Guidelines and tips for OTTR cDNA library generation and purification

1. Input templates:

- If input template molecules have 3' phosphate, remove it to increase efficiency of capture. Other 3′ extensions, e.g. tRNA aminoacylation, should also be removed. 5′ phosphates do not need to be removed, but larger 5′ modifications may influence capture efficiency.
- If input has been eluted from a denaturing gel slice in buffer containing SDS, precipitate using a salt that reduces detergent carryover like NaCl.
- Ideally the input template pool should be stored and resuspended in buffer without EDTA or other chelator. The workflow is optimized for input nucleic acids resuspended in water or dilute Tris buffer (e.g. 10 mM). If your sample is stored in TE, add no more than 0.5 μL input template volume to the reaction.
- If possible, check input absorbance spectrum, for example using a Nanodrop. Absorbance peaks at 230, 250 and 270 nm are from inhibitory salts, ethanol, and phenol, which can be removed by dialysis or clean & concentrate columns.
- If your input pool has secondary structure that could decrease accessibility of the template 3′ end, denaturation can be added prior to the workflow; for example heat for 2 min at 60ºC then move to ice for 3 min. This is best done without divalent cations in the buffer to minimize fragmentation, especially for tRNAs.
- OTTR protocols have been validated using an input range of 0.02 ng to 10 ng size-selected small RNA. Low input will increase adaptor dimer formation, which increases the need for careful size selection of cDNA to remove adaptor dimer *prior* to PCR (as well as post-PCR sequencing library size selection). Overly high input will allow tandem input template copying and introduce bias among input 3′ nucleotides.

2. Library generation and purification:

- Perform an OTTR reaction with a positive control RNA oligonucleotide in the input size range. Then its cDNA can be a guide for size selection by denaturing PAGE.
- Especially with low-input applications, perform a post-PCR size selection step to remove adaptor dimer products.

3. Custom primers

Primer duplexes (PD):

- cDNA 5′ adaptor primer DNA has a +1 3′ overhang and a +3 5′ overhang with respect to its complementary RNA. The 5′ Cy5 or IR800 modification reduces unwanted use of primer for cDNA library generation. The 5′ dye is also useful to visualize cDNA for gel-based size selection.

- Primer-complement strand can be entirely RNA or chimeric. The 5′ six nucleotides should be ribonucleotides; fewer ribonucleotides reduces library synthesis efficiency. Use of many contiguous 2′O-Methyl ribonucleotides in the 3′ region reduces unwanted use of primer duplex strands as input template.

4. Custom adaptor templates (3′ AT):

- Different combinations of ribonucleotides and deoxyribonucleotides can be used.
- 3′ end must be C but can be ribonucleotide, deoxy, or dideoxy (the latter suppresses any ability of 3′ AT oligonucleotide to prime a cDNA).
- 5′ end Cy5 modification inhibits additional cDNA synthesis after copying of the 3′ AT, keeping library products at their intended length. It also reduces artifact adaptor trimers arising from primer extension across two adaptor templates. Cy5 is a strong block to additional template jumping, but other bulky 5′ modifications can be used if some extra template jumping is tolerable.