Standard operating procedure for stranded, ribodepleted RNA-seq library preparation from total RNA

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RNA-seq library preparation

- *Stranded, ribodepleted and indexed RNA-seq libraries were prepared from 1 µg total RNA using the TruSeq Stranded Total RNA Ribo-Zero H/M/R Gold Kit and TruSeq RNA Single Indexes Sets A and B (Illumina) and subjected to paired-end sequencing (2 x 100 bp) in a multiplexed design on a HiSeq 2500 Sequencing System (Illumina).*
- *The protocol was adapted according to the TruSeq Stranded Total RNA Sample Preparation Guide (Illumina), LS (Low Sample) protocol option.*

Samples

- Total RNA samples with average RIN values ranging from 7.9 to 8.8 depending on the tissue type were used for RNA-seq library preparation.

Equipment

- HiSeq 2500 Sequencing System plattform (Illumina).
- Biometra Thermocycler T1
- DNA/RNA UV Cleaner UVC/T-M-AR (Biosan)
- Vortex Genie 2 (Scientific Industries)
- Benchtop microcentrifuge (Hettich, Centrifuge Mikro 120)
- 96 well plate centrifuge (Hettich, Centrifuge 32)
- Agilent 2100 Bioanalyzer (Agilent Technologies)
- NanoDrop 1000 spectrophotometer (PeqLab, VWR)
- Qubit fluorometer (Invitrogen, Thermo Fisher Scientific)
- Magnetic Stand 96 (Invitrogen, Cat # AM10027, Thermo Fisher Scientific)

Plastics

- 96-well 0.3 ml PCR plates, non-skirted (Abgene, Cat # AB0600, Thermo Fisher Scientific)
- Basic adhesive seals for 96 well plates (Abgene, Cat # AB0580, Thermo Fisher Scientific)
- Adhesive PCR plate seals (Abgene, Cat # AB0558, Thermo Fisher Scientific)
- RNase/DNase-free tubes

Kits & Chemicals

- TruSeq Stranded Total RNA Ribo-Zero H/M/R Gold Kit (Illumina, Cat # 20020598)
- TruSeq RNA Single Indexes Set A and B (Illumina, Cat # 20020492 and Cat # 20020493)
- HiSeq PE Cluster kit v4 –cBot (Illumina, Cat # PE-401-4001)
- HiSeq SBS kit v4, 50 cycles (Illumina, Cat # FC-401-4002)
- Quant-it Qubit dsDNA HS Assay kit (Invitrogen, Cat # Q32854Thermo Fisher Scientific)
- SuperScript II reverse transcriptase (Invitrogen, Cat # 18064-014**,** Thermo Fisher Scientific)
- Agilent RNA 6000 Nano kit (Agilent Technologies, Cat # 5067-1511)
- Agilent DNA 1000 kit (Agilent Technologies, Cat # 5067-1504
- Freshly prepared 70% Ethanol
- Freshly prepared 80% Ethanol
- RNAClean XP Beads (Beckman Coulter, Cat # A63987)
- AMPure XP Beads (Beckman Coulter, Cat # A63881)
- EB Buffer, 10 mM Tris-HCl, pH8.5 (Qiagen, Cat # 9086) with 0.1% Tween 20
- Roti Nucleic acid free, solution for removal of nucleic acid contamination from surfaces (Roth, Cat # HP69.1)
- Ultra Pure nuclease-free Water

Ribo-Zero Deplete and Fragment RNA

- *This process depletes the cytoplasmic mitochondrial ribosomal RNA (hereinafter rRNA) from the total RNA (total RNA).*
- *After rRNA depletion, the remaining RNA is purified, fragmented and primed for cDNA synthesis.*

Preparations

1. Remove the following components from -15°C to -25°C storage and allow to thaw at room temperature:

- Elute, Prime, Fragment High Mix
- rRNA Removal Mix - Gold
- rRNA Binding Buffer
- Resuspension buffer (can be stored at 2-8°C after thawing)

2. Remove the following components from the 2°C to 8°C storage and bring them to room temperature:

- Elution Buffer
- rRNA Removal Beads
- RNA Clean XP beads (let stand for at least 30 min).

3. Pre-program the thermal cycler

- Set the heating lid of the cycler to 100 °C.
- Program the cycler:
  - 68°C, 5 min >save as RNA denaturation,
  - 94°C 8 min, 4°C Hold > save as Elution 2 - Frag - Prime

4. Set the temperature of the centrifuge to 15-25°
5. Apply the BRP, DFP, RCP, RRP barcode labels on new 0.3 ml PCR plates

Prepare the BRP plate (Bind rRNA Plate, binding the rRNA)

1. Dilute the total RNA with nuclease-free ultra pure water to a final volume of 10 µl in the 96-well 0.3 ml PCR plate with the BRP barcode, i.e. for 1 µg in 10 µl = 0.1 µg/µl initial concentration.
2. Add 5 µl rRNA Binding Buffer RBB into each well of the BRP plate.
3. Add 5 µl of rRNA Removal Mix Gold RRMG into each well of the BRP plate. Carefully mix the entire volume in each well of the BRP plate, pipette up and down for 6 times to mix thoroughly.
4. Seal the BRP plate with adhesive seal
5. Place the remaining rRNA Binding Buffer RBB and rRNA Removal Mix Gold RRMG back to storage of -15°C to -25°C
6. Place the sealed BRP plate in the pre-programmed thermal cycler, close the lid and start the RNA Denaturation Program.
7. Incubate the BRP plate at room temperature for 1 min.

Prepare the RRP plate (rRNA removal plate, rRNA depletion)

1. Vortex the rRNA Removal Bead RRB Tubes equlibrated at room temperature vigorously to resuspend the beads
2. Add 35 µl rRNA Removal Beads RRB into each well of the 96-well 0.3 ml PCR plate with the RRP label.

- NOTE: This step should not be omitted! It is important for an optimal reaction that the samples from the BRP plate are added to the beads and not vice versa!

3. Remove the adhesive seal from the BRP plate
4. Transfer the total volume (20 µl) from each well of the BRP plate into a corresponding well of the RRP plate containing the rRNA Removal Beads RRB
5. Set the pipette to 45 µl, then with the pipette tip to the bottom of the wells quickly pipette 20 times to mix thoroughly
- Caution: Fast thorough mixing is important, insufficient mixing may compromise depletion efficiency.
- To prevent foaming, pipette with the tip of the pipette at the bottom of the well, and do not pipette the entire volume. Too much foam formation leads to sample loss!
6. Incubate the RRP plate at room temperature for 1 min.
7. Place the RPP plate in the magnetic stand for 1 min.
8. Transfer the total supernatant from each well of the RRP plate to a corresponding well of the 96-well 0.3 ml PCR plate labelled RCP (Caution: do not touch beads!).
9. Place the RCP plate in the magnetic stand for 1 min.
- If any beads have remained in the wells of the RCP plate, place the RCP plate in the magnetic position for 1 min, then transfer the supernatant into a new 0.3 ml plate. Repeat this as often as necessary until no beads are present anymore. The last of these plates is then the RCP plate used for Clean Up RCP.
10. Return the rRNA Removal Beads to 2°C to 8°C storage

Clean up of the RCP plate (RNA clean up plate, purification of the depleted RNA)

1. Vortex the RNAClean XP beads until they are well dispersed, then add 99 µl of the well mixed RNAClean XP beads into each well of the RCP plate containing the depleted RNA. Mix the entire volume thoroughly by up and down pipetting (10 times)
- NOTE: In case of degraded total RNA, add 193 µl of the well mixed RNAClean XP beads into each well of the RCP plate
2. Incubate the RCP plate for 15 min at room temperature
3. Place the RCP plate in the magnetic stand for 5 min at room temperature, be sure that all beads are bound to the side of the wells
4. Remove and discard the supernatant from each well of the RCP plate
- Leave the RCP plate in the magnetic stand while the following washing steps are performed with 70% Ethanol.
5. Add 200 µl freshly prepared 70% Ethanol to each well without swirling the beads
6. Incubate the RCP plate at room temperature for 30 sec in the magnetic stand, then remove and discard all of the supernatant from each well. Caution: Do not touch the beads!
7. Let the RCP plate dry at room temperature for 15 min in the magnetic stand, then remove it from the magnetic stand
8. Centrifuge the thawed elution buffer ELB (room temperature) at 600xg for 5 sec.
1. Add 11 µl Elution Buffer ELB into each well of the RCP plate. Mix the entire volume gently by up-down pipetting (10x)
9. Incubate the RCP plate at room temperature for 2 min.
10. Place the RCP plate in the magnetic stand for 5 min.
11. Return the elution buffer ELB to the 2°C to 8° C storage
12. Transfer 8.5 µl of the supernatant from each well of the RCP plate into a corresponding well of a new 96 well 0.3 ml PCR plate labelled with the DFP bar code
13. Add 8.5 µl Elute, Prime, Fragment High Mix EPH in each well of the DFP plate. Pipette gently up-down to mix the entire volume, 10x. (Elute, Fragment, Prime, High Mix contains random hexamers for the RT and serves as 1st strand cDNA synthesis buffer)
14. Sealing the DFP plate with adhesive seal
15. Return Elute, Prime, Fragment High Mix EPH to the -15°C to -25°C storage and the RNAClean XP Beads to 2°C - 8°C storage

Incubation of the DFP plate (1) - (depleted RNA fragmentation plate, fragmentation of depleted RNA)

1. Place the sealed DFP plate in the pre-programmed thermocycler, close the lid and start the program Elution 2 - Frag - Prime.
   - For inserts >120-200 bp with a mean size of 150 bp or if degraded RNA is present, other program conditions must be selected!
2. Remove the DFP plate from the cycler when 4°C has been reached and centrifuge briefly
3. Continue immediately with Synthesize First Strand cDNA

Synthesis of First strand cDNA

- *The RNA fragments are reverse transcribed with random hexamer primers in first strand cDNA.*
- *The addition of Actinomycin D (First Strand Synthesis Act D mix, FSA) prevents interfering DNA-dependent synthesis during RNA-dependent synthesis, which improves strand specificity. CAUTION: FSA contains Actinomycin D (collect waste separately); Actinomycin D is VERY TOXIC*

Preparations

- Thaw First Strand Synthesis Act D Mix FSA at room temperature
- Program the thermal cycler: > save as Synthezise 1st Strand
  - 25°C, 10 min;
  - 42°C 15 min,
  - 70 °C 15 min,
  - hold 4°C
- Preheating the lid of the cycler to 100 °C

Add FSA

1. Remove the adhesive seal from the DFP plate
2. Centrifuge the thawed First Strand Synthesis Act D Mix FSA briefly for 5 sec at 600 g
3. Add 50 µl SuperScript II reverse transcriptase (SS II) to the FSA. If the entire FSA mix is not required, pipette 1 µl SS II to 9 µl FSA. Mix gently but thoroughly and centrifuge briefly. Mark on the FSA tube that SS II has been added.

- NOTE: If SS II has been added to FSA, this solution must not be thawed more than 6 times. However, as more than 6 thawing cycles are likely to occur, aliquots must be taken beforehand and the aliquots stored at -15 to -25°C.

4. Add 8 µl FSA with SS II into each well of the DFP plate. Mix the entire volume gently by up-down pipetting (6x)
5. Seal the DFP plate with adhesive seal and centrifuge briefly.
6. Return FSA Mix Tube and SS II immediately after use to-15°C to -25°C storage

Incubation of the DFP Plate (2)

1. Insert the DFP plate into the pre-programmed thermal cycler, close the lid and start the Synthesize 1st Strand program.
2. When the cycler has reached 4°C, remove the DFP plate and continue <u>immediately</u> with Synthesize Second Strand cDNA

Synthesis of Second Strand cDNA

- *The RNA template is removed and a replacement strand is synthesized by inserting dUTP instead of dTTP, ds cDNA is formed.*
- *The dUTP integration quenches the secondary strand during amplification, because the polymerase stops synthesis after this nucleotide. Ampure XP beads are used to separate the ds cDNA from the 2nd strand reaction mix.*
- *At the end of the process blunt-ended cDNA is present.*

Preparations

- Thaw Second Strand Marking Master Mix SSM, Resuspension buffer RSB and End repair control CTE at room temperature.
- Allow AMPure XP beads to equilibrate to room temperature for at least 30 minutes.
- Set the thermal cycler to 16 °C
- Preheat the cycler lid to 30°C
- Apply the ALP (Adapter Ligation Plate) barcode label to anew 0.3 ml PCR plate

Add SMM

1. Remove the adhesive seal from the DFP plate
2. When using the Inline Control Reagent:
- Centrifuge the thawed End Repair Control CTE Tubes at 600 xg for 5 min
- Dilute the CTE to 1/50 in Resuspension Buffer RSB before use (e.g. 2 µl CTE + 98 µl RSB). Remaining diluted RSB is discarded after use.
- Pipette 5 µl diluted End Repair Control CTE into each well of the DFP plate.
3. Centrifuge the thawed Second Strand Marking Master Mix SSM briefly for 5 sec at 600 g
4. Add 20 µl Second Strand Marking Master Mix SSM into each well of the DFP plate, carefully mix 6x with the pipette
5. Seal the DFP plate with adhesive seal
6. Return remaining Second Strand Marking Master Mix SSM to -15 to -25°C storage after use

Incubation of the DFP Plate (3)

1. Place the DFP plate in the preheated thermal cycler, close the lid and incubate for 1 h at 16°C.
2. Remove the DFP plate from the cycler and place it on the laboratory bench
3. Remove the adhesive seal from the DFP plate
4. Allow the DFP plate to equilibrate to room temperature

Clean Up of the DFP plate

1. Vortex AMPure XP beads until they are fully dispersed
2. Pipette 90 µl well-mixed AMPure XP beads into each well of the DFP plate (which already contains 50 µl of cDNA), mix the whole volume carefully but thoroughly with the pipetting up-down, 10x.
3. Incubate the DFP plate at room temperature for 15 min.
4. Place the DFP plate in the magnetic stand, incubate for 5 min at room temperature until all beads are adhered to the side of the wells
5. Remove 135 µl supernatant from each well and discard, caution: do not touch beads!
   - Leave DFP plate in magnetic stand during subsequent washing steps with 80% Ethanol
6. Add 200 µl freshly prepared 80% Ethanol to each well of the DFP plate without swirling the beads
7. Incubate DFP plate for 30 sec at room temperature
8. For second 80% Ethanol-Wash: repeat steps 6 and 7

9. Allow the DFP plate to dry at room temperature for 15 min, then remove the plate from the magnetic stand
10. Centrifuge Resuspension Buffer RSB for 5 sec at 600xg
11. Add 17,5 µl Resuspension Buffer RSB into each well of the DFP plate, mix gently but thoroughly, 10x with pipetting up-down
12. Incubate the DFP plate for 2 min at room temperature
13. Place the DFP plate in the magnetic stand and incubate for 5 min at room temperature.
14. Transfer 15 µl of the supernatant (ds cDNA) from the wells of the DFP plate into a corresponding well of a new 0.3 ml 96-well plate labeled ALP (Adapter Ligation Plate)

SAFE STOPPING POINT

The protocol can be safely interrupted at this point. Seal the ALP plate with adhesive seal. It can be stored at 15- 25 °C for up to 7 days.

Adenylation of 3' ends

- *A single A nucleotide is attached to the 3' ends of blunt-end fragments to prevent self ligation and ligation with other fragments (concatenated artefacts).*
- *A corresponding T nucleotide at the 3' end of the adapter provides the complementary overhang for the ligation of the adapter to the fragment.*
- *This strategy reduces chimera formation.*

Preparations

- Thaw A-Tailing Mix ATL and A-Tailing Control CTA at room temperature, divide into application aliquots and freeze
- Bring Resuspension Buffer RSB from 4°C to room temperature
- Thaw the frozen ALP plate at room temperature and centrifuge for 1 min at 280xg, remove the adhesive seal.
- Pre-program the thermal cycler: Preheat the lid to 100°C, 37°C 30 min, 70°C 5 min, hold 4°C > save as ATail70,

Add and adenylate ATL (A Tailing Mix)

1. Spin the A-Tailing Control CTA 5 sec at 600 xg for 5 sec, dilute immediately before use: 1/100 (1 µl CTA + 99 µl Resuspension Buffer RSB). Discard the remaining diluted CTA after use.
2. Add 2,5 µl diluted A-Tailing Control (or Resuspension Buffer if no CTA is used) into each well of the ALP plate
3. Add 12.5 µl A-Tailing Mix ATL into each well of the ALP plate, with the pipette set to 30 µl and mix 10x by up-down pipetting
4. Seal the ALP plate with adhesive seal
5. PLace the ALP plate into the thermal cycler and start the pre-programmed program ATail70
6. When 4°C is reached, remove the ALP plate from the cycler and proceed <u>immediately</u> with Ligate Adapters

Ligation of Adapters

- *Ligation of the multiple Index Adapter to the ends of the ds cDNA*

Preparations

- Thaw selected RNA Adapter Index Tube and one Tube Stop Ligation Buffer STL as well as Ligations Control CTL at room temperature
- Split and freeze Ligase Control into application aliquots. DNA-Ligase Mix LIG in application aliquots and freeze, LIG should not be taken out of the freezer before the instructions of the protocol require it.
- Bring the Resuspension Buffer RSB to room temperature.
- Allow AMPure XP Beads to equilibrate for at least 30 min at room temperature.
- Preheat the thermal cycler to 30 °C, preheat lid to 100 °C
- Apply CAP (Clean up ALP Plate) Barcode label to a new 0.3 ml PCR plate
- Apply PCR Barcode label to a new 0.3 ml PCR plate

Add and ligate LIG (Ligation Mix)

1. Centrifuge RNA index adapter, Ligation Control CTL and Stop Ligation Control STL for 5 sec at 600 xg
2. Take the LIG Mix LIG out of -20°C storage immediately before use
3. Remove the adhesive seal from the ALP plate
4. Dilute Ligation Control CTL 1/100 (1 µl Ligase Control + 99 µl Resuspension Buffer) immediately before use. Discard remaining dilution after use.
5. Add 2.5 µl diluted Ligation Control CTL (or Resuspension Buffer) into each well of the ALP plate
6. Add 2.5 µl Ligation Mix LIG into each well of the ALP plate,
7. Immediately after use return LIG Mix to -20°C storage
8. Add 2.5 µl RNA Index Adapter into each well of the ALP plate, mixing 10x with setting the pipette to 40 µl
9. Seal the ALP plate with adhesive seal
10. Centrifuge the ALP plate 1 min at 280xg

Incubate ALP (ligate adapters)

1. Incubate the ALP plate for 10 min at 30 °C in a preheated thermal cycler
2. Remove the ALP plate from the cycler

Add STL (Stop Ligation control, inactivation of ligation)

1. Remove the adhesive seal from the ALP plate
2. Pipette 5 µl Stop Ligation Buffer STL into each well, carefully mix the entire volume 10x with the pipette

Clean-up ALP (purification of the ligated fragments, separation of the remaining adapters)

1. Vortex AMPure XP Beads thoroughly for at least 1 min until they are well dispersed
2. Add 42 µl well mixed AMPure XP beads into each well, mix the whole volume 10x with the pipette
3. Incubate the ALP plate at room temperature for 15 min.
4. Place the ALP plate in the magnetic stand for at least 5 min until the liquid is clear
5. Carefully remove 79.5 µl supernatant from each well of the ALP plate and discard (caution: do not swirl up beads), the ALP plate remains in the magnetic stand!
6. Add 200 µl freshly prepared 80% Ethanol into each well, do not swirl the beads!
7. Incubate the ALP plate at room temperature for at least 30 sec, then remove all supernatant from wells, do not swirl the beads!

8. Repeat washing steps 6 and 7
9. Allow the ALP plate to dry in the magnetic stand for 15 min
10. Remove the ALP plate from the magnetic stand
11. Add 52,5 µl Resuspension Buffer RSB into each well, mix the entire volume with the pipette (10x) until the beads are completely resuspended
12. Incubate the ALP plate at room temperature for 2 min.
13. Place the ALP plate back in the magnetic stand and incubate at room temperature for at least 5 min until the liquid is clear
14. Add 50 µl supernatant from each well of the the ALP plate into the corresponding well of the new PCR plate labelled with CAP barcode, be careful not to swirl up the beads.
15. Vortex the AMPure XP beads thoroughly until they are well dispersed
16. Add 50 µl of well mixed AMPure XP beads into each well of the CAP plate for a second clean-up, carefully mix the entire volume 10x with the pipette.
17. Incubate the CAP plate at room temperature for 15 min.
18. Place the CAP plate in the magnetic stand for at least 5 min until liquid is clear
19. Remove 95 µl supernatant from each well of CAP plate (leave in magnet position) and discard (Caution: do not swirl up the pellet).
20. Add 200 µl freshly prepared 80% Ethanol, do not swirl up the pellet.
21. Incubate CAP plate for at least 30 sec at room temperature in the magnetic stand, remove all supernatant, do not swirl up pellet
22. Repeat washing steps 20-21
23. Let the CAP plate dry in the magnetic stand for 15 min, then remove it from the magnetic stand
24. Add 22.5 µl Resuspension Buffer RSB into each well of the CAP plate, carefully mix with pipette the entire volume (10x) until the beads are completely resuspended
25. Incubate the CAP plate for 2 min at room temperature
26. Incubate the CAP plate in the magnetic stand at room temperature for at least 5 min until the liquid is clear
27. Transfer 20 µl supernatant from each well of the CAP plate into a corresponding well of a new PCR plate, caution: do not swirl beads.

SAFE STOPPING POINT

The protocol can be safely interrupted at this point. Seal the PCR plate with adhesive seal. It can be stored at 15- 25 °C for up to 7 days.

Enrichment of DNA Fragments

- *Selective enrichment of DNA fragments with adaptors at both fragment ends and library amplification by PCR.*
- *The number of cycles should be minimized to avoid bias in library representation.*

Preparations

- Thaw the PCR Master Mix PMM and PCR Primer Cocktail PPC at room temperature and centrifuge both for 5 sec at 600 g.
- Bring the resuspension buffer RSB to room temperature.
- Allow AMPure XP Beads to equilibrate for at least 30 min at room temperature.
- If the PCR plate has been frozen, thaw it at room temperature, then centrifuge for 1 min at 280xg, remove the adhesive seal.
- Pre-program the thermal cycler: >save under PCR
  - Heating cover at 100 °C
  - 98°C 30 sec denaturation
  - 15 cycles: 98°C, 10 sec- 60°C, 30 sec- 72°C, 30 sec-
  - 72°C, 5 min
  - Hold 4°C
- Apply TSP1 (Target sample plate) barcode label to a new 0.3 ml PCR plate

Preparing the PCR

1. Pipette 5 µl PCR Primer Cocktail into each well of the PCR plate.
2. Add 25 µl PCR Master Mix to each well of the PCR plate, mix 10x with a pipette set to 40 µl
3. Seal the PCR plate with adhesive seal
4. Place the PCR plate into the cycler, start the PCR program

Clean-up PCR (purification)

1. Remove the adhesive seal from the PCR plate
2. Vortex AMPure XP beads thoroughly until they are well dispersed
3. Add 50 µl well mixed AMPure XP beads into each well containing 50 µl of the amplified library; mix the entire volume 10x gently, but thoroughly with the pipette.
4. Incubate PCR plate at room temperature for 15 min.
5. Let the PCR plate stand in the magnetic stand for at least 5 min at room temperature until the liquid is clear
6. Remove 95 µl supernatant from each well of the PCR plate (in the magnetic stand) and discard Caution: do not swirl up pellet.
7. Add 200 µl freshly prepared 80% Ethanol, do not swirl up the pellet.
8. Incubate the PCR plate at room temperature for at least 30 sec in the magnetic stand, remove all supernatant, do not swirl up the pellet
9. Repeat washing steps 7-8
10. Let the PCR plate dry for 15 min in the magnetic stand, then remove it from the magnetic stand
11. Add 32,5 µl Resuspension Buffer RSB into each well of the PCR plate, carefully mix with pipetting the entire volume (10x) until the beads are completely resuspended
12. Incubate the PCR plate for 2 min at room temperature
13. Incubate the PCR plate in the magnetic stand at room temperature for at least 5 min until the liquid is clear
14. Transfer 30 µl of the clear supernatant from each well of the PCR plate to a corresponding well of the TSP1 plate, caution: do not swirl beads.

SAFE STOPPING POINT

The protocol can be safely interrupted at this point. Seal the TSP1 plate with adhesive seal. It can be stored at 15- 25 °C for up to 7 days.

Validation of the library

- Quantification of the library on the Qubit fluorimeter by analysing 1 µl of resuspended library using the Quant-it Qubit DNA HS assay kit
- Quality control of the library on the Agilent Bioanalyzer by analysing 1 µl of resuspended library on the Bioanalyzer 2100 with the Agilent DNA-1000 Assay kit

Normalization and Pooling of the libraries

- Generation of DNA templates for cluster formation.
- Multiplex libraries are normalized to 10 nM in the DCT (Diluted Cluster Template) plate and then equal volumes are pooled in the PDP (Pooled DCT plate) plate.

Preparations

- Apply the DCT Barcode Label on the 96 well MIDI plate
- Apply the PDP Barcode Label to the 96 0.3 ml PCR Plate
- Thaw the TSP1 plate at room temperature, centrifuge for 1 min at 280xg, remove the adhesive seal

Prepare the DCT plate

1. Transfer 10 µl sample of the library from each well of the TSP1 plate to a corresponding well of the DCT plate.
2. Normalize the concentration of the Sample Library in each well of the DCT plate to 10 nM: Dilute with EB Buffer (10mM Tris-Cl, pH 8.5) containing 0.1% Tween 20, mix well by pipetting 10 times up and down
   - NOTE: Depending on the concentration of the Sample Library, the final volume of the normalized Sample Library may be 10-400 µl!
   - For calculating of required volume: Use the Conversion_microgram_nmol.xls, LibraryDilutionCalculator_AI_043007.xls
3. Proceed further depending on the library type:
   - Non-pooled library: Protocol ends here, continue with cluster generation, seal DCT plate with adhesive seal, store at -15 to -25°C.
   - Pooled Library: Prepare the PDP plate

Prepare the PDP plate

1. Determine the number of samples (sample libraries) to be combined in a pool
2. Pool 2-24 samples: transfer 10 µl of each normalized library to be pooled into a well of the 0.3 ml PDP plate. The total volume in each well of the PDP plate is 10 times the number of combined libraries (20-240 µl).
3. Mix well with pipetting 10x
4. Seal the PDP plate with adhesive seal, freeze at -15 to -25°C

Normalizing and pooling in tubes

1. Transfer 10 µl sample Library from TSP1 plate to a 0.5 ml tube
2. Normalize the sample library to 10 nM: dilute with Tris-Cl 10mM, pH8.5 with 0.1% Tween 20, mix well with a pipette, mix thoroughly, 10x
3. Freeze the tubes at -15 to -25°C if no multiplexing occurs

4. For multiplexing: add 10 µl of each normalized library to a new tube (ATTENTION: do not add two libraries with the same index in the same tube), mix the entire volume 10x with the pipette, freeze the tube at -15 to -25°C

Sequencing of the libraries

The RNA-seq libraries were subjected to paired-end sequencing (2 x 100 bp) in a multiplexed design on a HiSeq 2500 Sequencing System (Illumina). The HiSeq PE Cluster kit v4 –cBot and TruSeq SBS kit v4 were used following the manufacturer's protocols. Samples are multiplexed with 12 samples per lane. Each sample is run on two separate lanes.