

Probe synthesis protocol

For use with oligo-pools generated by the CARPDM pipeline

Before beginning

The following reagents are needed for this protocol, along with the Twist oligo pool:

Reagent	Supplier	Catalogue #
Phusion High-fidelity DNA Polymerase (2U/ μ L)	Thermo Fisher Scientific	F530
FastDigest LguI	Thermo Fisher Scientific	FD1934
Bio-16-UTP (10mM)	Thermo Fisher Scientific	AM8452
HiScribe T7 High Yield RNA Synthesis Kit	New England Biolabs	E2040S
Monarch RNA Cleanup Kit	New England Biolabs	T2040
RNAse-Free Dnase I	New England Biolabs	M0303
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs	N0447L
MinElute PCR Purification Kit	Qiagen	28006
QIAquick Nucleotide Removal Kit	Qiagen	28306
Amplification primers from CARPDM output	IDT	N/A

Equipment

Beyond standard plasticware and pipettes, the facility performing this protocol will require:

- Thermal cycler with a heated lid that can accommodate 0.2mL strip tubes

- Microcentrifuge for purifications

- System to quantify DNA/RNA concentration (E.g. Nanodrop, Qubit)

- System to examine RNA quality after transcription (E.g. BioAnalyzer, Urea-PAGE)

Probe Synthesis Protocol

Part A – Oligo pool amplification

1. Resuspend oligo pool to 10ng/μL concentration and amplification primers to 100uM using 10mM Tris pH = 8 buffer.
 - a. Before opening, centrifuge the tubes containing the lyophilized DNA for 30 seconds at 5kg to ensure none adhered to the lid or sides.
2. Create the following Master Mix:

Reagent	Stock	Final	μL/reaction	MM μL
Phusion HF Buffer	5 X	1 X	10 μL	168 μL
T7 amplification primer	100 μM	1 μM	0.5 μL	8.4 μL
Pool amplification primer	100 μM	1 μM	0.5 μL	8.4 μL
dNTP	10 mM	0.2 mM	1 μL	16.8 μL
Phusion DNA Polymerase	2 U/μL	0.02 U/μL	0.5 μL	8.4 μL
Oligo Pool	10 ng/μL	0.02 ng/μL	0.1μL	1.68 μL
H2O			37.4 μL	628.32 μL
Totals:			50 μL	840 μL

3. Aliquot 50uL of the above Master mix into 16 separate PCR reactions. Load these reactions to a thermal cycler and run the following protocol:

Temperature	Time	Cycles
98°C	30s	
98°C	10s	12
60°C	30s	
72°C	15s	
72°C	10m	
12°C	∞	

4. Purify the reactions according to the protocol listed below:
 - a. First, pool four reactions into a single 1.5mL tube (200uL final volume), add 1mL of Buffer PNI and mix thoroughly. Repeat for all remaining reactions. There should be four tubes, each with 200uL of sample and 1mL buffer PNI.
 - i. Two of these tubes will be run over a single column.
 - b. Load 700uL of PNI/sample mix from one tube to one column, then load 700uL of another tube to another column.
 - c. Centrifuge both at 3.8kg for one minute, discard flowthrough.

- d. Load the remaining 500uL from each tube to separate columns, then add another 200uL from each of the other tubes to separate columns.
 - e. Centrifuge both at 3.8kg for one minute, discard flowthrough.
 - f. Load 700uL of each tube to separate columns.
 - g. Centrifuge both at 3.8kg for one minute, discard flowthrough.
 - h. Load the final 200uL of each tube to separate columns.
 - i. Centrifuge both at 3.8kg, discard flowthrough.
 - j. Add 750uL Buffer PE to each column.
 - k. Centrifuge both at 3.8kg, discard flowthrough.
 - l. Dry spin at 17.9kg for one minute.
 - m. Aspirate residual ethanol from the rim surrounding the silica filter using a P20 pipette.
 - n. Add 30µL Buffer EB directly to the filter of each column. Let sit for one minute at room temperature.
 - o. Elute by centrifuging at 17.9kg for one minute.
 - p. Combine these purification reactions into a single pool.
5. Quantify DNA concentration using your method of choice (e.g., Nanodrop, Qubit).

Part B: LguI Restriction Enzyme Treatment

1. Determine the volume of the amplified oligo pool required to add 1.5µg to the reaction.
2. Use this value to determine the amount of water to be added per reaction. The final reaction should be 50uL, including the template and Master mix.
3. Construct the following master mix

Reagent	Stock	Final	µL/reaction	MM µL
FastDigest Buffer	10 X	1 X	5 µL	21 µL
LguI	25 X	1 X	2 µL	8.4 µL
H2O			Up to 50uL with template	Per reaction volume × 4 × 1.05

4. Aliquot the master mix into four separate reactions. Add the required volume of template.
5. Incubate according to the following conditions:

Temperature (°C)	Time
37	3 hours
65	5 minutes
12	Hold

6. Pool all samples into a 1.5mL tube and purify with MinElute PCR purification kit according to the below protocol:
 - a. Add 1mL Buffer PB to 200uL sample. Mix thoroughly.

- b. Load 700uL of mix to column.
 - c. Centrifuge at 17.9kg for one minute.
 - d. Discard flowthrough.
 - e. Repeat the prior three steps until the entire sample is bound.
 - f. Add 750uL buffer PE to the column.
 - g. Centrifuge at 17.9kg for one minute.
 - h. Discard flowthrough.
 - i. Dry spin at 17.9kg for one minute.
 - j. Aspirate residual ethanol from the rim surrounding the silica filter using a P20 pipette.
 - k. Add 10µL Buffer EB directly to the filter of each column. Let sit for one minute at room temperature.
 - l. Elute by centrifuging at 17.9kg for one minute.
7. Quantify DNA concentration using your method of choice (e.g., Nanodrop, Qubit).

Part C: In-vitro transcription

1. Determine the volume of the purified LguI-treated pool needed for 1µg of input into transcription. Mix this volume with an amount of nuclease-free H₂O to yield 6.5µL of input for the reaction.
 - a. If the purified pool does not have a high enough concentration, it is still possible to run the transcription reaction, though final yields may be lower.
2. Assemble the following reaction using reagents from the NEB HiScribe T7 transcription kit and separately purchased biotinylated UTP:

Reagent	Stock	Final	µL/reaction
Reaction Buffer	10 X	0.75 X	1.5 µL
ATP	100 mM	7.5 mM	1.5 µL
GTP	100 mM	7.5 mM	1.5 µL
CTP	100 mM	7.5 mM	1.5 µL
UTP	100 mM	5 mM	1 µL
Bio-16-UTP	10 mM	2.5 mM	5 µL
T7 Polymerase Mix	13.33 X	1 X	1.5 µL
H ₂ O			Up to 20uL with template
Total:			20 µL

3. Incubate for 16 hours at 37°C.
 - a. It is possible to incubate for less time, though the final yield may be lower.
4. Add 70µL Nuclease-free H₂O, 10uL Dnase Buffer I, and 2uL RNase-Free Dnase I.
5. Incubate for 15 minutes at 37°C.

6. Purify with the Monarch RNA cleanup kit according to the following protocol:
 - a. Add 200 μ L binding buffer to 100 μ L sample and mix thoroughly.
 - b. Add 300 μ L ethanol, flick to mix.
 - c. Load the entire sample to the column.
 - d. Spin one minute at 16kg.
 - e. Discard flowthrough.
 - f. Add 500uL wash buffer.
 - g. Spin one minute at 16kg.
 - h. Discard flowthrough.
 - i. Repeat the prior three steps once for a total of two washes.
 - j. Dry spin for one minute at 16kg.
 - k. Transfer to RNase-free microfuge tube.
 - l. Add 50uL Nuclease-free H₂O.
 - m. Let sit for 1min.
 - n. Elute by spinning for 1 minute at 16kg.
7. Quantify RNA concentration using your method of choice.

Part D: Quality Control

For this portion, run the RNA sample on a BioAnalyzer small RNA Assay. A smear above the 80nt point indicates the stochastic incorporation of biotinylated UTP into the probes.

Alternatively, one can run 100ng of the probe pool on a 12.5% 19:1 Acrylamide: Bisacrylamide with 8M Urea gel for two hours at 220V and post-stain with SYBR Gold. Include a GeneRuler ULR Ladder for sizing (Thermo Fisher Scientific, Catalogue # SM1211). We recommend the Owl P10DS Dual Gel Electrophoresis System. However, other platforms should be amenable, though voltage and run times will have to be optimized.