TWIST PROTOCOL – TARGET ENRICHMENT

List and order of reagents for completion to the stop-point.

• Primer 6
• ProtoScript II Reaction Mix
• ProtoScript II Enzyme Mix
• E.coli RNAse H
• NEB second strand synthesis reaction buffer
• NEBNext second strand synthesis Enzyme mix
• DNA Purification beads
• Ethanol Absolute
• 10x Fragmentation buffer
• 5x Fragmentation Enzyme
• Ligation MasterMix
• TWIST universal adapters
• TWIST UDI Primer (96-well plate)
• 10x High-Fidelity PCR Buffer
• 10mM dNTP Mix
• 50mM MgSO4
• Platinum TAQ Polymerase

First strand synthesis

1. If Primer 6 is unopened
    a. Add 666µL of MQ-H2O to make a concentration of 50uM.
2. Create a MM consisting of (**per sample**);
    a. 25µL of ProtoScript II Reaction Mix
    b. 5µL ProtoScript II Enzyme Mix.
3. Add 5µL of diluted Random Primer 6 to each well or 0.1mL tube for each sample.
4. Add 15µL of the sample to each PCR tube or well to make 20µL. This is the **Primer Annealing Solution**.
5. Heat the **Primer Annealing Solution** in a thermocycler (**ARON, TWIST 1**);
    a. Step 1. 95°C for 5 minutes
    b. Step 2. 20*C.
    c. Lid at 105°C.

    Immediately place on ice when the thermocycler reaches 20°C.

6. Add 30µL reagents from **Step 2 (MM)** to the **Primer Annealing Solution**, to make a volume of 50µL.
7. Incubate both solutions in a thermocycler at (**ARON, TWIST 2**);
    a. Step 1. 25°C for 5 minutes.
    b. Step 2. 42°C 1 hour.
    c. Step 3. 8°C for 5 minutes.
    d. Step 4. 4°C hold.
    e. Lid at 105°C

    This completes first strand synthesis (ssRNA → cDNA). Place plate or tubes on ice.

E.coli RNAse H

1. Add 1µL E.coli RNAse H to each sample on ice. Then incubate in the thermocycler at 37°C for 30 minutes, lid at should be off. (If the Lid>37°C, wait for it to reach <37°C. **ARON, TWIST 3**). *This step removes all RNA still left in the samples*.

Second strand synthesis

1. Make a new MM consisting of (per reaction);
    a. 18µL nuclease free H_2O
    b. 8µL of NEB second strand synthesis reaction buffer
    c. 4µL NEBNext second strand synthesis Enzyme mix
2. Add 30µL of the new MM to each tube of the **first strand synthesis** for a total of 81µL.
3. Incubate the **second strand synthesis solution** in the thermocycler at 16°C for 1 hour and move it to the bench (**ARON, TWIST 4)**.

    This completes second strand synthesis (cDNA → dsDNA).

    Note: Read **!** below

Purification 1

! Take out the DNA Purification beads and leave them on the bench until RT. **Before use, mix thoroughly**, and again after 2-4 samples.

! Prepare 80% Ethanol solution by adding 8mL Ethanol Absolut to 2mL MQ-H2O.

1. Take samples and transfer into new Eppendorf 1.5mL tubes.
2. Add 96µL of DNA Purifications beads to each **second strand synthesis solution** and incubate for 5 minutes RT.
3. Place samples on a magnetic plate for 1 minute, until supernatant is clear.
4. When the supernatant is clear, without removing the tube from the magnet, discard the supernatant without disturbing the pellet.
5. Wash the bead pellet by adding 200µL freshly prepared 80% ethanol (without disturbing the pellet), then incubate it for 1 minute. Remove and discard the Ethanol.
6. Repeat **Step 5** once for a **total of 2** washes, keeping the samples on the magnet.
7. Remove the remaining ethanol making sure you **do not disturb the bead pellet**.
8. Airdry the pellet on the magnetic plate for 5 minutes, **making sure not to overdry the samples**.
9. Remove the tubes from the magnetic plate and add 27µL of Nuclease free H2O to each sample.
10. Homogenize the samples by pipetting up-and-down.
11. Incubate at room temperature for 2 minutes.
12. Place the tubes on the magnet and let them stand for 3 minutes until they form a bead pellet.
13. Transfer 25µL of the clear supernatant containing the cDNA to a clean PCR 0.2mL strip-tube, making sure you do not disturb the pellet.

DNA fragmentation, end repair, and dA-Tailing.

1. Program the thermocycler and set the temperature of the heated lid to **70°C**. The conditions are as follows (**ARON, TWIST 5)**;
    a. Step 1 – 4°C, HOLD
    b. Step 2 – 32°C, 2 minutes
    c. Step 3 – 65°C, 30 minutes.
    d. Step 4 – 4°C, HOLD

2. Take 25µL of the cDNA from **Purification** Step 13 into 96-well PCR plate or strip-tubes, and place on ice.
3. Prepare **Enzyme Fragmentation MM** (E-MM) in an Eppendorf 1.5mL tube on ice with the following volumes (**per reaction)**;
    a. Nuclease free water – 10µL
    b. 10x Fragmentation buffer – 5µL
    c. 5x Fragmentation Enzyme – 10µL

    For a total volume of 25µL per reaction.

4. Add the E-MM to **each** 25µL cDNA sample (Step 2) and mix gently then centrifuge the samples.
5. Take plate/tubes to the pre-chilled thermocycler and activate the next cycle.
    a. Immediately prepare reagents for the next step!
    b. When you take out the plate, start "ARON, TWIST 6" to ensure low lid temperatures.

Ligation, Twist universal adapters

1. Create a **Ligation MM** by adding in a new 1.5mL tube on ice **(per reaction)**;
    a. Add 30µL of Ligation MasterMix.
    b. Add 17.5µL H_2O.

    For a total volume of 47.5µL per reaction.

2. Add 2.5µL TWIST universal adapters into each of the sample well/tube, containing the PCR products from Step 5 of **DNA fragmentation, end repair, and dA-Tailing**.
3. Mix well by pipetting and keep on ice.
4. Following, add 47.5µL of the **Ligation MM** from Step 1. Mix by gently pipetting.
5. Seal the tubes/plate and pulse-spin to ensure that the solution is at the bottom of the tube.
6. Incubate the ligation reaction in the thermocycler at (**ARON, TWIST 6)**;
    a. 20°C, 15 minutes
    b. The lid should be ~20°C and turned OFF.

Purification 2

1. Vortex the DNA purification beads until they are **WELL** mixed.
    a. *Optional:* Transfer the ligated sample to 1.5mL Eppendorf's, by first centrifugation at 1200rpm 30 seconds.
2. Add 80µL of homogenized DNA Purification beads to the ligated sample from previous Step 6 (**Ligation, Twist universal adapters**).
3. Mix well by pipetting up-and-down, **ALOT**.
4. Incubate the samples at 5 minutes RT.
5. Place the samples on a magnetic plate for 1 minute, or until clear.
6. Without removing the tubes/plate, remove and discard the supernatant **without disturbing the pellet**.
7. Wash the bead pellet by adding 200µL by adding freshly prepared 80% Ethanol.
8. Incubate for 1 minute.
9. Remove and discard the ethanol without disturbing the pellet.
10. Repeat the wash twice for a total of **3** washes.
11. Carefully remove the remaining ethanol without disturbing the pellet.
12. Airdry the pellet for 5 minutes, making sure not to overdry it.

13. Remove the tubes from the magnetic plate, and add 17µL of H_2O, mix by pipetting until homogenized.
14. Incubate at RT for 2 minutes.
15. Place the plate/tube on a magnetic plate, let it stand for 3 minutes until the beads form a pellet.
16. Transfer 15µL of the clear supernatant containing the ligated and indexed libraries to a clean well on the 96-well thermocycling PCR plate, making sure you **DO NOT** disturb the pellet. Immediately proceed to the next step.

PCR amplification using TWIST UDI primers, Purification, and QC

1. Program the thermocycler with the following conditions (**ARON, TWIST 7**);
    a. Step 1. 98℃ for 45 seconds.
    b. Step 2. 98℃ for 15 seconds, 60℃ for 30 seconds, 72℃ for 30 seconds, for 12 cycles.
    c. Step 3. 72℃ for 1 minute.
    d. Step 4. 4℃, HOLD.
    e. Lid at 105℃.

2. Add 10µL of TWIST UDI Primer provided in a 96-well plate to each of the cDNA libraries (15µL from step 16, **Purification 2)**, do not use perforated wells, and follow the numbering system of a normal PCR Plate.
3. Add;
    a. ddH2O - 41.9µL
    b. 10x High-Fidelity PCR Buffer – 5µL
    c. 10mM dNTP Mix – 1µL
    d. 50mM MgSO4 – 2µL
    e. Platinum TAQ Polymerase – 0.1µL

    *For a total of 50µL, for **TWO** samples in a MM.*

4. Add 25µL of the above MM to the cDNA libraries for a total of 50µL.
5. Pulse-spin the sample/tube and transfer to the thermocycler program described on Step 1.
6. Remove the samples from the block when the program is complete and proceed to Purification.
7. Vortex the DNA Purification beads until they are **mixed well**. Add 50µL of homogenized beads to each ligation sample from step 6.
8. Mix well by pipetting up-and-down.
9. Incubate for 5 minutes RT.
10. Place the samples on a magnetic plate for 1 minute, or until clear.
11. With the plates on the magnet, remove and discard the clear supernatant.
12. Gently wash the bead pellet by adding 200µL of freshly prepared 80% ethanol, without disturbing the pellet.
13. Incubate for 1 minute.
14. Remove and discard the ethanol.
15. Repeat the wash once, for a total of two washes while keeping the samples on magnets.
16. Carefully remove the remaining supernatant without disturbing the pellet.
17. Airdry the pellet for 5 minutes, **without overdrying the pellet**.
18. Remove the plates/tubes from the magnetic plate.
19. Add 22µL of H2O to each sample.
20. Mix until homogenized by pipetting.
21. Incubate at room temperature for 2 minutes.
22. Place the plates or tubes on a magnetic plate, wait 3 minutes until the beads form a pellet.

23. Transfer 20µL of the clear supernatant containing the amplified indexed libraries on the 96-well thermocycler plate, **without disturbing the pellet.**

Stop Point: *If you do not not intend to run the bioanalyzer next, store the supernatant in strip-tubes at -20°C, otherwise run the BioAnalyzer and store the samples in -20°C.*

24. Check the concentration of each sample via Qubit, and then perform quality control via BioAnalyzer.
    a. If the sample is more than 10ng/µL, dilute the sample.
    b. The average fragment size should be 200-450 bp.
    c. The average fragment size should be written down and saved for the calculations in the end of this protocol.

List and order of reagents for completion until the next stop point.

• Blocker Solution
• Universal Blockers
• Hybridization mix
• TWIST Fixed or Custom panel
• Hybridization enhancer

Hybridize captured probes with pools

1. Pool all samples into a single 1.5mL Eppendorf tube to create the **Library Pool**.
2. Pulse-spin the **Library Pool**.
3. Dry the **Library Pool** in a vacuum concentrator without heat. Check the samples every 30 minutes to ensure no overdrying.
    a. This could be a stopping point if you want – the dried library pool can be stored in -20°C for up to 24 hours.
    b. If not, immediately proceed to the next step.
4. Resuspend the dried **Library Pool** in
    a. 5µL Blocker solution and
    b. 7µL universal blockers.
5. Start a heating block to 65°C.
6. Preheat the Thermocyler to 95°C and the lid to 105°C (**ARON, TWIST 8)**.
7. Heat Hybridization mix **reagent** to 65°C in the heating block for 10 minutes, or until all precipitate has dissolved.
    a. Let the mix cool to RT before proceeding.
8. Prepare the **Probe solution** as follows in a 0.2mL tube;
    a. 20µL Hybridization mix
    b. 4µL TWIST Fixed or Custom panel.
    c. 4µL DNAse free water.
9. Mix the **Probe solution** by flicking the tube.
10. Heat the **Probe solution** in the thermal cycler at 95°C for 2 minutes, then immediately take out the tube and cool on ice for 5 minutes (**ARON, TWIST 8)**.
11. While the P**robe solution** is cooling on ice, put the resuspended **Library pool** in the thermal cycler at 95°C for 5 minutes, lid 105°C (**ARON, TWIST 8)**.
12. When 5 minutes has past, let the **Probe solution** and resuspended **Library pool** rest in room temperature for 5 minutes.
    a. While waiting, program the thermal cycler to 70°C HOLD with the lid at 85°C (**ARON, TWIST 9)**.

13. Vortex **only** the **Probe solution**, then pulse-spin both solutions.
14. Transfer 28uL of the **Probe solution** into the **Library Pool**. Mix by vortexing the tube, then pulse spin.
    a. Ensure there are no bubbles in the solution, pulse-spin until bubble-free.
15. Put the tube in the thermocycler, add 30uL Hybridization Enhancer to the reaction, carefully placing it on top of the liquid, and with no mixing.
16. Seal the tube tightly.
17. Incubate the **Hybridization Reaction** at 70°C for 16 hours in the thermocycler, with the lid at 85°C **(ARON, TWIST 9)**.
    a. Make sure you are back in the laboratory 1 hour before completion of the PCR.

List of reagents needed for completion.

• DNA Purification beads
• Streptavidin beads
• Equinox Amp mix (2x)
• Amplification primers
• Binding buffer
• Wash Buffer 1
• Wash Buffer 2
• Amplification, ILMN

Bind Hybridized targets to Streptavidin Beads.

1. One hour before the 16-hour PCR is done, take both DNA Purification beads and Streptavidin beads out from the fridge and place on the bench.
2. Set a heating block to 48°C.
3. Thaw the Equinox Amp mix (2x) and the amplification primers on ice.
4. Take 1mL Binding buffer and aliquot into a 1.5mL tube.
5. Take 300µl Wash Buffer 1 and aliquot into a 1.5mL tube.
6. Take 800 µL Wash Buffer 2 and aliquot into a 1.5mL tube.
7. Put all three tubes in the heating block at 48°C.
    a. Let sit until all precipitate is dissolved. When done;
    b. Take the Binding buffer and Wash Buffer 1 from the heating block and let cool down on the bench. **!** Leave Wash Buffer 2 in the heating block.
8. Vortex the Streptavidin beads, then add 100µL beads to a clean 1.5mL tube.
    a. Add 200uL Binding buffer to the beads and mix well by pipetting.
9. Place the tube on a magnetic stand for 1 minute. When the supernatant is clear, remove it with a pipette without disturbing the pellet.
10. Repeat this wash and mix with Binding buffer twice for a total of 3 washes.
    a. Ensure no Binding buffer is remaining, then;
11. Add 200uL Binding buffer and resuspend the pellet by pipetting.
12. When the 16-hour incubation at 70°C is done, **without removing the tube from the thermocycler**, carefully;
    a. Take all the **Hybridization reaction** and quickly add to the Streptavidin beads tube, and mix until homogeneous, **do not vortex**.
13. Place the tube on a shaker at RT for ~30 minutes, shaking enough to keep the solution mixed.
14. Remove the tube from the shaker and pulse-spin to get all the solution to the bottom, then place the tube on the magnetic stand and let sit for 1 minute, or until supernatant is clear.

Purification 3.

1. Remove the clear supernatant without disturbing the pellet. Remove the tube from the magnetic stand and add 200µL **room temperature** Wash Buffer 1.
2. Mix by pipetting until homogeneous.
3. Pulse spin to ensure all liquid is at the bottom.
4. To get rid of background binding, transfer the **Hybridization Reaction** and beads to a new 1.5mL Eppendorf Tube.
5. Place the new tube on the magnetic stand and let sit for 1 minute, or until supernatant is clear.
6. Remove and discard the clear supernatant with a pipet without disturbing the pellet. Remove the tube from the stand.
7. Add 200µL of the 48°C Wash Buffer 2, to the pellet. Mix by pipetting, then pulse spin the tube.
8. Incubate at 48°C for 5 minutes, then place on the magnetic stand for 2 minutes, or until supernatant is clear.
    a. The beads will stick to the walls making the supernatant possibly not clear, be careful when pipetting **not to touch the walls** of the tube.
9. Remove and discard the supernatant without disturbing the pellet.
10. Repeat the washing twice for a total of **3** washes.
11. Pulse-spin the dry tube to ensure all liquid is at the bottom.
12. Place the tube on the magnetic stand, with a 10µL pipette;
    a. Remove the remaining supernatant without disturbing the pellet.
    b. Proceed immediately to the next step, **do not** let the beads dry.
13. Immediately remove the tube from the magnetic stand and add 45µL DNAse free H_2O. Mix by pipetting up and down. Place on ice.
    a. This solution will be referred to as the Streptavidin Binding Bead Slurry**,** or just **Slurry**.

Post-capture PCR amplify, purify, and perform QC.

Prepare the beads, thermal cycler, and PCR mi

1. Program the thermal cycler to the following (**ARON, TWIST 10)**;
    a. 98°C for 45 seconds.
    b. 98°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, for 16 cycles.
    c. 72°C for 1 minute.
    d. 4°C HOLD.
2. Make sure the **Slurry** is thoroughly mixed. Otherwise, mix well by pipetting.
3. Take 22.5µL of the **Slurry** and transfer into a 0.2mL PCR-tube.
    a. The extra **Slurry** should be frozen and stored at -20°C.
4. Prepare a PCR MM accordingly directly **into** the **Slurry**:
    a. 22.5µL **Slurry** (Already in the tube)
    b. 2.5µL Amplification, ILMN
    c. 25µL Equinox Library Amp Mix (2x)
    d. The total volume is now 50µL, mix well by pipetting.
5. Pulse-spin the tube and transfer to the thermocycler and start the program (**ARON, TWIST 10**). When the program is done, proceed immediately to purification.
    a. The mixture will now be referred to as **Capture Reaction**.

Purification 4

1. Vortex the room-temperature DNA purification beads until well mixed.

2. Add 50µL DNA Purification beads to the **Capture Reaction**. Mix by pipetting.
    a. The streptavidin and DNA binding beads are both in the solution.
3. Incubate in room temperature for 5 minutes, then place on a magnetic holder. Wait for 1 minute, or until the supernatant is clear.
4. With the tube still on the magnet, remove and discard the supernatant without disturbing the pellet.
5. Add 200µL freshly prepared 80% ethanol without disturbing the pellet.
6. Let sit for 1 minute, then remove and discard the supernatant without disturbing the pellet.
7. Repeat the wash once for a total of **2** washes.
8. Carefully remove any remaining ethanol with a 10µL pipette, without touching the pellet.
9. Open the lid and let the pellet dry on the magnetic stand for 5 minutes.
10. Remove the **Capture Reaction** from the magnet and add 32µL DNAse free water.
    a. Mix by pipetting.
11. Incubate in room temperature for 2 minutes, then place the tube on the magnetic holder, wait 1 minute, or until the supernatant is clear.
12. With the tube on the magnetic holder, transfer 30µL of the clear supernatant containing the enriched libraries to a clean 1.5mL Eppendorf tube, carefully without disturbing the pellet.
13. Read the concentration on the Qubit. Calculate the nM of the sample, which has to be above 4 for Illumina MiSeq, according to the equation provided below.

$$\frac{[Concentration\ of\ the\ sample]}{660 * (average\ fragment\ size)} 10^6 = nM\ (nanomolar)$$