FlexPrep UHT Library Preparation Kit:
  - Extra DNA Purification Beads are supplied in the library preparation kit

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and pulse-spin.
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes.
- Vortex the pre-equilibrated DNA Purification Beads until well mixed.
- Prepare 500 µl fresh 80% ethanol for each sample to be processed.

ALIQUOT AND CONCENTRATE THE LIBRARIES

1.1 Pool 16 µl of 8 FlexPrep library pools (containing 12 libraries each) into a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate to generate a final pool of 96 libraries.

1.2 Quantify the final pool of 96 libraries using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantification Assay.

1.3 Use the concentration of the final pool of libraries to calculate the volume (in µl) needed for library mass input between 4 µg to 10 µg for each capture.

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

1.5 Pulse-spin the tube to collect all liquid to the bottom of the tube.

1.6 Add 1.8x homogenized DNA Purification Beads to the tube(s) containing the final pool of libraries. Mix well by vortexing.

1.7 Incubate for 5 minutes at room temperature.

1.8 Pulse-spin to ensure all the solution is at the bottom of the tube(s) and place the tube(s) on a magnetic plate or rack for 3 minutes or until the solution is clear.

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

1.10 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

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

1.12 Carefully remove all remaining ethanol using a 10-µl pipette, making sure to not disturb the bead pellet.
NOTE: If necessary, pulse-spin to ensure complete removal of ethanol.

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

1.14 Remove the tube(s) from the magnetic plate or rack and add 7 µl Universal Blockers and 5 µl Blocker Solution. Mix by pipetting until homogenized. Do not remove the solution from the beads.

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

SAFE STOPPING POINT: The concentrated pre-hybridization solution may be stored at -20°C for up to 24 hours before proceeding to Step 2.

PROCEED TO STEP 2: HYBRIDIZE CAPTURE PROBES WITH POOLS

STEP 2 HYBRIDIZE CAPTURE PROBES WITH POOLS

Use the concentrated pre-hybridization solution from Step 1 to perform the hybridization reaction.

Test the compatibility of your thermal cycler and PCR tubes or plates by incubating them at 95°C for up to 5 minutes to ensure they do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

Reagents Required

- Concentrated pre-hybridization solution from Step 1.14
- Twist fixed or custom panel
- Twist custom secondary (spike-in) panel(s) (optional)
- Twist FlexPrep Hybridization Reagents:
  - Hybridization Mix
  - Hybridization Enhancer

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix, and then pulse-spin.
- Set a heat block to 65°C.
- Program a 96-well thermal cycler to 95°C and set the heated lid to 105°C.

PREPARE THE PROBE SOLUTION

2.1 Heat the Hybridization Mix at 65°C in the heat block for 10 minutes or until all precipitate is dissolved, then cool to room temperature on the benchtop for 5 minutes.

2.2 Prepare a probe solution in a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate as indicated in the table below. Mix by flicking the tube(s).

REAGENT	VOLUME PER REACTION
Hybridization Mix	20 µl
Twist Fixed or Custom Panel	4 µl
Optional: Secondary Panel (in place of water)	4 µl
Water (up to total volume)	(0-4) µl
Total	**28 µl**

NOTES:

- If using optional Secondary Panel (spike-in) content, add 4 µl of probes in place of water.
- Hybridization Mix is very viscous. Pipette slowly to ensure accurate pipetting.
- Small white particles may be present in the Twist Fixed or Custom Panel tube(s). This will not affect the final capture product.

PERFORM THE HYBRIDIZATION REACTION

2.3 Heat the probe solution (from Step 2.2) to 95°C for 2 minutes in a thermal cycler with the lid at 105° C, then immediately cool on ice for 5 minutes.

2.4 While the probe solution is cooling on ice, heat the tube containing the concentrated pre-hybridization solution (from Step 1.14) at 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then equilibrate both the probe solution and concentrated pre-hybridization solution to room temperature on the benchtop for 5 minutes.

2.5 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.

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

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

2.8 Pulse-spin the tube(s) to ensure there are no bubbles present.

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

2.9 Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C.
NOTE: Halting hybridization between 15–17 hours will not affect downstream capture quality.

PROCEED TO STEP 3: BIND HYBRIDIZED TARGETS TO STREPTAVIDIN BEADS

STEP 3 BIND HYBRIDIZED TARGETS TO STREPTAVIDIN BEADS

Reagents Required

- Hybridization reactions (from Step 2.9)
- From the Twist FlexPrep Wash Buffers:
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- From Twist FlexPrep Binding Beads:
  - Streptavidin Binding Beads

Before You Begin

- Preheat the following tubes at 48°C until all precipitate is dissolved:
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- For each hybridization reaction:
  - Equilibrate 800 µl Binding Buffer to room temperature
  - Equilibrate 225 µl Standard Wash Buffer 1 to 68°C
  - Leave 700 µl Wash Buffer 2 at 48°C
- Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes
- In preparation for Step 4 (Post-Capture PCR Amplify, Purify, and Perform QC), thaw on ice:
  - 2x Twist Library Amp Mix (from the Twist FlexPrep UHT Library Preparation Kit)
  - Amplification Primers, ILMN
- Equilibrate DNA Purification Beads (Step 1) to room temperature for at least 30 minutes

PREPARE THE BEADS

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

3.2 Add 100 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

3.3 Add 200 µl Binding Buffer to the tube(s) and mix by pipetting.

3.4 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

3.5 Repeat the wash (Steps 3.3 and 3.4) two more times, for a total of three washes.

3.6 After removing the clear supernatant from the third wash, add a final 200 µl Binding Buffer and resuspend the beads by vortexing until homogenized.

3.7 Heat the resuspended beads at 68°C for at least 10 minutes before continuing to Step 3.8.

BIND THE TARGETS

3.8 After the hybridization (Step 2.9) is complete, open the thermal cycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of preheated Streptavidin Binding Beads from Step 3.7. Mix by pipetting and flicking.

⚠ IMPORTANT: Rapid transfer directly from the thermal cycler at 70°C is a critical step for minimizing off-target binding. Do not remove the tube(s) of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binding Beads. Allowing to cool to room temperature for less than 5 minutes will result in as much as 10–20% increase in off-target binding.

3.9 Incubate the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 5 minutes at 68°C, agitation is not required.
NOTE: Do not vortex. Aggressive mixing is not required.

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

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

3.12 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.
NOTE: Some Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product.

3.13 Remove the tube(s) from the magnetic stand and add 200 µl 68°C Standard Wash Buffer 1. Mix by pipetting.

3.14 Incubate the tube(s) for 5 minutes at 68°C.

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

3.16 Transfer the entire volume from Step 3.15 (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.

⚠ IMPORTANT: This step reduces background from non-specific binding to the surface of the tube.

3.17 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube(s) from the magnetic stand and add 200 µl of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube(s).

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

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

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

3.21 Repeat the wash (Steps 3.17–3.20) two more times, for a total of three washes.

3.22 After the final wash, use a 10-µl pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.
NOTE: Before removing the supernatant, the bead pellet may be briefly spun to collect supernatant at the bottom of the tube or plate and returned to the magnetic plate.

3.23 Remove the tube(s) from the magnetic stand and add 45 µl water. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

PROCEED TO STEP 4: POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

STEP 4 POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

Reagents Required

- Streptavidin Binding Bead slurry (from Step 3.23)
- Ethanol
- Molecular biology grade water
- Reagents thawed and equilibrated in Step 3:
  - DNA Purification Beads
  - 2x Twist Library Amp Mix
  - Amplification Primers, ILMN
- Agilent Bioanalyzer High Sensitivity DNA Kit (or equivalent)
- Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay

Before You Begin

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

PREPARE THE BEADS, THERMAL CYCLER, AND PCR MIX

4.1 Program a thermal cycler with the following conditions. Set the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	# OF CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	*Varies. Use table to the right.*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

PANEL SIZE	# OF CYCLES MULTIPLEX
>100 Mb	5
50–100 Mb	7
10–50 Mb	8
1–10 Mb	9
500–1,000 kb	11
100–500 kb	13
50–100 kb	14
<50 kb	15

NOTE: Number of amplification cycles may vary depending on sample type

4.2 If the Streptavidin Binding Bead slurry has settled, mix by pipetting.

4.3 Transfer 22.5 µl of the Streptavidin Binding Bead slurry to a 0.2-ml thin-walled PCR strip-tube(s). Keep on ice until ready to use in the next step.

NOTE: Store the remaining 22.5 µl water/Streptavidin Binding Bead slurry at –20°C for future use.

4.4 Prepare a PCR mixture by adding the following reagents to the tube(s) containing the Streptavidin Binding Bead slurry. Mix by pipetting.

REAGENT	VOLUME PER REACTION
Streptavidin Binding Bead Slurry	22.5 µl
Amplification Primers, ILMN	2.5 µl
2x Twist Library Amp Mix	25 µl
Total	**50 µl**

PCR AMPLIFY

4.5 Pulse-spin the tubes, transfer them to the thermal cycler, and start the cycling program.

4.6 When the thermal cycler program is complete, remove the tube(s) from the block and immediately proceed to the Purify step.

PURIFY

4.7 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

4.8 Add 90 µl (1.8x) homogenized DNA Purification Beads to the tube(s) from Step 4.6. Mix well by vortexing.
NOTE: It is not necessary to recover supernatant or remove Streptavidin Binding Beads from the amplified PCR product.

4.9 Incubate for 5 minutes at room temperature.

4.10 Place the tube(s) on a magnetic plate for 1 minute or until the supernatant is clear.

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

4.12 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

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

4.14 Carefully remove all remaining ethanol using a 10-µl pipette, making sure to not disturb the bead pellet.
NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

4.15 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

4.16 Remove the tube(s) from the magnetic plate and add 32 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each capture reaction. Mix by pipetting until homogenized.

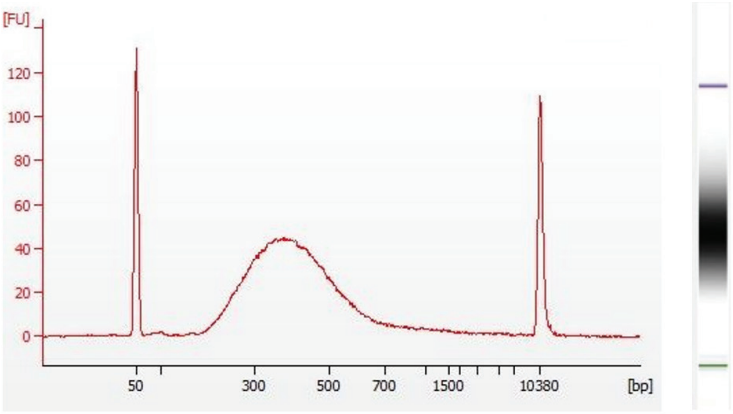
4.17 Incubate at room temperature for 2 minutes.

4.18 Place the plate or tube(s) on a magnetic plate and let stand for 3 minutes or until the beads fully pellet.

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

PERFORM QC

4.20 Validate and quantify the enriched libraries using an Agilent Bioanalyzer High Sensitivity DNA Kit and a Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay.
NOTE: When using the Agilent Bioanalyzer High Sensitivity DNA Kit, load 0.5 µl of the final sample. Average fragment length should be 400 bp to 450 bp using a range setting of 150 bp to 1,000 bp. Final concentration may vary and is dependent on panel size and the number of PCR cycles.



Electropherogram generated by an Agilent High Sensitivity DNA analysis of the enriched libraries that were prepared as described. Note the single prominent peak.

NOTE: Due to the inline barcodes found on the Twist FlexPrep Normalization Adapters, 5% to 10% PhiX is needed in order to get a balanced nucleotide composition on an Illumina sequencing platform.

STOPPING POINT: If not proceeding immediately, store the enriched libraries at -20°C for up to 24 hours.

STEP 5 SEQUENCING ON AN ILLUMINA PLATFORM

Sequence the enriched libraries on an Illumina platform. Sequencing protocols and settings will depend on the application and instrumentation used.

Please contact customersupport@twistbioscience.com for recommendations.

END OF WORKFLOW