RNA_3v2

GENERATING STRAND-SPECIFIC cDNA LIBRARY FOR NGS SEQUENCING (Transcriptome Analysis)

Protocol written by Sabine Stöcker & Ramón Ramos Barrales. Mod. By LC Feb 2017

1. Preparation of RNA

For the detailed RNA preparation, please refer to the first part of the "*Pombe* total RNA extraction and RT-qPCR protocol" (Version 3, 10.7.2013)

Day 1-3 Growth & Harvest

Day 1: Set up pre-cultures. Grow for 1 day at 30°C.

Day 2: Set up main cultures (25ml, OD_{600}(start) = 0.02 – 0.05). Grow for 12-16h at 30°C.

Day 3: Harvest the cultures (OD_{600} = 0.4 – 0.8), wash the cells in H_2O and freeze down in liquid nitrogen. Store at -80°C.

Day 4-5 RNA extraction and DNase treatment

Day 4 RNA extraction: Resuspending the cells in TRIzol and breaking the cells up with the Precyllis 24 (Peqlab). The DNA and RNA are purified from the cell lysate in two "chloroform" washes. Afterwards they are precipitated with isopropanol and the pellet is washed 2x in ethanol. Finally the pellet is resuspended in 100µl of Nuclease free H_2O and can be stored at -80°C or immediately used for the DNase treatment.

Day 5 DNase treatment: Determine the RNA concentration using the Nano-Drop and dilute 20µg of RNA in 36µl of H_2O. Perform the DNase treatment by adding the TURBO DNA-free 10x buffer and DNase I to the diluted samples. The reaction is stopped after 1 hour by adding the TURBO DNase inactivation reagent. The samples can be stored at -80°C or directly used for the transcriptome analysis.

2. RNA library preparation

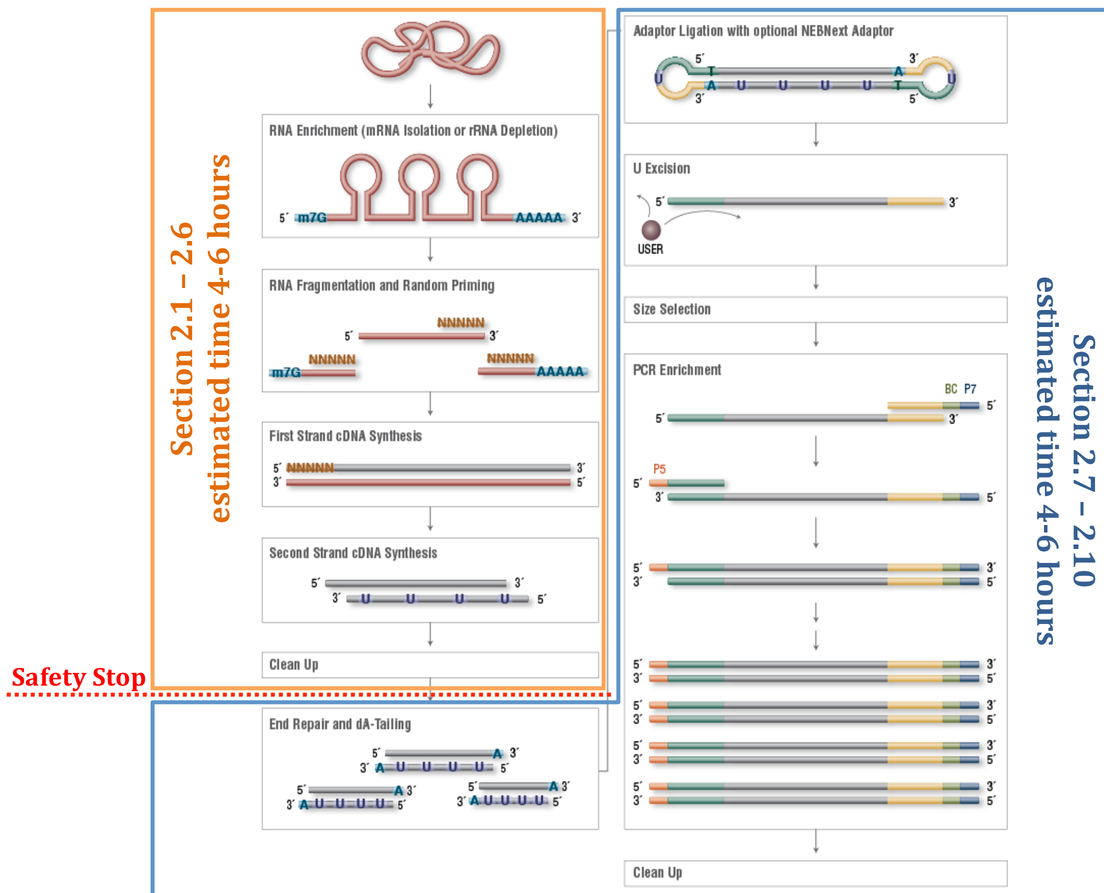
The following protocol is optimized from the *“NEBNext®Ultra™Directional RNA Library Prep Kit for Illumina®”* (#E7420) instruction manual, using the **High-Fidelity 2x Master Mix** (Section 2).

If you are using the **Hot Start HiFi PCR Master Mix**, please make sure to adjust the settings of the final PCR enrichment (Section 2.9). For the adjustments please refer to Appendix A.

Note: The protocol is optimized for approximately 200 bp RNA inserts. To generate libraries with longer RNA insert sizes, please refer to the original manual of the *“NEBNext®Ultra™Directional RNA Library Prep Kit for Illumina®”*.

Requirements:

- NEBNext®Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)
- NEBNext Multiplex (NEB #E7335)
- Magnetic rack + transparent tape (Tesa-Film)
- 80% EtOH (in Nuclease free H_2O)
- 0.1x TE, pH 8.0
- 10 mM Tris-HCl, pH 7.5 - 8.0
- Agencourt®AMPure®XP beads (Beckmann Coulter, Inc. #A63881)
- Actinomycin D dissolved in DMSO to 5 µg/µl (Sigma, #A1410)



2.1 RNA Sample Preparation

The RNA sample should be …
… free of salts (e.g., Mg^{2+}, or guanidinium salts)
… free of organics (e.g., phenol and ethanol)
… treated with DNase. The DNase should be removed after treatment.

1. Measure the RNA concentration using the NanoDrop
2. Dilute 1 µg of total RNA in 50µl of nuclease-free H_2O in a nuclease-free 0.2ml PCR tube
3. Keep the sample on ice

2.2 Preparation of First Strand Reaction Buffer and Random Primer Mix

Note: AMPure XP Beads are required throughout the protocol. Allow beads to reach room temperature prior to use. Redissolve them by rotating them in the cold room

Prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) as follows in a nuclease-free tube:

Component	Volume
• NEBNext First Strand Synthesis Reaction Buffer (5X)	8 µl
• NEBNext Random Primers Nuclease-free water	2 µl
Nuclease-free H_2O	10 µl
Total Volume	20 µl

Note: Keep the mix on ice.

2.3 mRNA Isolation, Fragmentation and Priming

Note: This step takes around 3 hours when handling 6 samples

1. Aliquot 15 µl of NEBNextOligo $d(T)_{25}$ beads into a nuclease-free 0.2 ml PCR tube.
2. Wash the beads by adding 100 µl of RNA Binding Buffer (2X) to the beads. Pipette the entire volume up and down 6 times to mix thoroughly.
3. Place the tubes on the magnetic rack (by taping it with the transparent tape on the magnetic rack) and incubate at room temperature for 2 minutes.
4. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads. Afterwards remove the tubes from the magnetic rack.
5. Repeat steps 2–4.
6. Resuspend the beads in 50 µl of RNA Binding Buffer (2X) and add the 50 µl of total RNA sample from Step 2.1.

Note: For the Steps 7 and 16 and 27 use the stored PCR program "*RNAseq_1_Isolation&Fragmentation*".

7. Place the tube on a thermal cycler and close the lid. Heat the sample at 65°C for 5 minutes and hold at 4°C to denature the RNA and facilitate binding of the poly-A mRNA to the beads. Remove the tube from the thermal cycler when the temperature reaches 4°C. Press continue!
8. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.
9. Place the tube on the magnetic rack and incubate at room temperature for 2 minutes to separate the poly-A mRNA bound to the beads from the solution.
10. Remove and discard all of the supernatant. Take care not to disturb the beads. Afterwards remove the tube from the magnetic rack.
11. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Pipette the entire volume up and down 6 times to mix thoroughly.
12. Place the tube on the magnetic rack at room temperature for 2 minutes.
13. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads. Afterwards remove the tube from the magnetic rack.
14. Repeat steps 11–13.
15. Add 50 µl of Tris Buffer (from kit) to each tube. Gently pipette the entire volume up and down 6 times to mix thoroughly.
16. Place the tube on the thermal cycler. Close the lid and heat the samples at 80°C for 2 minutes, then hold at 25°C to elute the Poly-A mRNA from the beads. Remove the tube from the thermal cycler when the temperature reaches 25°C. Press continue!
17. Add 50 µl of RNA Binding Buffer (2X) to the sample to allow the mRNA to re-bind to the beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.
18. Incubate the tube at room temperature for 5 minutes.
19. Place the tube on the magnetic rack at room temperature for 2 minutes.
20. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads. Afterwards remove the tube from the magnetic rack.
21. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.
22. Place the tube on the magnetic rack at room temperature for 2 minutes.
23. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads. Afterwards remove the tubes from the magnetic rack.
24. Wash the beads by adding 200 µl of Tris Buffer (from kit). Gently pipette the entire volume up and down 6 times to mix thoroughly.
25. Place the tube on the magnetic rack at room temperature for 2 minutes.
26. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads. Afterwards remove the tubes from the magnetic rack.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip remove all of the Tris Buffer. **Caution:** Do not disturb beads that contain the

mRNA.

27. Fragmentation - Elute mRNA from the beads by adding 15 µl of the First Strand Synthesis Reaction Buffer and Random Primer mix (2X) prepared in Section 2.2 incubating the sample at 94°C for 15 minutes. Immediately, place the tubes on the magnetic rack. In the meantime, you can prepare the first strand reaction mixture (2.4.2)

Note: For RNA insert sizes > 200nt, refer to the original manual of the "*NEBNext®Ultra™Directional RNA Library Prep Kit for Illumina®*".

28. Collect the purified mRNA by transferring 10 µl of the supernatant to a clean nuclease-free PCR Tube.
29. Place the tube on ice and proceed **directly** to first strand cDNA synthesis.

2.4 First Strand cDNA Synthesis

Note: This step takes around 1 hour when handling 6 samples

1. Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.

Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.

2. To the fragmented and primed mRNA (10 µl from above Section 1.2, Step 28) add the following components and mix by gentle pipetting:

Component	Volume
• Murine RNAse Inhibitor	0.5 µl
Actinomycin D (0.1 µg/µl)	5 µl
• ProtoScript II Reverse Transcriptase	1 µl
Nuclease free water	3.5 µl
Final volume	10 µl (+ previous 10 µl)

3. Incubate the sample in a preheated thermal cycler using the stored program "*RNAseq_2_First Strand Synthesis*".

Note: For longer RNA fragments (> 200 nt), please increase the incubation at 42°C from 15 minutes to 50 minutes in the PCR program of step 3.

4. Immediately, proceed to the second strand cDNA synthesis (section 2.5).

Note: Already start the stored "*RNAseq_3_Second Strand Synthesis"* program in the thermal cycler and leave the lid open to cool down (for the second strand Synthesis the lid heat should be ≤ 40°C). To allow for faster cooling down of the lid, fan it.

2.5 Perform Second Strand cDNA Synthesis

Note: This step takes around 1h15min when handling 6 samples

1. Add the following reagents to the First Strand Synthesis reaction (20 µl):

Nuclease-free water	48 µl
• Second Strand Synthesis Reaction Buffer (10X)	8 µl
• Second Strand Synthesis Enzyme Mix	4 µl
Total volume	80µl

2. Mix thoroughly by gentle pipetting.
3. Incubate the samples in a preheated Thermal Cycler using the stored program "*RNAseq_3_Second Strand Synthesis*".

2.6 Purify the Double-stranded cDNA using AgencourtAMPure XP Beads

Note: This step takes around 45min when handling 6 samples

1. Vortex AMPure XP Beads to resuspend.
2. Add 144 µl (1.8X) of resuspended AMPure XP Beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps. Quickly spin the tubes and remove all residual EtOH.
7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 60 µl of 10 mM Tris-HCl buffer. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack

until the solution is clear.

9. Remove 55.5 µl of the supernatant and transfer to a clean nuclease-free PCR tube.

Safety Stop: If you need to stop at this point in the protocol samples can be stored at –20°C. Or directly proceed to 2.7.

2.7 Perform End Prep of cDNA Library & Adaptor Ligation

Note: This step and step 2.8 take around 2.5 hours when handling 6 samples

Note: AMPure XP Beads are required throughout the protocol. Allow beads to reach room temperature prior to use. Redissolve them by rotating them in the cold room

1. Mix the following components in a sterile nuclease-free tube:

Component	Volume
Purified double stranded cDNA (Step 9, Section 2.6)	55,5 µl
• NEBNext End Repair Reaction Buffer (10X)	6,5 µl
• NEBNext End Prep Enzyme Mix	3 µl
Total volume	65 µl

2. Incubate the sample in a thermal cycler using the stored program "*RNA_seq_4_End prep&Adaptor ligation*".
3. Remove the samples from the thermal cycler then the temperature reaches 4°C. Deactivate the heat lid and leave the lid open for cooling down. Press continue!
4. Proceed **immediately** to the following Adaptor Ligation.
5. Dilute the •NEBNext Adaptor for Illumina (15 µM) to 1.5 µM with a 10-fold dilution (1:9) with 10 mM Tris-HCl for immediate use.

Note: The Adaptor is provided in the NEBNext Multiplex.

6. Add the following components **directly** to the end prep reaction mixture.

Caution: Do not pre-mix the components to prevent adaptor-dimer formation!

Component	Volume
End Prep Reaction	65 µl
• Blunt/TA Ligase Master Mix	15 µl
Diluted NEBNext Adaptor	1 µl
Nuclease-free Water	2,5 µl
Total volume	83,5 µl

7. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
8. Incubate 15 minutes at 20°C in a thermal cycler (no heated lid). For this, proceed with the

"*RNA_seq_4_End prep&Adaptor ligation*" program.

2.8 Purify the Ligation Reaction Using AMPure XP Beads

Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in the original manual of the "*NEBNext®Ultra™Directional RNA Library Prep Kit for Illumina®*".

1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl (add 16.5 µl and check if the volume is enough). It is important to ensure the final volume is 100 µl prior to adding AMPure XP Beads.

Note: X refers to the original sample volume of 100 µl from the above step.

2. Add 100 µl (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments (**Caution:** do not discard the beads).
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol, and air-dry beads for 5 minutes while the tube is on the magnetic rack with the lid open, having the flame on.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

9. Remove the tube from the rack. Elute DNA target from the beads with 52 µl of 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
10. Transfer 50 µl supernatant to a clean PCR tube. Discard beads.
11. To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
12. Incubate for 5 minutes at room temperature.
13. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments (**Caution:** do not discard the beads!! In this steps the beads are especially sticky!).

Note: If you are selecting for larger size fragments (> 200 nt) please refer to the original manual of the "*NEBNext®Ultra™Directional RNA Library Prep Kit for Illumina®*".

14. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
15. Repeat Step 14 once for a total of 2 washing steps.
16. Briefly spin the tube, and put the tube back in the magnetic rack.
17. Completely remove the residual ethanol, and air-dry beads for 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

18. Remove the tube from the rack. Elute DNA target from the beads with 22 µl of 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

19. Without disturbing the bead pellet, transfer 17 µl of the supernatant to a clean PCR tube and proceed **directly** to PCR enrichment.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the NEBNext High-Fidelity 2X PCR Master Mix in the subsequent PCR step.

2.9 PCR Enrichment of Adaptor Ligated DNA

Note: This step takes around 1 hour

Note: NEBNext Multiplex Oligos for Illumina (NEB#E7335 and #E7500) now have new primer concentrations (10 µM). Please check oligo kit lot numbers to determine how to set up your PCR reaction.

Follow Section 2.9.1 if you are using the following oligos (10 µM primer):

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335) lot 0091412
NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500) lot 0071412
NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600) all lots

Follow Section 2.9.2 if you are using the following oligos (25 µM primer):

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335) lots 0071402 or 0081407
NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500) lots 0051402 or 0061407

2.9.1 PCR Library Enrichment

If you are using the **Hot Start HiFi PCR Master Mix**, please make sure to adjust the settings of the final PCR enrichment (Section 2.9). For the adjustments please refer to Appendix A.

If fewer than 12 indexes are used in a lane for sequencing, it is recommended to use the following indexes:

Pool of 2 samples: Index #6 and #12
Pool of 3 samples: Index #4, #6 and #12
Pool of 6 samples: Index #2, #4, #5, #6, #7 and #12

1. To the cDNA (17 µl) from Step 19 Section 2.8 add the following components and mix by gentle pipetting:

Note: The primers used in this step are provided by the "NEB Next Multiplex Oligos for Illumina".

Component	Volume
• NEB Next USER Enzyme	3 µl
• NEB Next **High-Fidelity 2X PCR Master Mix**	25 µl
• Index (X) Primer (1 Index Primer per sample)	2,5 µl
• Universal PCR Primer	2,5 µl
Total volume	50 µl

2. Incubate the samples in a pre-heated thermal cycler using the stored program "*RNAseq_5_PCR enrichment*".

Note: The number of PCR cycles should be adjusted based on RNA input. It is important to limit the number of PCR cycles to avoid over-amplification. If over-amplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace (section 3).

3. Proceed to Section 2.10 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

2.9.2 PCR Library Enrichment

If you are using the **Hot Start HiFi PCR Master Mix**, please make sure to adjust the settings of the final PCR enrichment (Section 2.9). For the adjustments please refer to Appendix A.

More information about the index primers (sequences) is shown in Appendix C.

1. To the cDNA (17 µl) from Step 19 Section 2.8 add the following components and mix by gentle pipetting:

Note: The primers used in this step are provided by the "NEB Next Multiplex Oligos for Illumina".

• NEB Next USER Enzyme	3 µl
• NEB Next **High-Fidelity 2X PCR Master Mix**	25 µl
• Index (X) Primer (1 Index Primer per sample)	1 µl
• Universal PCR Primer	1 µl
Total volume	50 µl

2. Incubate the samples in a pre-heated thermal cycler using the stored program "*RNAseq_5_PCR enrichment*".

Note: The number of PCR cycles should be adjusted based on RNA input. It is important to limit the number of PCR cycles to avoid over-amplification. If over-amplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace (section 3).

3. Proceed to Section 2.10 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

2.10 Purify the PCR Reaction using Agencourt AMPure XP Beads

Note: This step takes around 1-1.5 hours

Note: X refers to the original sample volume from the above step.

1. Vortex Agencourt AMPure XP Beads to resuspend.
2. Add 45 µl (0.9X, so 18 µl in the repetition step) of resuspended AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

8. Remove the tube from the rack. Elute the DNA target from the beads into 23 µl 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place it in the magnetic rack until the solution is clear.
9. Transfer 20 µl of the supernatant to a clean PCR tube.

Note: It is recommended to repeat the Purification of the PCR enrichment reaction to delete all

ions and components that could disturb the quality control on a Bioanalyzer®(*Agilent High Sensitivity Chip*).

10. Repeat Step 1-7.
11. Remove the tube from the rack. Elute the DNA target from the beads into 18 µl 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place it in the magnetic rack until the solution is clear.
12. Transfer 15 µl of the supernatant to a clean PCR tube.

Note: For a higher DNA concentration, fewer TE Buffer is used for the elution.

13. Samples can be stored at -20°C.

3. Quality Control & Sequencing

Bring the samples to Dr. Stefan Krebs (krebs@genzentrum.lmu.de; Blum Lab, Laboratory for Functional Genome Analysis, Gene Centre of LMU, Feodor-Lynen-Straße 25, 81377 München) for the quality control and the sequencing.

Note: If the PCR enrichment was too low, they will repeat the purification and perform another round of PCR enrichment for you. If the quality control is good they will directly proceed to the sequencing.

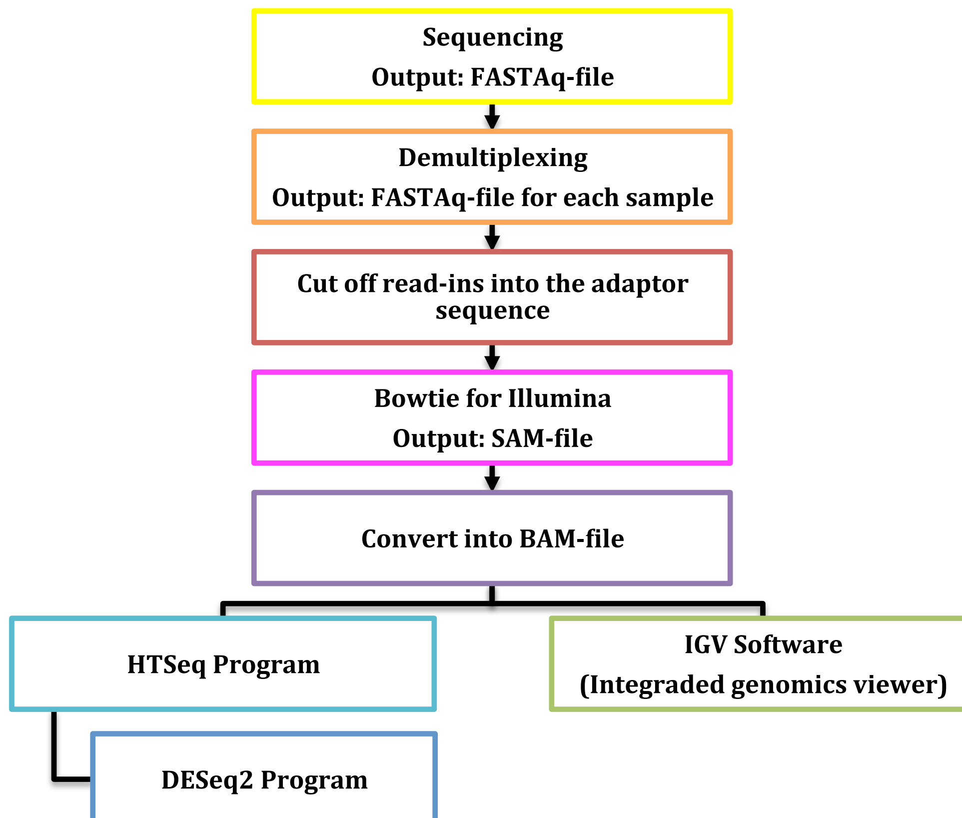
If you want to have the results of the bioanalyzer, please ask Dr. Krebs for sending it to you.

After the sequencing, Dr. Krebs will up-load the raw data on the Galaxy-platform.

4. Transcriptome Analysis

For the analysis of the sequencing data, please refer to the "SQA_1v1 RNAseq Analysis with Galaxy" protocol.

Overview of the workflow:



Appendix A – Hot Star HiFi PCR Master Mix

PCR Program Settings for the **Hot Start HiFi PCR Master Mix** used for the final PCR enrichment in section 2.9:

CYCLE STEP	TEMP	TIME	CYCLES
USER Digestion	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12-15
Annealing	65°C	75 seconds	
Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

Note: The number of PCR cycles should be adjusted based on RNA input. If 100 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. It is important to limit the number of PCR cycles to avoid over-amplification. If over-amplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace (section 3).

Appendix B – PCR program settings

- RNAseq_1_Isolation&Fragmentation

CYCLE STEP	TEMP	TIME	Step*
Pause at	65°C	-	7
Incubate	65°C	5 minutes	
Pause at	4°C	-	
Head Lid	110°C	-	16
Pause at	80°C	-	
Incubate	80°C	2 minutes	
Pause at	25°C	-	
Pause at	94°C	-	28
Incubate (Fragmentation)	94°C	15 minutes	
Store	4°C	∞	

*From Section 2.3

- RNAseq_2_First Strand Synthesis

CYCLE STEP	TEMP	TIME
Head Lid	105°C	-
Pause at	25°C	-
Incubate	25°C	10 minutes
Incubate	42°C	15 minutes
Incubate	70°C	15 minutes
Store	4°C	∞

- RNAseq_3_Second Strand Synthesis

CYCLE STEP	TEMP	TIME
Head Lid	≤40°C	-
Pause at	16°C	-
Incubate	16°C	1 hour
Store	4°C	∞

- RNAseq_4_End Prep&Adaptor Ligation

CYCLE STEP	TEMP	TIME	Step*
Head Lid	175°C	-	End Prep
Pause at	20°C	-	
Incubate	20°C	30 minutes	
Incubate	65°C	30 minutes	
Pause at	4°C	-	
Deactivate Head Lid			Adaptor Ligation
Pause at	20°C	-	
Incubate	20°C	15 minutes	
Store	8°C	∞	

*From Section 2.7

- RNAseq_5_PCR Enrichment for the **NEBNext High-Fidelity 2X PCR Master Mix**

CYCLE STEP	TEMP	TIME	CYCLES
USER Digestion	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12
Annealing	65°C	30 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

Note: The number of PCR cycles should be adjusted based on RNA input. If 100 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. It is important to limit the number of PCR cycles to avoid over-amplification. If over-amplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace (section 3).

Appendix B – Index Primers

NEB#	Product	Index Primer Sequence	Expected Index primer sequence Read
#E7311A: 0.010ml **#E7311AA: 0.020ml**	NEBNext Index 1 Primer for Illumina	5´CAAGCAGAAGACGGCATACGAGAT-**CGTGAT**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ATCACG
#E7312A: 0.010ml **#E7312AA: 0.040ml**	NEBNext Index 2 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**ACATCG**-GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	CGATGT
#E7313A: 0.010ml **#E7313AA: 0.040ml**	NEBNext Index 3 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**GCCTAA**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3-´	TTAGGC
#E7314A: 0.010ml **#E7314AA: 0.040ml**	NEBNext Index 4 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**TGGTCA**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TGACCA
#E7315A: 0.010ml **#E7315AA:0.040ml**	NEBNext Index 5 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**CACTGT**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ACAGTG
#E7316A: 0.010ml **#E7316AA: 0.040ml**	NEBNext Index 6 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**ATTGGC**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GCCAAT
#E7317A: 0.010ml **#E7317AA: 0.040ml**	NEBNext Index 7 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**GATCTG**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CAGATC
#E7318A: 0.010ml **#E7318AA: 0.040ml**	NEBNext Index 8 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**TCAAGT**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ACTTGA
#E7319A: 0.010ml **#E7319AA: 0.040ml**	NEBNext Index 9 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**CTGATC**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GATCAG
#E7320A: 0.010ml **#E7320AA: 0.040ml**	NEBNext Index 10 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**AAGCTA**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TAGCTT
#E7321A: 0.010ml **#E7321AA: 0.040ml**	NEBNext Index 11 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**GTAGCC**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GGCTAC
#E7322A: 0.010ml **#E7322AA: 0.040ml**	NEBNext Index 12 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**TACAAG**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CTTGTA