Department of Genomic Medicine
Cancer Immune Monitoring and Analysis Center
Division of Cancer Medicine

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center
Making Cancer History®

CIMAC LIBRARY PREPARATION USING TWIST KIT

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Title:	CIMAC LIBRARY PREPARATION USING TWIST KIT	
Applies to:	CIMAC Libraries from DNA	
Reference:	Twist User Guide	
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1. PURPOSE

TWIST NGS target enrichment kit consists of three main components to prepare the samples from sample preparation up to sequencing. The main components are mechanical shearing, library preparation, and target enrichment. The kit is able to prepare NGS libraries from samples types such as FFPE, fresh frozen tissues, blood, etc. The time required to prepare NGS libraries can take up to 24 – 72 hrs depending on the target enrichment protocol used. Twist has a rapid target enrichment protocol and an overnight enrichment protocol. In this workflow, the overnight enrichment protocol will be used.

2. GUIDELINES

2.1. Before beginning NGS library preparation, ensure that there is enough reagent starting from fragmentation up to target enrichment.
2.2. Ensure that the quality and quantity of the samples are sufficient before starting.
2.3. Do not step the quality check during the entire set up to prevent sequencing failed samples.
2.4. Read the instructions entirely before performing the protocol to ensure protocol performance.

3. REAGENT

3.1. Twist Library Preparation Kit, Mechanical Fragmentation, 96 Samples **P/N: 101281**

Contents	Volume	Source
10x ERA Buffer		-20
5x ERA Enzyme Mix		-20
DNA ligation Mix		-20
Amplification primers (ILMN)		-20
DNA Purification beads		2-8C
Twist Universal Adapter		-20

3.2. Reagents not included in the kit.

Reagents	Source	Catalog #	Storage Temperature

4. SUPPLIES/ EQUIPMENT

Supplies	Reference Source	Catalog #
Eppendorf LoBind Microcentrifuge tube 1.5 mL	Millipore Sigma	Z666548
Pipette tips 20 µl	Rainin or compatible	RT-L20F
Pipette tips 200 µl	Rainin or compatible	RT-L200F
Pipette tips 1000 µl	Rainin or compatible	RT-L1000F

Equipment	Reference Source	Catalog #
Eppendorf™ 5424 Microcentrifuges	Fisher Scientific	05-400-002
Thermo Scientific™ Precision™ Water Baths	Fisher Scientific	TSCIR19
Pipet-Lite LTS pipette 100-1000 µl	Rainin	L-1000
Pipet-Lite LTS pipette 20-200 µl	Rainin	L-200
Pipet-Lite LTS pipette 2-20 µl	Rainin	L-20
Heat Block		

5. SPECIAL SAFETY PRECAUTIONS

5.1. **Personal Protective Equipment**

5.1.1. Always wear proper PPE to prevent any injuries.

5.2. **Chemical and Hazardous waste disposal.**

5.2.1. Chemical and Hazardous waste must be disposed of in accordance to the laws and regulations. Do not pour down the drain.

6. QUALITY CONTROL

6 Determine quality and quantity of the extracted Genomic DNA with:

6.1.1. Quant-it Picogreen dsDNA Assay kit

6.1.2. Genomic DNA ScreenTape Analysis

6.1.3. ThermoFisher, NanoDrop One

7. PROCEDURE

Before Starting: Make sure the Covaris LE-220 sonicator is degassed and is at 4C.

7 Procedure

7.1. Mechanical Fragmentation – Covaris

7.1.1. Determine the gDNA concentration of the samples and dilute each gDNA sample in TE buffer with low EDTA to 5 ng/ul in a final volume of 50 ul. The resulting dilution should yield a total 250 ng per sample.

7.1.2. Transfer the diluted gDNA into their respective AFA covaris tube in the plate and seal the plate.

7.1.3. Place the plate into the Covaris Instrument and select the "Twist Fragmentation protocol".

7.1.4. Click the edit button and select the designated wells on the screen.

7.1.5. Finally, press the run button to begin fragmenting the samples.

7.2. QC Check

7.2.1. Run the fragmented samples on the Tapestation using the D1000 High Sensitivity Tape or high sensitivity DNA kit on the Bioanalyzer.

7.2.2. Ensure that the fragment size distribution range is between 200 – 250 bp.

7.2.3. If the samples are outside of the range, refragment the samples in increments of 15 – 60 seconds until the mode is in range.

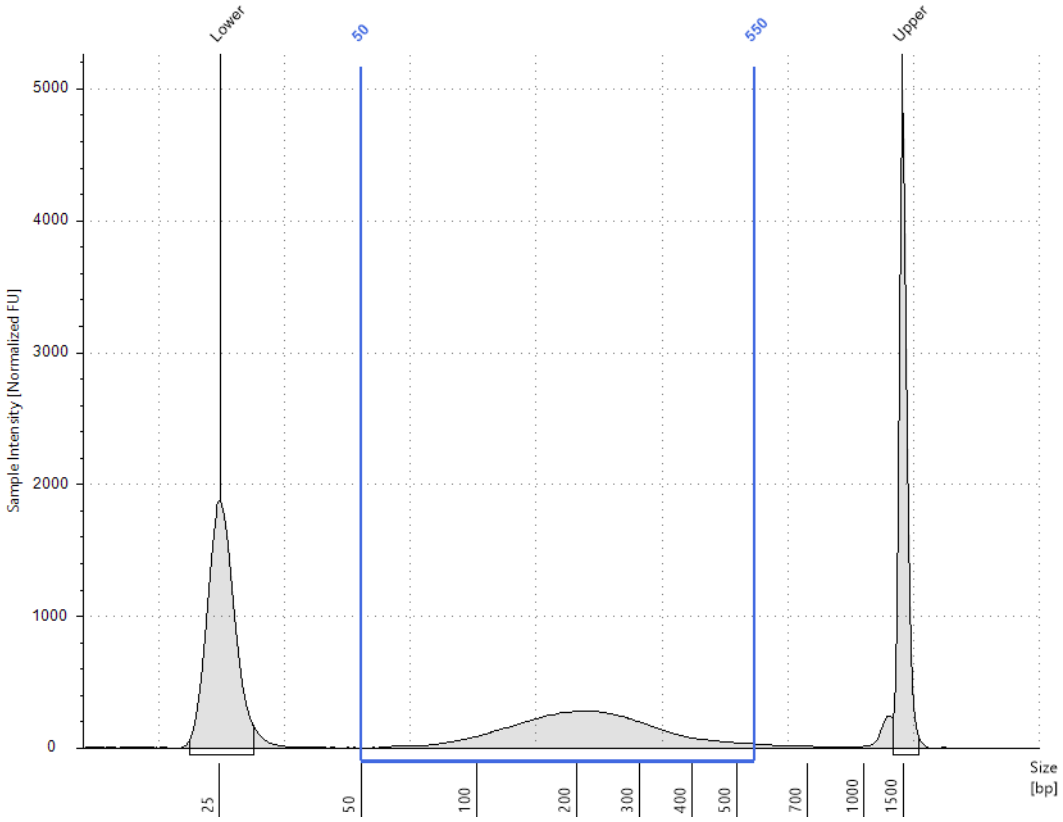

Figure 1. D1000 Tape QC

7.2.4. If not proceeding immediate to the library preparation protocol, store the DNA samples at -20C.

7.3. Library Preparation – End Repair and A-Tailing

7.3.1. Before beginning:

Thaw the 5x Enzyme Mix on ice and mix by flicking the tube. Do not Vortex.

Thaw the 10x ERA buffer on ice and mix by vortexing. If white precipitates are

seen, mix vigorously until the precipitate dissolves.

7.3.2. Run the PCR program "Twist ER and A-Tail" to pre-chill the thermal cycler at 4C. Pause the program once there's only 10 seconds left. This will pause the temperature for 10 minutes.

PCR Program: Twist ER and A-Tail

Temperature	Time
4C	HOLD
20C	30 minutes
65C	30 minutes
4C	HOLD

7.3.3. Using the concentration from the tapestation result, dilute the gDNA samples to 5 ng/ul with water in a final volume of 10 ul and total DNA input of 50 ng.

Note: If the concentration is below 5 ng/ul, the sample can be diluted into a final volume of 35 ul instead. Remove the 25 ul of water required in the ERA reaction.

7.3.4. Prepare an ERA reaction mastermix in a 1.5-ml microcentrifuge tube on ice using the volumes listed below.

Reagent	Volume per reaction (ul)*
Water	25
10x ERA Buffer	5
5x ERA Enzyme Mix	10
Total	40

Note: Prepare a master mix for multiple reaction and keep in mind samples that will require no water in their mastermixes.

7.3.5. Add 40 ul of ERA reaction Mastermix to each 10 ul gDNA sample well or 15 ul of mastermix without water if the sample has 35 ul gDNA sample.

7.3.6. Pulse-spin the sample plate or tubes and immediately transfer to the pre-chilled thermal cycler.

7.3.7. Continue the thermal cycler program to step 2-4.

7.3.8. When the thermal cycler program is complete, remove the samples from the block and place them on ice.

7.3.9. Proceed immediately to adapter ligation.

7.4. Library Preparation – Adapter Ligation

7.4.1. Thaw the reagents on ice before beginning and equilibrate DNA beads to room temperature for at least 30 minutes before using.

7.4.2. Add 5 ul of Twist Universal Adapters into each sample well containing the dA-tailed DNA fragments from step 7.3.8. Mix gently by pipetting and keep on ice.

7.4.3. Prepare the ligation mastermix in a 1.5 mL tube on ice as indicated below.

Reagent	Volume per reaction (ul)*
Water	15
DNA Ligation Buffer	20
DNA Ligation Mix	10
Total	45

***Prepare a mastermix for multiple reactions.**

7.4.4. Add 45 ul of the ligation master mix to the samples from step 7.4.2 and mix by pipetting.
7.4.5. Seal the plate and pulse-spin to ensure all solution is at the bottom of the tube.
7.4.6. Run the ligation program "Twist A-ligation" (20C for 15 minutes) with the lid open or off.
Note. While the program is running, prepare 1mL of 80% ethanol per sample to use for purification. Thaw the reagents to be used in the pre-capture PCR.

7.5. Library Preparation – Adapter Ligation Purification

7.5.1. Vortex the pre-equilibrated DNA purification beads until well mixed.
7.5.2. Remove the samples from the cycler once the program is complete and add 80 ul (0.8x) of homogenized DNA purification beads to each ligation samples. Mix well by vortexing.
7.5.3. Incubate the samples for 5 minutes at room temperature.
7.5.4. Place the samples on a magnetic plate for 1 minute.
7.5.5. Once the supernatant is clear, remove and discard the supernatant.
7.5.6. Wash the bead pellet by adding 200 ul of freshly made 80% ethanol**.** Incubate for 1 minute then remove and discard the ethanol without disturbing the pellet.
7.5.7. Repeat step 7.5.6 for a total of two washes, while keeping the samples on the magnetic plate.
7.5.8. Carefully remove all remaining ethanol using a 10 ul pipette. Make sure to not disturb the pellet.
7.5.9. 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.
7.5.10. Remove the plate or tubes from the magnetic plate and add 17 ul of low TE buffer. Mix by pipetting until homogenized.
7.5.11. Incubate at room temperature for 2 minutes.
7.5.12. Place the plate on a magnetic plate and let satand for 3 minutes or until the beads form a pellet
7.5.13. Transfer 15 ul of the clear supernatant containing the ligated and indexed libraries to a new plate without disturbing the bead pellet.
7.5.14. Discard the plate with the bead pellet and proceed to PCR amplification.

7.6. Library Preparation – Pre-Capture PCR

Note: Amplification primers, ILMN tubes 100220 100583 contained in kit1 are not required in this step. Using these primers will result In a failed PCR amplification.

7.6.1. Thaw the Twist UDI primers and KAPA HiFi HotStart ReadyMix on ice before starting.
7.6.2. Determine the indexes to be used per sample and store the information in the worksheet.
7.6.3. Add 10 ul of specific Twist UDI primer from the provided 96-well plate to each of the respective gDNA libraries from step 2.18 and mix well by gentle pipetting.
7.6.4. Add 25 ul of KAPA HiFi HotStart ReadyMix to the gDNA libraries from step 7.6.3 and mix well by pipetting.
7.6.5. Pulse-spin the sample plate and immediately transfer to the thermal cycler. Start the "Twist Pre-Cap" PCR program.

Program: Twist Pre-Cap PCR

Step	Temperature	Time	# of Cycles
1. Initialization	98C	45 seconds	1
2. Denaturation	98C	15 seconds	8
Annealing	60C	30 seconds	
Extension	72C	30 seconds	
3.Final Extension	72C	1 minute	1
4. Final Hold	4C	Hold	-

7.6.6. **SAFE STOPPING POINT.** Store the samples overnight at 4C or -20C for long term storage. Proceed with purification.

7.7. Library Preparation – Pre-Capture PCR Purification

7.7.1. Vortex the pre-equilibraited DNA purification beads until mixed.
7.7.2. Add 50 ul (1x) of homogenized DNA purification beads into each amplified sample from step 7.6.6. Mix well by vortexing.
7.7.3. Incubate the sample for 5 minutes at room temperature.
7.7.4. Place the samples on a magnetic plate for 1 minute.
7.7.5. Remove and discard the supernatant without disturbing the pellet once the supernatant is clear.
7.7.6. Wash the bead pellet by adding 200 ul of freshly made 80% ethanol**.** Incubate for 1 minute then remove and discard the ethanol without disturbing the pellet.
7.7.7. Repeat step 7.7.6 for a total of two washes, while keeping the samples on the magnetic plate.
7.7.8. Carefully remove all remaining ethanol using a 10 ul pipette. Make sure to not disturb the pellet.
7.7.9. 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.
7.7.10. Remove the plate from the magnetic plate and add 22 ul of water to each sample. Mix by pipetting until homogenized.
7.7.11. Incubate at room temperature for 2 minutes.
7.7.12. Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
7.7.13. Transfer 20 ul of the clear supernatant containing the amplified indexed libraries to a new plate or tube without disturbing the beads.
7.7.14. SAFE STOPPING POINT. If not proceeding immediately, store the amplified libraries at 4C overnight or -20C for long term storage.

7.8. Library Preparation – Pre-Capture PCR Quality Check

7.8.1. Run the purified samples in a D1000 tape and ensure that the concentration values is above 80 ng/ul and the average fragment length should be between 375 and 425 bp using a range setting of 150-1,000 bp.
Note: You can proceed with concentrations lower than 80 ng/ul but it could result in low library diversity after hybridization.

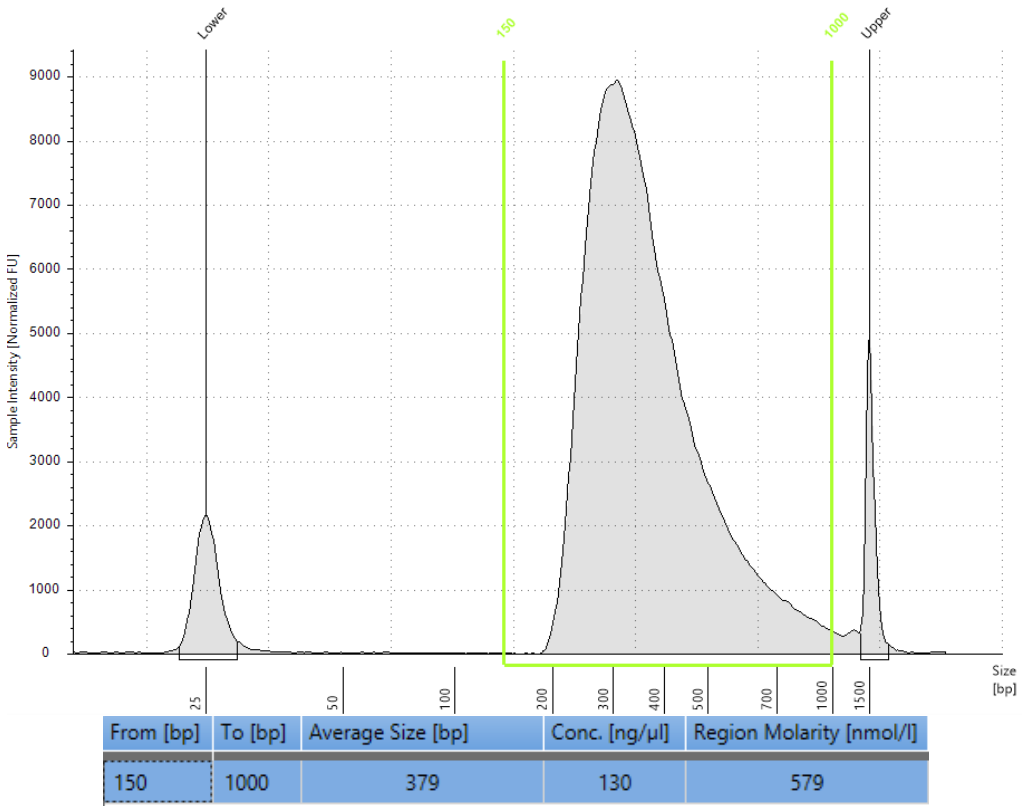

Figure 2. Expected Pre-Capture PCR QC.

7.9. Target Enrichment – Combine and Dry Down the Library

7.9.1. Determine the number of samples to be combined in a single pool.

7.9.2. Divide the amount of each indexed library per pool by the concentration from the D1000 tapestation quality check. Use the table below.
For example: 8-multiplex sample pool will require 187.5 ng per sample to a total mass of 1,500 ng.

Number of Indexed Samples per pool	Amount of each indexed library per pool	Total Mass per pool
1	500 ng	500 ng
2	500 ng	1,000 ng
3	500 ng	1,500 ng
4	375 ng	1,500 ng
8	187.5 ng	1,500 ng

Note: More than 1,500 ng total DNA can be used. However, do not exceed more than 4ug of DNA as this may lead to reduced performance.
If the amount of library is insufficient, smaller amount can be used but it will lead to decrease in library complexity.

7.9.3. Transfer and combine the calculated volumes from each amplified indexed libraries to their respective location in a 96 well plate. Mix by pipetting.

7.9.4. Seal the plate and pulse-spin the plate to minimize the amount of bubbles present.

7.9.5. Dry the indexed library pool(s) using a vacuum concentrator using low or no heat.

7.9.6. SAFE STOPPING POINT. If not proceeding immediately to the next step, store the dried indexed library pool at -20 for up to 24 hours.

7.10. Target Enrichment – Hybridization

7.10.1. Before beginning, thaw all required reagents on ice. Set a heat block to 65C.

7.10.2. Heat the hybridization mix at 65C in the heatblock for 10 minutes or until all precipitate is dissolved. Once dissolved, cool the hybridization mix to room temperature for 5 minutes.

7.10.3. Start the thermal cycler program at 95C hold.

7.10.4. Prepare a probe solution in a clean 96 well plate by following the table below.

Reagent	Volume (ul)
Hybridization Mix	20
Twist Fixed Panel	4
Water	4
Total	28

Note: Hybridization mix is very viscous. Pipette slowly to ensure accurate pipetting.

7.10.5. Resuspend the dried indexed library pool from step 7.9.6 by adding the reagents below. Mix by flicking the tubes.

Reagent	Volume (ul)
Dried Indexed Library Pool	-
Blocker Solution	5
Universal Blockers	7
Total	12

7.10.6. Heat the probe solution from step 7.10.3 to 95C for 2 minutes in a thermal cycler with a heated lid at 105C, then immediately cool on ice for 5 minutes.

7.10.7. While the probe solution is cooling on ice, heat the plate containing the resuspended indexed library pool at 95C for 5 minutes in a thermal cycler with lid at 105C. Then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.

7.10.8. Start the thermocycler program at 70C hold.

7.10.9. Carefully mix the probe solution by pipetting and then transfer entire amount (28ul) of the probe solution to the resuspended indxed library pool. Mix well by vortexing.

7.10.10. Cover the plate and pulse-spin the plate to ensure all solution is at the bottom.

7.10.11. Remove the seal and add 30 ul of hybridization enhancer to the top of the entire capture reaction.

7.10.12. Pulse-spin the plate to ensure that there are no bubbles present.

7.10.13. Seal the plate tightly and incubate the hybridization reaction at 70C for 16 hours in a thermal cycler with the lid at 85C (On).

7.11. Target Enrichment – Capture

7.11.1. Before beginning:

- Preheat the binding buffer wash buffer 1 and wash buffer 2 at 48C until any precipitate is dissolved.
- Equilibrate Streptavidin and DNA purification beads to room temperature for at least 30 minutes

For each hybridization reaction:

- Equilibrate 200 ul of Wash Buffer 1 to room temperature
- Preheat 700 ul Wash Buffer 2 to 48C

7.11.2. Vortex the pre-equilibrated Streptavidin beads until mixed.
7.11.3. Add 100 ul of the Streptavidin beads to a 1.5 mL microcentrifuge tube. Prepare one tube for each hybridization reaction.
7.11.4. Add 200 ul of Binding Buffer to the tubes and mix by pipetting.
7.11.5. Place the tube on a magnetic stand for 1 minute and remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.
7.11.6. Repeat the wash (steps 7.11.4 and 7.11.5) two more times for a total of three washes.
7.11.7. After removing the clear supernatant from the third wash, add a final 200 ul of Binding Buffer and resuspend the beads by vortexing until homogenized.
7.11.8. After hybridization is complete (step 7.10.3), open the thermal cycler lid directly transfer the volume of each reaction into the corresponding 1.5 ml tube of washed streptavidin beads from step 7.11.7. Mix by pipetting and flicking.
Note: Do not allow the hybridization reaction to cool less than 70C before transferring the solution. Rapid transfer directly from the thermal cycler at 70C is important in minimizing off-target binding.
7.11.9. Mix the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 30 minutes at room temperature on a shaker, rocker, or rotator at a speed sufficient to keep the solution mixed.
Note: Do not vortex.
7.11.10. 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).
7.11.11. Place the tube(s) on a magnetic stand for 1 minute.
7.11.12. Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.
Note: Residual hybridization enhancer may be visible after supernatant removal. It will not affect the final capture product.
7.11.13. Remove the tube(s) from the magnetic stand and add 200 µl Wash Buffer 1. Mix by pipetting.
7.11.14. Pulse-spin to ensure all solution is at the bottom of the tube(s).
7.11.15. Transfer the entire volume from Step 7.11.14 (~200 µl) into a new 1.5-ml microcentrifuge tube or a new 96 well plate, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.
7.11.16. Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
7.11.17. 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).
7.11.18. Incubate the tube(s) for 5 minutes at 48°C.
7.11.19. Place the tube(s) on a magnetic stand for 1 minute.
7.11.20. Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
7.11.21. Repeat the wash (Steps 7.11.17–7.11.20) two more times, for a total of three washes.
7.11.22. 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.
7.11.23. 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.

7.12. Post-Capture PCR

7.12.1. Before beginning, prepare 500 ul of 80% ethanol for each Streptavidin Binding Bead slurry.

7.12.2. Mix and spin the Streptavidin Binding Bead slurry with streptavidin beads before starting.

7.12.3. Transfer 22.5 µl of the Streptavidin Binding Bead slurry to a 0.2-ml thin-walled PCR strip-tube(s). Keep on ice until ready to use in the next step. Store the remaining 22.5 µl water/Streptavidin Binding Bead slurry at –20°C for future use.

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

Reagent	Volume (ul)
Streptavidin Binding Bead Slurry	22.5
Amplification Primers, ILMN	2.5
KAPA HiFi HotStart ReadyMix	25
Total	50

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

Program: Twist Post Cap PCR

Step	Temperature	Time	# of Cycles
1. Initialization	98C	45 seconds	1
2. Denaturation	98C	15 seconds	8
Annealing	60C	30 seconds	
Extension	72C	30 seconds	
3.Final Extension	72C	1 minute	1
4. Final Hold	4C	Hold	-

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

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

7.12.8. Add 50 µl (1.0x) homogenized DNA Purification Beads to the tube(s) from Step 7.12.6. Mix well by vortexing.

7.12.9. Incubate for 5 minutes at room temperature and place the tube(s) on a magnetic plate for 1 minute.

7.12.10. 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.

7.12.11. 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.

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

7.12.13. Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet.

7.12.14. Air-dry the bead pellet on a magnetic plate for 5–10 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

7.12.15. Remove the tube(s) from the magnetic plate and add 32 µl water or Buffer EB to each capture reaction. Mix by pipetting until homogenized.

7.12.16. Incubate at room temperature for 2 minutes.

7.12.17. Place the plate or tube(s) on a magnetic plate and let stand for 3 minutes or until the

beads fully pellet.

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

7.13. Perform QC.

7.13.1. Validate and quantify each enriched library using D1000 or D1000HS

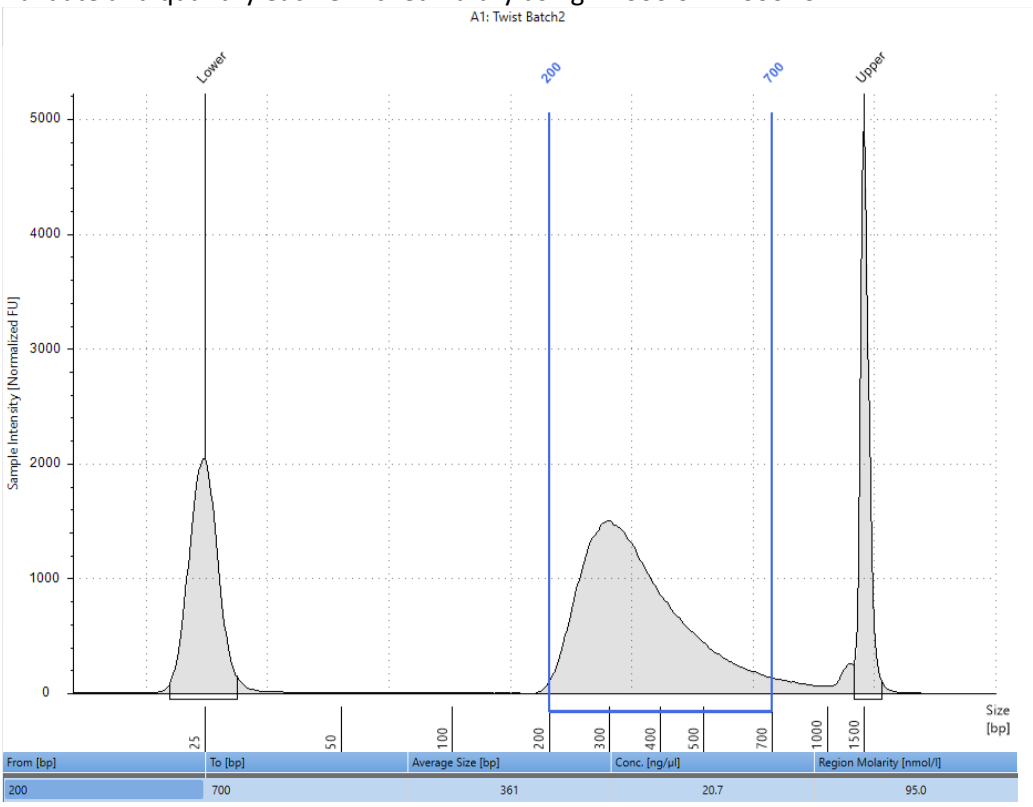

Figure 3. Expected Post-Capture PCR QC.

7.13.2. Perform qPCR for each pool.

7.13.3. Based on the qPCR result, combine equimolar values for each pool.

7.13.4. Perform qPCR and run the tape station for the final pool.

7.13.5. Sequence the final pool using the NovaSeq.

8. INTERPRETATION OF RESULTS*

Picogreen: DNA yield depends on the amount of starting material used for extraction. If there is little to no DNA in the eluate, inefficient cell lysis may have occurred. Follow the QIAamp DNA FFPE Tissue Handbook for troubleshooting.

Tapestation: A high DNA Integrity Number (DIN) indicates high quality gDNA. A low DIN indicates low quality and fragmented DNA. Note that DNA isolated from FFPE Samples is usually lower in molecular weight than DNA from fresh or frozen samples.

Nanodrop: A260/A280 ratio – "Pure" DNA will have a ratio of ~1.8. A higher ratio indicates presence of residual RNA. A lower than expected ratio indicates presence of contaminants which absorb strongly at 280 nm. A260/A230 ratio - Expected Range of 2.0-2.2. A lower than expected ratio indicates presence of contaminants (i.e. EDTA, Carbohydrates, Phenol, TRIzol)

which absorb at 230 nm.

9. RELATED/SUPPORTING DOCUMENTS

- Protocol_NGS_MechFragUniversalAdapterSystem_11Sep19_Rev1
- Twist_Protocol_NGS_SamplePrep_CovarisFFPE_10Sep19_Rev1.1
- Twist_Protocol_NGS_HybridizationTE_31OCT19_Rev1
- Environmental Protection Website: http://inside.mdanderson.org/departments/facilities/emergency-safety/environmental-protection.html