create template molecules for in vitro transcription. Second, an in vitro transcription produces RNA from these template molecules as well as another ~100-fold amplification. Third, a reverse transcription reaction generates single-stranded DNA from this RNA. Fourth, the RNA template is removed via alkaline hydrolysis. We utilize an RNA intermediate for two reasons: (i) in vitro transcription can produce very large quantities of nucleic acid in very small volumes, reducing the amount of material that must be used, and (ii) the alkaline susceptibility of RNA allows it to be easily removed from the final DNA probes. Because this reaction involves the use of an RNA intermediate, we recommend using the higher standards of laboratory cleanliness often required for handling RNA. Specifically, all surfaces and pipettes should be cleaned daily using RNase removal solutions and separate stocks of all buffers should be kept solely for RNA work.

4.1 Required Reagents

The following protocols will require the following reagents

1. 20× EvaGreen (Biotium; 31000)
2. 2× Phusion hot start polymerase master mix (New England Biolabs; M0536S)
3. Tris–EDTA (TE) pH 8 buffer (Ambion; AM9849)
4. DNA binding buffer (Zymo Research; D4004-1-L)
5. DNA wash buffer (Zymo Research; C1016-50)
6. Oligo binding buffer (Zymo Research; D4060-1-40)
7. 100-μg capacity silicon columns (Spin-V; Zymo Research; D4003-2-48)
8. RNA binding buffer (Optional; Zymo Research; R1013-2-100)
9. RNA prep buffer (Optional; Zymo Research; R1060-2-100)
10. RNA wash buffer (Optional; Zymo Research; R1003-3-24)
11. Quick HiScribe T7 polymerase kit (New England Biolabs; E2050S)
12. RNasin plus (Promega; N2611)
13. Maxima H− reverse transcriptase (ThermoScientific; EP0751)
14. 10 m*M* mix of dNTPs (New England Biolabs; N0447S)
15. 0.5 *M* EDTA (Ambion; AM9261)
16. 1 N NaOH (VWR; JT5635-2)
17. Nuclease-free water (Ambion; AM9932)
18. 100% Ethanol (KOPTEC; VWR; 89125-186)
19. D/RNAaseFree (VWR; 47751-044)
20. 1.5-mL LoBind tubes (Eppendorf; 022431021)

21. PCR tubes

The following protocols will require the following equipments

1. Tabletop centrifuge
2. qPCR machine or thermocycler
3. 37°C incubator or water bath
4. 50°C water bath
5. 95°C water bath
6. Vacuum manifold (optional)
7. Gel electrophoresis equipment for polyacrylamide gels (optional)
8. Vacuum concentrator (optional)

4.2 Amplification of In Vitro Template

The first step in this protocol is to use PCR to amplify template molecules for the in vitro transcription. We recommend running this reaction as a limited-cycle PCR, ie, monitor the status of the reaction in real time with a qPCR machine and remove the samples immediately before the final amplification plateau. We recommend limiting the number of PCR cycles because we have found that over amplification of complex libraries can produce molecules that miss-prime on other molecules, forming long concatemers that both reduce the yield of proper encoding probes and which could produce spurious signals.

Step 1: Design the primers. This PCR will not only amplify the library, it will also add the T7 promoter to these molecules to allow in vitro transcription of these templates. The sequence of the forward primer is the same as that designed in Section 3.5. However, the sequence of the reverse primer, also designed in Section 3.5, must include a T7 promoter, TAATACGACTCACTATAGGG, at the 5′ end. Example primers can be seen in Table 3. The forward primer will also be used as the primer for reverse transcription in Section 4.4; thus, it is recommended to order this primer at a relatively large synthesis scale, such as 100 nmol or 250 nmol.

Table 3 Example Primers

Forward primer	CGCGGGCTATATGCGAACCG
Reverse primer with T7 promoter	*TAATACGACTCACTATAGGG* CGTGGAGGGCATACAACGC

These primers are compatible with the encoding probe template listed in Table 2. Note that the 5′ G in the reverse primer has been removed so as not to create a G quadruplet with the terminal GGG of the T7 promoter.

The T7 promoter ends in a G-triplet, and the presence of a G quadruplet, ie, four Gs in a row, often significantly lowers synthesis yields; thus, we recommend removing any 5′-terminal G nucleotides in the sequence of the reverse primer. The presence of the terminal G nucleotides in the T7 promoter region will serve as replacements for these nucleotides in the priming region. Resuspend forward primer to 200 μ*M* and the reverse primer to 100 μ*M* both in TE.

Step 2: Prepare the PCR. In a 1.7-mL Eppendorf tube, mix the following: 40 μL 20× Eva Green, 2 μL 200 μ*M* forward primer, 4 μL 100 μ*M* reverse primer, 1 μL of 80 ng/μL complex oligopool, 353 μL nuclease-free water, and 400 μL 2× Phusion hot start polymerase master mix. Aliquot 50 μL volumes into 16 PCR tubes.

Step 3: Amplify the template. Run the following protocol on a qPCR machine: (i) 98°C for 3 min, (ii) 98°C for 10 s, (iii) 65°C for 10 s, (iv) 72°C for 15 s, and (v) measure the fluorescence of each sample. Repeat cycle steps (ii) through (v) until the rate at which the sample amplifies decreases, which is a sign that it is approaching the final amplification plateau. Due to the complexity of these oligonucleotide pools, it is very unlikely that, once denatured, each molecule will find and rehybridize to its complement as opposed to partially hybridize with the common priming regions of a different molecule; thus, it is recommended that samples be removed after the elongation step—while the instrument is at 72°C—and before it reaches the 98°C-melting step of the next cycle. If a qPCR machine is not available, we recommend determining the appropriate number of cycles to run by quantifying the yield of small-scale PCR reactions run for different numbers of cycles.

Step 4: Purify the template. We utilize column purification to remove enzyme, nucleotides, and primers. In a 15-mL Falcon tube, mix the following: 800 μL of the PCR reaction generated in Step 3 and 4 mL of DNA binding buffer. Pull this mixture across a 100-μg capacity column using either a vacuum manifold or a centrifuge. Wash the column twice with 300 μL DNA wash buffer, spinning the column in a tabletop centrifuge at maximum speed for 30 s each time. Elute the template by adding 170 μL nuclease-free water to the column, transferring the column to a fresh 1.7-mL Eppendorf tube, and spinning at maximum speed for 30 s. Set aside 10 μL of this reaction for quality control.

Step 5 (Optional): Quality control for template reaction. Two important quality control steps can be performed at this point. First, it is useful to measure the concentration of the template with a spectrophotometer, such as the

Nanodrop. The concentration should be between 10 and 50 ng/μL. The second quality control step is gel electrophoresis and will be described in Step 5 of Section 4.4.

4.3 In Vitro Transcription

The second step of this protocol is a high-yield in vitro transcription reaction that further amplifies the template molecules created in Section 4.2 as well as converts them into RNA.

Step 1: In vitro transcription. In a fresh 1.7-mL Eppendorf tube, mix the following: 160 μL of the in vitro template created in Section 4.2, 176 μL of nuclease-free water, 250 μL of the NTP buffer mix provided with the Quick HiScribe T7 polymerase kit, 25 μL of RNasin Plus, and 25 μL T7 polymerase (from the same HiScribe kit). Incubate the reaction in a 37°C incubator for 12–16 h. Often the reaction is complete after 6–8 h, but it is typically convenient to leave this reaction overnight. Remove 20 μL for quality control.

Step 2 (Optional): Quality control for the in vitro transcription. To confirm that the in vitro transcription was successful, purify the reaction and measure its concentration with a spectrophotometer. To purify, mix 20 μL of the in vitro reaction with 30 μL nuclease-free water, 100 μL RNA binding buffer, and 150 μL 100% ethanol. Pass across a 100-μg capacity spin column in a tabletop centrifuge. Wash this column once with 400 μL RNA prep buffer, and then twice with 200 μL RNA wash buffer, each time with a 30-s spin at the maximum speed of the tabletop centrifuge. Elute the RNA with 100 μL nuclease-free water. If successful, the concentration of the in vitro transcription should be between 0.5 and 2 μg/μL. Purified RNA can also be run on a gel as described in Step 5 of Section 4.4.

4.4 Reverse Transcription and Purification

In this step of the protocol, the large quantities of RNA produced by the high-yield in vitro transcription are converted to single-stranded DNA using a reverse transcription reaction. This RNA template is then removed via alkaline hydrolysis, and the final encoding probes are purified and concentrated.

Step 1: Reverse transcription. To the unpurified in vitro transcription created in Section 4.3, add the following and mix well: 200 μL 10 m*M* dNTP mix, 120 μL 200 μ*M* forward primer, 240 μL 5× Maxima buffer, 24 μL RNasin Plus, and 24 μL Maxima H− reverse transcriptase. Incubate

in a 50°C water bath for 1 h. It is important to use a water bath, not an air incubator, to insure that the temperature of the sample rises to 50°C quickly.

Step 2: Alkaline hydrolysis. Split the above reaction into two 1.7-mL Eppendorf tubes and add the following to each: 300 μL 0.5 *M* EDTA and 300 μL 1 N NaOH. Incubate in a 95°C water bath for 15 min.

Step 3: Purification of ssDNA probe. Combine the two aliquots above into a single 50-mL Falcon tube and add the following: 4.8 mL Oligo binding buffer and 19.2 mL 100% ethanol. Mix well and split equally between eight 100-μg capacity spin columns. Pull the sample across the columns with a vacuum manifold or via centrifugation. Wash the columns once with 750 μL DNA wash buffer. Elute the columns using 100 μL of nuclease-free water. Combine eluates and set aside 10 μL for quality control.

Step 4: Concentration of probe. Use a vacuum concentrator to dry the samples. This process could take several hours. Resuspend the dried pellet in 24 μL of nuclease-free water, or if desired, the hybridization buffer described in Section 5.1. Store probe at −20°C and avoid unnecessary freeze-thaw cycles. If a vacuum concentrator is not available, it is also possible to concentrate the probe using ethanol precipitation.

Step 5 (Optional): Quality control of in vitro template, RNA, and probe. We recommend running the in vitro template, the RNA, and the final probe on a 15% TBE-urea polyacrylamide gel to identify both RNase contamination and low conversion of the reverse transcription primer to full-length probe. Large smearing both in the RNA band and the probe band can indicate RNase contamination. Failure to efficiently convert the reverse transcription primer into probe is revealed by a bright band running at the 20-nt length corresponding to the primer. Increasing the amount of RNA template in the reverse transcription often improves the fraction of primer converted into probe. We routinely obtain ∼75% or greater incorporation of the reverse transcription primer into probe with the above protocol.

5. SAMPLE PREPARATION AND STAINING

The preparation and staining of samples for MERFISH follows closely the typical protocols used for smFISH (Raj et al., 2008). However, there are a few places in which we have modified these protocols to optimize MERFISH staining. Again, RNase contamination can destroy samples, so care should be taken to work in an RNase-free environment.