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

Protocol written by Lucia Martin Caballero on January 2018

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 (50ml, 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.

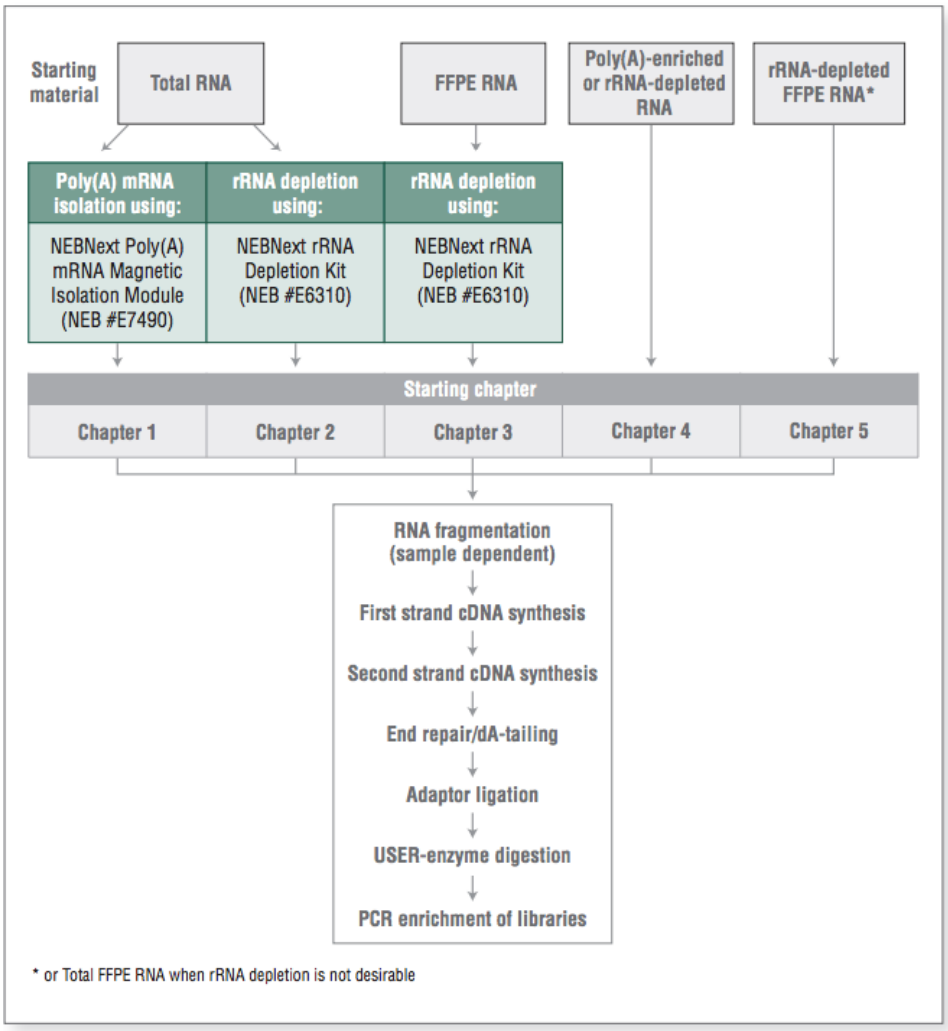
2. RNA library preparation

The following protocol is optimized from the "*NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®*" (#E7760/#E7765) instruction manual.

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™ II Directional RNA Library Prep Kit for Illumina®*

Requirements:

- NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina or customer supplied oligos
- PCR tube Magnetic rack (Ask Magdalena, she has one)
- 80% EtOH (in Nuclease free H_2O, freshly prepared)
- Agencourt®AMPure®XP beads (Beckmann Coulter, Inc. #A63881)
- PCR machine



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)
… free of divalent cation chelating agents (e.g. EDTA, EGTA, citrate)
… treated with DNase. The DNase should be removed after treatment.

1. Measure the RNA concentration using the Qubit. In the past it was done in the nanodrop and worked fine.
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, pipet out the required volume with a cut pipette tip (sticky!) and let them reach RT.

9. In a nuclease-free eppi, prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) as follows:

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

10. Mix thoroughly by pipetting up and down several times

Note: Keep the mix on ice until the mRNA is purified.

2.3 mRNA Isolation, Fragmentation and Priming Starting with Total RNA

1. To wash the Oligo dT Beads, add the following to a nuclease-free eppi. If preparing multiple libraries, beads for up to 10 samples can be added to a single 1.5 ml tube for subsequent washes. [Careful: volumes below are specified per library!!!]

Oligo $d(T)_{25}$ Beads	20 µl
RNA Binding Buffer (2X)	100 µl
Total Volume	120 µl

2. Wash the beads by pipetting the entire volume up and down 6 times to mix thoroughly.
3. Place the tubes on the magnetic rack and incubate at room temperature for 2 minutes (or until the solution is clear).
4. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
5. Remove the tubes from the magnetic rack.
6. Add 100 µl RNA Binding Buffer (2X) and repeat steps 2-5. (Volume per sample!!)
7. Resuspend the beads in 50 µl of RNA Binding Buffer (2X) and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl RNA Binding Buffer (2X) per sample.
8. Add 50 µl beads to each RNA sample from step 2.1.2, and mix thoroughly by pipetting up and down several times.

Note: For the Steps 9 and 18 and 29 use the stored PCR program "*New_RNAseq_1_Isolation&Fragmentation*".

9. Place the tube on a thermal cycler and close the lid and start the protocol 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!
10. Mix thoroughly by pipetting up and down several times. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.
11. 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.
12. Remove and discard all of the supernatant. Take care not to disturb the beads. Afterwards remove the tube from the magnetic rack.
13. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.
14. Place the tube on the magnetic rack at room temperature for 2 minutes.
15. 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.
16. Repeat steps 13-15.
17. Add 50 µl of Tris Buffer (from kit) to each tube. Gently pipette the entire volume up and down 6 times to mix thoroughly.
18. Place the tube on the thermal cycler. Close the lid and continue the saved protocol to do the first elution of the mRNA from the beads.
19. Remove the tube from the thermal cycler when the temperature reaches 25°C. Press continue!
20. 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.
21. Incubate the tube at **room temperature for 5 minutes**.
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 tube from the magnetic rack.
24. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.

25. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

Note: It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.

26. Place the tube on the magnetic rack at room temperature for 2 minutes.
27. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads that contain the mRNA.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps.

28. Afterwards remove the tubes from the magnetic rack.

Note: The next step provides a fragmentation incubation time resulting in an RNA insert size of ~200 nt. For RNA insert sizes > 200 nt, refer to original manual.

29. **Fragmentation -** Elute mRNA from the beads by adding 11.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer mix (2X) prepared in Section 2.2 and pipette up and down several times to resuspend the beads. Put into thermocycler and continue program.
30. Once the machine has reached 4°C, immediately transfer the tube to ice for 1min.
31. Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (around 2mins).
32. Collect the fragmented mRNA by transferring 10 µl of the supernatant to a clean nuclease-free PCR Tube. **Avoid transferring the magnetic beads!**

Note: If for some reason the supernatant volume recovered is less than 10 µl, bring the volume to 10 µl by adding the first strand synthesis reaction buffer and random primer mix (2x) prepared in 2.2 and continue with the protocol.

33. Place the tube **on ice** and proceed **directly** to first strand cDNA synthesis.

2.4 First Strand cDNA Synthesis

1. Assemble the first strand cDNA synthesis reaction **on ice** by adding the following components into fragmented and primed RNA from the previous step, and mix by thoroughly by pipetting up and down several times:

Fragmented and primed RNA (From 2.3)	10 µl
• NEBNext Strand Specificity Reagent	8 µl
• NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total volume	20 µl

2. 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 2.

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

Note: Already start the stored "*RNAseq_3_Second Strand Synthesis-New"* 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. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis reaction product from 2.4.

First-Strand synthesis product	20 µl
Nuclease-free water	48 µl
• NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10x)	8 µl
• NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Total volume	80µl

2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down several times.
3. Incubate the samples in a preheated Thermal Cycler using the stored program "*RNAseq_3_Second Strand Synthesis*".

2.6 Purification of 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. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a 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 and the flame on.

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 53 µl of 0.1X TE buffer (from kit). Mix well on a vortex mixer or by pipetting up and down. Briefly 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 50 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 End Prep of cDNA Library

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. Assemble the end prep reaction on ice by adding the following components to second strand synthesis product from 2.6.

Purified double stranded cDNA (Section 2.6)	50 µl
• NEBNext Ultra II End Repair Reaction Buffer (10X)	7 µl
• NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total volume	60 µl

2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

3. *Incubat*e the sample in a thermal cycler using the stored program "*RNA_seq_4_End prep&Adaptor ligation-New*".
4. 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!
5. Proceed **immediately** to the following Adaptor Ligation.

2.8 Adaptor Ligation

1. Dilute the •NEBNext Adaptor (provided in the NEBNext Multiplex kit) prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptor on ice. For starting RNA amount of 1 µg, dilute the adaptor 5-fold.
2. Add the following components **directly** to the end prep reaction mixture.

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

End Prep Reaction	60 µl
Diluted NEBNext Adaptor (1/5 diluted)	2,5 µl
• NEBNext Ligation Enhancer	1 µl
• NEBNext Ultra II Ligation Master Mix	30 µl
Total volume	93,5 µl

3. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

4. 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-New*" program.
5. After the 15 minutes, press continue. Add 3 µl of • USER enzyme to the ligation mixture from the previous step, resulting in 96.5 µl.
6. Mix well and incubate put the tube back to the PCR machine and press continue.
7. After the 15 minutes, proceed immediately to Purification of the Ligation Reaction.

2.9 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.

1. Add 87 µl (0.9X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
2. Incubate for 10 minutes at room temperature.
3. Quickly spin the tube in a microcentrifuge and place the tube on the 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).
4. 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.
5. Repeat Step 4 once for a total of 2 washing steps.
6. Briefly spin the tube, and put the tube back in the magnetic rack.
7. 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.

8. Remove the tube from the rack. Elute DNA target from the beads with 17 µl of 0.1X TE (from the kit). Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and

incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

Safety Stop: If you need to stop at this point in the protocol samples can be stored at –20°C. Or directly proceed to 2.10.

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.

2.10 PCR Enrichment of Adaptor Ligated DNA

Note: Check and verify that the concentration of your oligos is 10 µM on the label.

Note: This protocol is optimized for the oligos we use (NEB#E7335 and #E7500)!. If you wish to use other oligos (such as NEB #E6609), please refer to the original manual.

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. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

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

Fw and Rev primers separate	
Component	Volume/library
Adaptor ligated DNA	15 µl
• NEBNext Ultra II Q5 Master Mix	25 µl
Universal PCR Primer/i5 primer	5 µl
Index (X) primer/i7 primer	5 µl
Total Volume	50 µl

Fw and Rev primers combined	
Component	Volume/library
Adaptor ligated DNA	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/Universal Primer Mix	10 µl
Total Volume	50 µl

2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.
3. Place the tube on a thermocycler and use the program "*RNAseq_5_PCR enrichment-New*".

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.

4. Proceed to Section 2.11 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

2.11 Purification of the PCR reaction using AMPure XP beads

1. Vortex Agencourt AMPure XP Beads to resuspend.
2. Add 45 µl (0.9X) 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 the 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 (from the kit) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. 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.

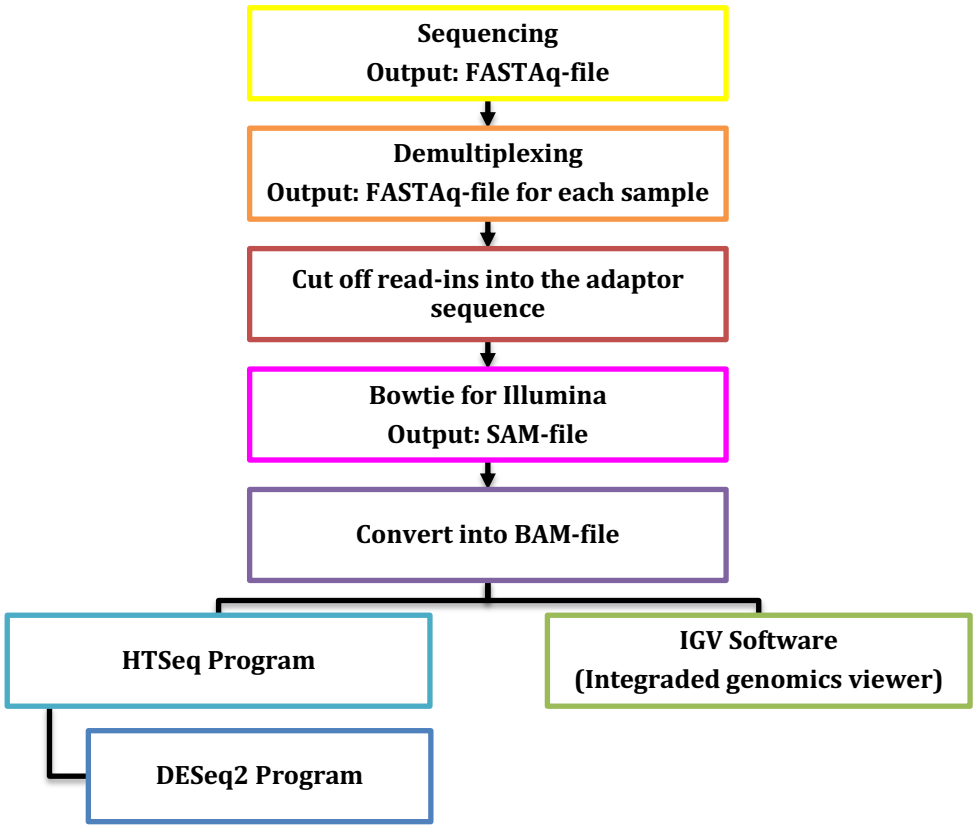
If you want to have the results of the bioanalyzer, please ask Dr. Krebs for sending it to you.

After the sequencing, Dr. Krebs will upload 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 B – PCR program settings

- RNAseq_1_Isolation&Fragmentation-New

CYCLE STEP	TEMP	TIME	Step
Pause at	65°C	-	9
Incubate	65°C	5 minutes	
Pause at	4°C	-	
Head Lid	110°C	-	18
Pause at	80°C	-	
Incubate	80°C	2 minutes	
Pause at	25°C	-	
Pause at	94°C	-	29
Incubate (Fragmentation)	94°C	15 minutes	
Store	4°C	∞	

- RNAseq_2_First Strand Synthesis (old program)

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 (old program)

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

- RNAseq_4_End Prep&Adaptor Ligation-New

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	
Pause at	37°C	-	
Incubate	37°C	15 minutes	
Store	4°C	∞	

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

CYCLE STEP	TEMP	TIME	CYCLES
Heat lid	105	-	-
Pause at	98°C	-	-
Initial Denaturation	98°C	30 seconds	8-9*
Denaturation	98°C	10 seconds	
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	1

* The number of PCR cycles should be adjusted based on RNA input. If over-amplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace (section 3). To readjust the number of PCR cycles, refer to the original manual.

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	5′-CAAGCAGAAGACGGCATACGAGAT-**AAGCTA**-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3′	TAGCTT

	Illumina		
#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