UF | ICBR Gene Expression & Genotyping UF | ICBR NextGen DNA Sequencing

RNAseq Frequently Asked Questions:

Q: Which ICBR core conducts RNAseq projects?

A: RNAseq projects should be submitted in the Gene Expression & Genotyping core, using the ICBR iLab portal. Once libraries have been constructed, the pooled library will be transferred to the NextGen Core for sequencing. No separate request in iLab is necessary if sequencing is requested along with library construction. To start the RNAseq project, please contact ICBR-Geneexpression@ad.ufl.edu (or yanp@ufl.edu) for more details and for planning your experiment.

Our library construction and sequencing services do not include data processing. Data analysis services can be requested separately from the ICBR Bioinformatics core (ICBR-Bioinformatics@ad.ufl.edu).

Q: Should customers make an appointment to discuss their RNA-seq project?

A: We suggest that you schedule a consultation meeting with us before you start your project. This helps us in better understanding the goals of your experiment. Our Bioinformatics experts can give you suggestions about the most appropriate experimental design to achieve your goals.

Q: What is the Illumina RNAseq workflow using NEB reagents?

1. Start with total RNA. RIN>8 is best, but Illumina RNAseq is quite forgiving.
2. cDNA synthesis:
   a. If you are only interested in polyA transcripts, use the poly (A) mRNA magnetic isolation module with oligo(dT) cDNA synthesis primers. Using the NEB poly (A) capture approach precludes the need for depleting ribosomal RNA, which would otherwise represent ~95% of your reads. For RNA fragmentation, the times can vary depending on the RNA quality. High quality RNA for 2x100 sequencing can be done by using the maximum fragmentation time in the manual
   b. If you are interested in all transcripts, including non-poly (A), long non-coding RNA, you would need to start with the RNA ribodepletion kit. NEB has off-the-shelf products for human/mouse/rat ribodepletion. For other species you'd need custom products such as those offered by NuGen (i.e., AnyDeplete) for which the ribosomal depletion happens after cDNA synthesis.
3. At this point, you'd be ready to attach the adaptors to the ds-cDNA fragments using the Ultra II RNA Library Prep Kit. The NEB manual is self-explanatory. When you get to the final amplification step with barcoded primers, you'll need to use primers from the NEBNext Multiplex Oligos set (Dual Index primer set 1). We would recommend using less amplification cycles than those recommended by NEB in the manual. If you are starting with one microgram of total RNA, you probably don't need more than ~7 cycles.
4. Perform QC on your libraries: Qubit, TapeStation and qPCR. With mass and size information, calculate molarity.
5. Depending on your familiarity with the library construction protocol, qPCR quantification may be skipped. However, we strongly suggest quantifying the final library pool using qPCR (the Gene Expression core can do it for $40 /sample or pool). qPCR measures the "functional" molecules in the library (i.e., molecules that contain full p5 and p7 adaptors).

6. Normalize concentrations and pool equimolarly.
7. The Gene Expression core will pass the pooled library to the NextGen for sequencing. Illumina recommends ~50 million reads per samples. Since one lane on the HiSeq produces ~300 million reads, you can do 6 samples per lane (barcoded!!). However, you can multiplex more or less samples per lane if you so desire, depending on your experimental goals.
8. For pre-constructed RNAseq libraries (i.e., libraries that have NOT been made in the Gene Expression Core, please submit directly to NGS core at least 10 ul at 5-10 nM of your pool for sequencing.

Q: How much RNA needed for RNASeq library prep?

Library complexity is very important in an RNA-seq experiment. If the starting RNA is limited, amplification artifacts are more likely to occur which affects the interpretation of the results. The standard protocol for library construction requires between 100 ng and 1 µg of total RNA. There are kits available for ultra-low RNA input that start with as little is 10 pg-10ng of RNA; however, the reproducibility increases considerably when starting with 1-2 ng. If possible, 1 to 1.5 ug of total RNA is preferred for sample QC and library prep.

Q: What are the RNA quality Requirements?

A: High sample quality is essential for successful RNA-Seq experiments. Customer should determine initial QC of total RNA sample with a NanoDrop. The 260/280 and 260/230 ratios need to be greater than 1.8. To determine total RNA quality we use Agilent Bioanalzyer or Tapestation. **7-10 ranges of RIN (>7) of samples are highly recommended by Kit.**

Q: What if customers do not have 50 ng input RNA? Does GE offer RNA library prep services?

A: If customer has less than 50 ng of input RNA, we offer an option for RNA amplification using Clontech's SMART-seq v4 Ultra Low Input RNA Kit to create amplified cDNA, followed by library preparation using Illumina's Nextera XT kit. The minimum input required for this kit is 10 pg, but >200 pg is preferred.

Q: Does GE accept FFPE RNA for RNA-seq?

A: For degraded and FFPE RNA, we offer the TruSeq Stranded Total RNA with Ribo-Zero Gold prep. This uses Ribo-Zero technology to deplete the rRNA (both cytoplasmic and mitochondrial), as the normal protocol will not work due to loss of the poly-A tails. We still require a Bioanalyzer report. We accept RINs of <7 for this protocol, but cannot guarantee the quality of sequencing results as the RNA integrity is so variable in FFPE.

Q: Will the GE Core send QC results before they start my library prep?

A: Usually 'Yes', the GE core will upload data to iLab which content RIN scores of customer's RNA samples when applicable along with concentration. Specially, if the quality and quantity of customer's samples do not meet the requirements for the library prep, we will let customer know, as well as give customer some possible options to make a decision on how to move forward with the submitted samples.

Q: How do customers submit replacement samples?

A: If after initial QC customer need to submit more sample (s) to the core, please upload the sample info of replaced sample(s) to iLab, and clearly indicate which samples replaced which samples.

Q: Is it OK to isolate samples in batches?

A: It is best if you can isolate samples at once, but it is common practice that researchers collect/extract RNAs at different times using the same protocol.

Q: Can customers get back the samples?

A: Yes. Please make a note in the comment box in iLab submission form that you want the samples back upon the completion of the project. We keep customers' samples up to 6 months. It is customers' responsibility to retrieve the samples on time.

Q: How long does the core keep customer's leftover samples for?

A: Due to limited space in the GE cores freezer we only keep customer's samples for 6 months. If customer would like samples returned to them then they should contact the core to schedule a day and time to come and pick them up.

Q: What is the turnaround time for an RNA-seq project?

A: Turnaround time depends largely on the volume of activity at the time of sample submission and the complexity of the project. Given typical workloads, library construction requires approximately 2-3 weeks, and sequencing-analysis often requires approximately 2 - 3 weeks after that. If data analysis service is requested,this would add an additional 1-2 weeks.

Q: How should I design my RNA-seq experiment?

A: You can schedule a consultation meeting by contacting .ICBR-GeneExpression@ad.ufl.edu or yanp@ufl.edu. We will schedule a meeting with the participation of members from the Gene Expression, NextGen and Bioinformatics cores.

Q: Which strand is sequenced for my strand-specific RNA-seq data?

A: When using paired-end sequencing, the forward read of the resulting sequencing data represents the "anti-sense strand" and the reverse read the "sense strand" of the genes.