Mouse Small RNA Library Prep (Illumina TruSeq with modifications)

General Practices

- *When not in the thermocycler, RNA samples should be kept on ice.*
- *For master mixes, always add 10-20% additional volume to account for dead volume.*

Total RNA Precipitation

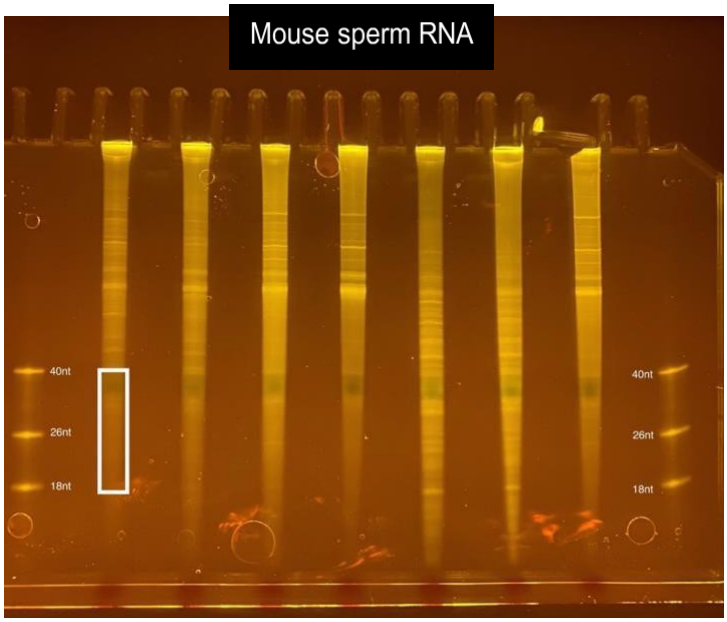
Starting with total RNA samples in isopropanol.

- Centrifuge at 14,000 RPM for 15 minutes at 4°C.
- Remove supernatant and wash pellet with 1 mL cold 70% ethanol. Centrifuge at 14,000 RPM for 5 minutes at 4°C.
- Remove supernatant while leaving pellet in place. Make sure all ethanol is removed by spinning down the tubes in a benchtop centrifuge and removing liquid using a gel-loading tip 2X.
- Reconstitute the RNA pellet in 8 µL of nuclease-free water. Transfer liquid to labelled PCR tubes. *Dry and reconstitute samples/pellets one at a time

Small RNA Isolation

- Add 8 µL (1 volume) of Invitrogen Gel-Loading Buffer II (THERMOFISHER SCIENTIFIC: CAT # AM8546G) to each tube.
- Make a denaturing RNA gel using an 18-well gel cassette.
    - Clean gel apparatus with tap water, D/RNase Free™ Decontaminant (ARGOS TECHNOLOGIES: CAT # D6002C), and DI water.
    - For 1 gel, use 13-15 mL of RNA gel solution (15% polyacrylamide, 7M Urea, 1X TBE stored at 4°C), 130-150 µL ($1/100^{th}$ vol) of 10% ammonium persulfate (stored at 4°C), and 13-15 µL ($1/1000^{th}$ vol) of TEMED (BIO-RAD: CAT # 1610800).
    - Use 1X TBE buffer in the gel apparatus. Clean wells of the gel with 1X TBE and a syringe in order to clear urea buildup from the wells. Clean wells at least twice before loading.
    - Run 2 µL of small RNA ladder (18 – 26 – 40 nt RNAs from IDT at 1 uM final conc.) flanking the samples with an empty lane in between (do NOT load ladder into the outermost lanes of the gel). Load samples into every other lane.
- Run gel at 14W constant wattage. For 2 gels run at 28W. It will take 25-30 minutes for the dye front to reach the end of the gel, at which point the run should be terminated.
- When gel is finished running, notch the gel in the upper-righthand corner to identify the correct orientation of the gel.
- Stain gel with 1 µL SYBR Gold Nucleic Acid Gel Stain (THERMOFISHER SCIENTIFIC: CAT # S11494) in enough 1X TBE to cover gel. Let rock for 5 minutes.
- Remove TBE with SYBR and add ~100ml (enough to cover gel) fresh 1X TBE to wash gel for 5 minutes on a rocker.

- Visualize gel on blue block and use a razorblade to cut a gel slice containing the fraction of RNAs that are 18-40 nt for each sample. Place individual gel slices in labelled 1.5 mL low-adhesion microcentrifuge tubes.
    - Take images of the RNA gels prior to cutting. These will be used later to evaluate differences in RNA input between samples and differentially amplify the cDNA libraries prior to sequencing.
    - Example: An image of total RNA from 7 mouse sperm samples. The white box is the fraction of small RNA that is excised from the gel.



- Crush the gel slices with a disposable pestle or pipette and add 700 µL of gel elution buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 300mM NaCl). Wrap tubes in parafilm and shake overnight at 1,000 RPM at room temperature.
- The next day, remove the eluted buffer from the tubes and place in 0.45 µm Costar® Spin-X® centrifuge tube filters (CORNING: CAT # 8170). Spin at 10,000 G for 1 minute at 4°C.
- Transfer flow-through to a 1.5 mL low-adhesion microcentrifuge tube.
- Add 1 µL of GlycoBlue™ Coprecipitant (THERMOFISHER SCIENTIFIC: CAT # AM9515) to the flow-through and mix well.
- Add 1.1-1.2X volume of isopropanol to the solution and mix well by vortexing.
- Store at -20°C for at least 1 hour before proceeding to the next step.
    - If precipitating small RNAs during the same day, place at -20°C for 15 minutes and then -80°C for 15 minutes.

Small RNA Precipitation + 3' Adapter Ligation (reagents from Illumina TruSeq small RNA kit)

- Centrifuge at 14,000 RPM for 15 minutes at 4°C.
- Preheat the thermocycler blocks to 70°C and 28°C.
- Remove supernatant and wash pellet with 1 mL cold 70% ethanol. Centrifuge at 14,000 RPM for 5 minutes at 4°C.
- Make a master mix of the 3' adapter solution – 3 µL per sample
    - 0.25 µL of the 3' adapter
    - 2.75 µL of nuclease-free water
- Remove supernatant while leaving pellet in place. Make sure all ethanol is removed by spinning down the tubes in a benchtop centrifuge and removing liquid using a gel-loading tip 2X.
- Reconstitute each small RNA pellet in 3 µL of the 3' adapter solution. Transfer solution to labelled PCR tubes on ice.
- After all pellets are reconstituted, heat at 70°C for 2 minutes. After the 2 minutes, immediately place the tubes on ice.
- Make a master mix of the 3' adapter ligation solution – 2 µL per sample.
    - 1 µL of Ligation Buffer (HML) (This reagent is in the core ligations kit)
    - 0.5 µL of RNase Inhibitor
    - 0.5 µL of T4 RNA Ligase 2, **truncated** (*sold separately from kit - NEB: CAT # M0242L)
- Add 2 µL of the 3' adapter ligation solution to each sample and mix well (total rxn volume 5 µL).
- Incubate at 28°C for 1 hour.
- Add 0.5 µL of Stop solution to sample tubes that are still in the thermocycler (total rxn volume 5.5 µL). Allow to incubate at 28°C for 15 additional minutes. Place sample tubes on ice when incubation concludes.

5' Adapter Ligation (reagents from Illumina TruSeq small RNA kit)

- Add 0.5 µL of RNA 5' Adapter to each sample
- Incubate tubes at 70°C for 2 minutes, then place immediately on ice before adding other reagents.
- Make a master mix of the following reagents:
    - 0.5 µL of 10 mM ATP
    - 0.5 µL of T4 RNA Ligase
- Add 1.0 µL of the 5' adapter solution to each sample tube (total rxn volume 7 µL).
- Incubate samples at 28°C for 1 hour.
- After the incubation, place the tubes on ice.

Reverse Transcription / cDNA

- Preheat thermocycler blocks to 70°C and 50°C.
- Thaw the 25 mM dNTP mix, the RNA RT Primer (RTP), the RNase Inhibitor, and the Ultra-Pure water from the Illumina kit on ice. Also thaw 5X First Strand Buffer, 100 mM DTT, and SuperScript™ II Reverse Transcriptase on ice.
- Add 0.5 µL of RNA RT Primer to each adapter-ligated RNA sample and mix well (total rxn volume 7.5 µL).
- Incubate tubes at 70°C for 2 minutes. Place tubes on ice following incubation.
- Before preparing the master mix below, work out how much volume of dNTPs is required and dilute the 25 mM dNTPs 1:1 with Ultra-Pure Water to create a 12.5 mM dNTP solution. If you need 9 µL, make 10 µL for extra. Mix and place on ice ready to add into master mix below.

- Prepare the master mix for the reverse transcription reaction – 5 µL per sample.
    - 2 µL 5X First Strand Buffer*
    - 1 µL 100 mM DTT*
    - 1 µL Ultra-Pure water
    - 0.5 µL 12.5 mM dNTP mix
    - 0.25 µL RNase Inhibitor
    - 0.25 µL SuperScript™ II Reverse Transcriptase*

*The 5X First Strand Buffer, 100 mM DTT, and the SuperScript™ II Reverse Transcriptase comes in the SuperScript™ II Reverse Transcriptase kit (THERMOFISHER SCIENTIFIC: CAT # 18064022).

- Add 5.0 µL of reverse transcription master mix to each sample (total rxn volume 12.5 µL).
- Incubate at 50°C for 1 hour. Place samples on ice following incubation. At this point, the small RNA has been transformed into cDNA. Save the cDNA until the samples have been sequenced and analyzed. The samples can be stored at -20°C.

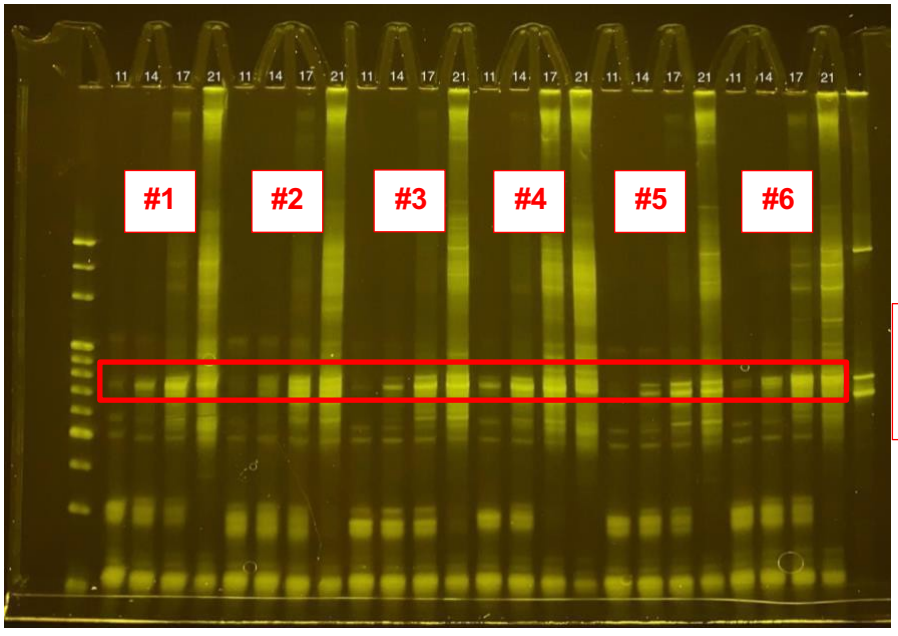
Optimization of Differential PCR Amplification of cDNA

To prevent over-amplification and formation of PCR artifacts in the sequencing libraries, the cDNA must be differentially amplified to control for differences in the amount of starting material of RNA.

1. First, return to the images of denaturing RNA gels that were taken during the "Small RNA isolation." For each type of sample (i.e., experimental condition or tissue type), select 2 samples that represent the differences in the amount of RNA starting material.
2. Determine the number of PCR cycles that will be tested on each representative sample. For high-input samples, try 11,14,17, and 20 cycles. For low-input samples, try 15, 18, 21, and 24 cycles.
3. Set up the PCR reactions for each sample.
    a. Go through the samples and assign the desired index number (1-48) to each sample.
        i. Do not use reverse primers 31 and 41 in the same sequencing run.
    b. Create a PCR master mix for PCR – 20.5 µL per sample
        i. 7.5 µL nuclease-free H_2O
        ii. 12.5 µL PCR mix (PML or KAPA HiFi *sold separately from kit)
        iii. 0.5 µL forward primer (RP1)
    c. For each sample, use:
        i. 20.5 µL of the master mix
        ii. 4 µL of the cDNA
        iii. 0.5 µL of reverse indexed primer (RPI1-48)
1. Run PCR programs on thermocycler, with N being the lowest number of cycles in the optimization range (11 in 11,14,17,20 or 15 in 15,18,21,24):

PML (Illumina Kit)	KAPA HiFi HotStart ReadyMix (ROCHE: CAT # 7958927001)
98°C 30 s N cycles of: 98°C 10 s 60°C 30 s 72°C 15 s 4°C ∞	95°C 3 min N cycles of: 98°C 20 s 60°C 15 s 72°C 15 s 4°C ∞

2. After the program finishes, remove 4 µL of solution into PCR tubes labelled with the sample name and the number of PCR cycles. Leave the rest of the solution in the thermocycler and run 3 additional cycles before removing another 4 µL of solution and placing it into separate labelled PCR tubes. Repeat until 20/24 cycles have been completed, depending on if the samples are low/high-input. Keep PCR product on ice.
3. Run a non-denaturing 8% polyacrylamide gel in order to visualize the PCR products.
    a. Make 8% polyacrylamide gel in a 26-well gel cassette
        i. 4 mL 30% polyacrylamide (BIORAD: CAT # 1610156)
        ii. 1.5 mL 10X TBE
        iii. 9.5 mL nuclease-free H_2O
        iv. 15 µL TEMED
        v. 150 µL 10% ammonium persulfate
    b. Flank samples with a High-Resolution RNA Ladder (HRL) on one side and a Custom RNA Ladder (CRL) on the other side. These ladders are supplied in the TruSeq kit and can be combined 1:1 with 6X DNA-Loading Dye (NEB: CAT # B7025S).
    c. Add 1 µL of 6X DNA-loading dye (NEB: CAT # B7025S) to each 4 µL sample and 2 µL of 6X DNA loading dye to each 12 µL sample.
    d. Load the PCR product from differing number of cycles adjacent to each other for each sample. For example, if sample "A" was amplified using 11, 14, 17, and 20 cycles, load the 11-cycle product from sample "A" in lane 1, the 14-cycle product from sample "A" in lane 2, etc.
    e. Run gel for 35 minutes (the dye front will run off the gel, but this is okay).
    f. Stain gel with 1 µL of SYBR Gold Nucleic Acid Gel Stain in 10 mL of 1X TBE.
    g. Visualize the gel on the blue block to determine what the optimal number of PCR cycles is for each sample. The goal is to maximize the amount of desired cloning product (143- 165 nt total library length corresponding to 18-40 nt inserts with adapters) and minimizing the amount of bulged PCR product (larger amplicons). For example, for samples #1 & 6 – 17 cycles has some bulged product, thus 16 cycles would be recommended. Samples #2, 3 & 5 – 17 cycles is perfect. Sample #4 – 14 cycles.



4. Return to the images of denaturing RNA gels that were taken during the "Small RNA isolation." Using the representative samples whose optimal number of PCR amplification cycles were determined in this section, select other samples which had similar levels of initial total RNA. Create groupings of samples which should undergo the same number of PCR amplification cycles.

Differential PCR Amplification of cDNA

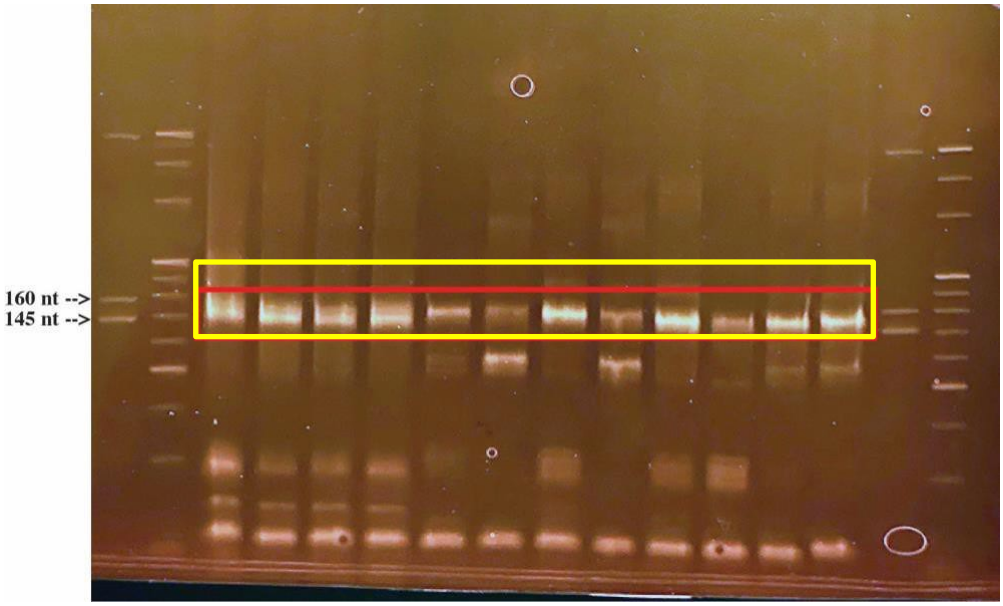
1. Prepare master mix – 20.5 µL per sample.
    a. 7.5 µL nuclease-free H_2O
    b. 12.5 µL PCR mix (PML or KAPA HiFi)
    c. 0.5 µL forward primer (RP1)
2. For each library, combine:
    a. 20.5 µL master mix
    b. 4 µL cDNA
    c. 0.5 µL reverse indexed primer (RPI1-48)
3. Split PCR reactions into groupings based on the optimal number of PCR cycles.
4. Run the PCR reaction with N being the optimal number of PCR cycles for a particular grouping:

PML (Illumina Kit)	KAPA HiFi HotStart ReadyMix (ROCHE: CAT # 7958927001)
98°C 30 s N cycles of: 98°C 10 s 60°C 30 s 72°C 15 s 72°C 10 min 4°C ∞	95°C 3 min N cycles of: 98°C 20 s 60°C 15 s 72°C 15 s 72°C 2 min 4°C ∞

5. Place libraries on ice.

Gel Purification and Isolation of Small RNA Libraries

1. Run libraries on an 18-well non-denaturing 8% polyacrylamide gel.
    a. Make 8% polyacrylamide gel:
        i. 4 mL 30% polyacrylamide
        ii. 1.5 mL 10X TBE
        iii. 9.5 mL nuclease-free H_2O
        iv. 15 µL TEMED
        v. 150 µL 10% ammonium persulfate
    b. Add 5 µL of 6X loading dye to each small RNA library before loading onto gel.
    c. Run gel for 35 minutes at 14 W per gel (the dye front will run off the gel, but this is okay).
    d. Stain gel with 5-10 µL ethidium bromide (BIORAD: CAT # 1610433) in 1X TBE.
2. Isolate the 143-165 nt fraction of each library.
    a. Visualize gel on the blue block and use a clean razor blade to excise the fraction (145nt bottom CRL band – 200nt bright band on HRL; yellow box. It is important not to cut the adapter dimer band that sits below 145nt marker).



b. Place the gel slice in a 1.5 mL low-adhesion microcentrifuge tube.
c. Crush the gel slice using a pestle and add 700 µL of gel elution buffer.
d. Wrap tubes in parafilm and shake overnight at 1,000 RPM at room temperature.
e. Transfer all eluted liquid to a 0.45 µm Costar® Spin-X® centrifuge tube filter (CORNING: CAT # 8170) and spin at 10,000 G for 1 minute at 4°C.
f. Transfer flow-through to a 1.5 mL low-adhesion microcentrifuge tube.
g. Add 3 µL of glycogen (AMBION: CAT # AM9510).
h. Add 1.1-1.2X volume of isopropanol and mix well before placing at -20°C for at least one hour.

3. Precipitate and reconstitute the small RNA library.
   a. Spin at 14,000 RPM for 15 minutes at 4°C.
   b. Remove supernatant, wash with 1 mL cold 70% ethanol, and spin again at 14,000 RPM for 5 minutes at 4°C.
   c. Remove supernatant, dry pellet (*Do NOT overdry pellet), and reconstitute in 6 µL of nuclease-free water.
4. Quantify the concentration of DNA using the Qubit fluorometer and the Qubit dsDNA HS Assay kit.
   a. Make working solution (199 µL of dsDNA HS buffer and 1 µL of dsDNA HS reagent for each sample or standard).
   b. Make Standard #1 (190 µL working solution, 10 µL standard #1 solution) and Standard #2 (190 µL working solution, 10 µL standard #2 solution).
   c. For each sample, combine 199 µL of working solution and 1 µL sample in the Qubit assay tubes.
   d. For small RNA libraries, concentrations above 1 ng/µL are ideal.
5. Keep libraries at –20°C until ready to sequence.

Set up the run on BaseSpace (any time before sequencing)

>Runs >New Run >Run Planning
>Name the run:
Example: 20221118_GL_smallRNA_condition_sperm
>Add a description
>Instrument Platform is NextSeq 1000/2000
>Secondary Analysis is BaseSpace
>Next
>Configuration Page
>Application: *leave as default*
>Library Prep Kit: Not Specified
>Index Adapter Kit: Not Specified
>Next
>Configuration
>Index Reads: 1 Index
>Read Type: Single Read
>Read Lengths: 75 / 6 / 0 / 0
(Read 1 / Index 1 / Index 2 / Read 2)
>Download Template
Add Samples and Indexes --> Save as CSV --> Import
>Next
>Run Review
>Confirm Read Lengths
>Save as Planned

Preparing the Library for Sequencing (on the day of sequencing)

1. Using this protocol, you can sequence a total of 20-50 libraries at one time depending on the read depth required for your experiment.
2. When you're ready to sequence, make dilutions of all your libraries so that you have > 2 µL at a concentration of 1 ng/µL for each library.
    a. If a library has a concentration below 1 ng/µL, do not dilute it.
3. Take 2 µL from each 1 ng/µL library dilution and combine in a single 1.5 mL low-adhesion microcentrifuge tube.
    a. If a library has a concentration below 1 ng/µL that is still detectable, add 2 ng of DNA to your pool. This applies for all libraries with a concentration > 0.4 ng/µL.
4. Use the qubit to quant your pooled libraries. This should be around 1 ng/µL.
5. Calculate the molarity of dsDNA in the pooled library solution based on the concentration of the solution. For small RNA libraries: # of nucleotides is 151

   = (conc ng/µL)/(151 * 607.4 +157.9) *10^6

   = **(concentration in ng/µL / 91,875.3) * 10^6 = nM**
6. Dilute the library to 2.2 nM using RSB with Tween-20.
7. Take 47.5 µL of the 2.2 nM library and add 2.5 µL of 2 nM PhiX for a 5% spike-in.
8. Take 18 µL of the combined library_PhiX solution and add 30 µL of RSB with Tween-20.
9. You will load 24 µL in the sequencing cartridge.

Sequencing

1. At this point:
    a. You have signed up to use the NextSeq 1000 with the sequencing core.
    b. The cartridge is thawed as recommended by the manufacturer.
    c. The flow cell was thawed at RT for at least 30 minutes.
    d. Your library is combined, diluted, and ready to be sequenced.
2. Login on the NextSeq 1000 and find your run that was pre-setup on BaseSpace.
3. Confirm that all your information was input correctly and follow the instructions given.
    a. Invert cartridge 10 times.
    b. Insert the flow cell and remove the gray placeholder.
    c. Pierce the foil and load 24 µL of your final library to the cartridge.
    d. Select your run on the sequencer and insert the cartridge. Hit load > next > run.
4. Wait for the sequencer to run through all initial checks and stay until you hear three consecutive popping noises. At this point the sequencer should run successfully.

Reagents and Materials

- 30% Polyacrylamide solution (BIORAD: CAT # 1610156)
- 6X DNA Loading Dye (NEB: CAT # B7025S)
- Costar Spin-X 0.45 µm centrifuge tube filter (CORNING: CAT # 8170)
- D/RNase Free™ Decontaminant (ARGOS TECHNOLOGIES: CAT # D6002C)
- Ethidium bromide (BIORAD: CAT # 1610433)
- Glycoblue Coprecipitant (THERMOFISHER SCIENTIFIC: AM9515)
- Glycogen (AMBION: CAT # AM9510)
- Invitrogen Gel-Loading Buffer II (THERMOFISHER SCIENTIFIC: CAT # AM8546G)
- KAPA HiFi HotStart ReadyMix (ROCHE: CAT # 7958927001)
- SuperScript II Reverse Transcriptase kit (THERMOFISHER SCIENTIFIC: CAT # 18064022)
- SYBR Gold Nucleic Acid Gel Stain (THERMOFISHER SCIENTIFIC: CAT # S11494)
- T4 RNA Ligase 2, Deletion Mutant (LUCIGEN: CAT # LRD1132K) this enzyme discontinued in Aug 2023, now use NEB CAT #: M0242L
- TEMED (BIO-RAD: CAT # 1610800)
- 10x TBE: 121.1g Tris, 61.83g Boric acid, 7.41g EDTA