RNA Imaging with MERFISH - Probe Construction

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Abstract

Staining hundreds to thousands of RNA species each of which requires tens of unique encoding probes requires a very large number of unique oligonucleotide sequences. The sheer number of unique sequences makes traditional solid phase oligonucleotide synthesis prohibitively expensive in most cases. For example, assuming a modest cost of $0.10/base, an encoding probe length of ~100 nt, and the need for ~50,000 unique oligos for a single MERFISH measurement, the cost of the needed oligos would be $500,000! To circumvent this astronomical cost, we have developed a high-throughput approach to generating these probes which utilizes array-derived synthesis of complex oligonucleotide pools. These arrayderived complex oligonucleotide pools that contain ~100,000 custom designed sequences can be purchased for only a few thousand dollars. The challenge to using these pools is that each individual sequence is provided in quantities far too small to be used directly for labeling. Thus, we developed an enzymatic amplification protocol to generate the encoding probes in high quantity sufficient for RNA FISH experiments using the array-derived complex oligonucleotide pools as templates.

Citation: Jeffrey R. Moffitt,Xiaowei Zhuang RNA Imaging with MERFISH - Probe Construction. **protocols.io** dx.doi.org/10.17504/protocols.io.meqc3dw
Published: 29 Mar 2018

Guidelines

The basic protocol involves four steps, which are described in detail in the following sections. First, the oligopool is amplified via PCR to 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.

Equipment:

1. Table top 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 poly-acrylamide gels (optional)

8. Vacuum concentrator (optional)

The protocol workflow is as follows:

Amplification of *in vitro* Template (Steps 1-8)

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, i.e. 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.

***In vitro* Transcription (Steps 9-14)**

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

Reverse Transcription and Purification of Encoding Probes (Steps 15-25)

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.

Materials

20X EvaGreen 31000 by Biotium

2X Phusion hot start polymerase master mix M0536S by New England Biolabs

Tris-EDTA (TE) pH 8 buffer AM9849 by Ambion

DNA binding buffer D4004-1-L by Zymo Research

DNA wash buffer C1016-50 by Zymo Research

Oligo binding buffer D4060-1-40 by Zymo Research

100-µg capacity silicon columns (Spin-V) D4003-2-48 by Zymo Research

RNA binding buffer (Optional) R1013-2-100 by Zymo Research

RNA prep buffer (Optional) R1060-2-100 by Zymo Research

RNA wash buffer (Optional) R1003-3-24 by Zymo Research

Quick HiScribe T7 polymerase kit E2050S by New England Biolabs

RNasin plus N2611 by Promega

Maxima H- reverse transcriptase EP0751 by Thermo Scientific

10 mM mix of dNTPs N0447S by New England Biolabs

0.5 M EDTA AM9261 by Ambion

1 N NaOH JT5635-2 by Vwr

Nuclease-free water AM9932 by Ambion

100% Ethanol (KOPTEC) 89125-186 by Vwr

D/RNAaseFree 47751-044 by Vwr

1.5 mL LoBind tubes 022431021 by Eppendorf

PCR tubes by Contributed by users

Protocol

Amplification of in vitro Template

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 the protocol for Design of Oligonucleotide Probes. However, the sequence of the reverse primer, 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 Reverse Transcription and Purification of Encoding Probes below; thus, it is recommended to order this primer at a relatively large synthesis scale, such as 100 nmol or 250 nmol. The T7 promoter ends in a G triplet, and the presence of a G quadruplet, i.e. 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 µM and the reverse primer to 100 µM both in TE.

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Table 3

Example primers. 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.

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

Amplification of in vitro Template

Step 2.

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

AMOUNT
1 µl Additional info: 80 ng/µL complex oligopool
AMOUNT
353 µl Additional info: nuclease free water
AMOUNT
4 µl Additional info: 20X Eva Green
AMOUNT
2 µl Additional info: 200 µM forward primer
AMOUNT
4 µl Additional info: 100 µM reverse primer
AMOUNT
1 µl Additional info: 80 ng/µL complex oligopool
AMOUNT
353 µl Additional info: nuclease free water
AMOUNT
400 µl Additional info: 2X Phusion hot start polymerase master mix

Amplification of in vitro Template

Step 3.

Amplify the template. Run the following protocol on a qPCR machine: 1) 98 °C for 3 minutes; 2) 98 °C for 10 s; 3) 65 °C for 10 s; 4) 72 °C for 15 s; 5) Measure the fluorescence of each sample. Repeat cycle steps 2 through 5 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 number of cycles.

Amplification of in vitro Template

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.

AMOUNT
800 µl Additional info: PCR reaction
AMOUNT
4 ml Additional info: DNA binding buffer

Amplification of in vitro Template

Step 5.

Wash the column with 300 µL DNA wash buffer, spinning the column in a table top centrifuge at maximum speed for 30 s. (1/2)

AMOUNT
300 µl Additional info: DNA wash buffer

Amplification of in vitro Template

Step 6.

Wash the column with 300 µL DNA wash buffer, spinning the column in a table top centrifuge at maximum speed for 30 s. (2/2)

AMOUNT
300 µl Additional info: DNA wash buffer

Amplification of in vitro Template

Step 7.

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.

AMOUNT
170 µl Additional info: Nuclease-free water

Amplification of in vitro Template

Step 8.

(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 ng/µL to 50 ng/µL. The second quality control step is gel electrophoresis and will be described in Section 'Reverse Transcription and Purification of Encoding Probes' below.

In vitro Transcription

Step 9.

in vitro transcription. In a fresh 1.7 mL Eppendorf tube, mix the following: 160 µL of the in vitro template created above; 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 hours. Often the reaction is complete after 6–8 hours, but it is typically convenient to leave this reaction overnight. Remove 20 µL for quality control.

AMOUNT
160 µl Additional info: in vitro template
AMOUNT
176 µl Additional info: nuclease free water
AMOUNT
250 µl Additional info: NTP buffer mix
AMOUNT
25 µl Additional info: RNasin Plus
AMOUNT
25 µl Additional info: T7 polymerase
TEMPERATURE
37 °C Additional info: Incubation

In vitro Transcription

Step 10.

(Steps 10-14 are 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 150µL 100% ethanol. Pass across a 100-ug-capacity spin column in a table top centrifuge.

AMOUNT
20 µl Additional info: in vitro reaction
AMOUNT
30 µl Additional info: nuclease-free water
AMOUNT
100 µl Additional info: RNA binding buffer
AMOUNT
150 µl Additional info: 100% ethanol

In vitro Transcription

Step 11.

Wash this column once with 400 µL RNA prep buffer with a 30 s spin at the maximum speed of the table top centrifuge.

AMOUNT
400 µl Additional info: RNA prep buffer

In vitro Transcription

Step 12.

Wash column with 200 µL RNA wash buffer with a 30 s spin at the maximum speed of the table top centrifuge. (1/2)

AMOUNT
200 µl Additional info: RNA wash buffer

In vitro Transcription

Step 13.

Wash column with 200 µL RNA wash buffer with a 30 s spin at the maximum speed of the table top centrifuge. (2/2)

AMOUNT
200 µl Additional info: RNA wash buffer

In vitro Transcription

Step 14.

Elute the RNA with 100 µL nuclease-free water. If successful, the concentration of the in vitro transcription should be between 0.5 µg/µL to 2 µg/µL. Purified RNA can also be run on a gel as described below (Step 24).

AMOUNT
100 µl Additional info: nuclease-free water

Reverse Transcription and Purification of Encoding Probes

Step 15.

Reverse transcription. To the unpurified in vitro transcription created in the Steps above, add the following and mix well: 200 µL 10 mM dNTP mix; 120 µL 200 µM forward primer; 240 µL 5X Maxima buffer; 24 µL RNasin Plus; 24 µL Maxima H- reverse transcriptase.

AMOUNT
200 µl Additional info: 10mM dNTP mix
AMOUNT
120 µl Additional info: 200 µM forward primer
AMOUNT
240 µl Additional info: 5X Maxima buffer
AMOUNT
24 µl Additional info: RNasin Plus
AMOUNT
24 µl Additional info: Maxima H- reverse transcriptase

Reverse Transcription and Purification of Encoding Probes

Step 16.

Incubate in a 50°C water bath for 1 hour.

TEMPERATURE
50 °C Additional info: Water bath

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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.

Reverse Transcription and Purification of Encoding Probes

Step 17.

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 minutes.

AMOUNT
300 µl Additional info: 0.5 M EDTA
AMOUNT
300 µl Additional info: 1N NaOH
AMOUNT
300 µl Additional info: 0.5 M EDTA
AMOUNT
300 µl Additional info: 1 N NaOH
TEMPERATURE
95 °C Additional info: Water bath

Reverse Transcription and Purification of Encoding Probes

Step 18.

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.

AMOUNT
4.8 ml Additional info: Oligo binding buffer
AMOUNT
19.2 ml Additional info: 100% ethanol

Reverse Transcription and Purification of Encoding Probes

Step 19.

Wash the columns once with 750 µL DNA wash buffer.

AMOUNT
750 µl Additional info: DNA wash buffer

Reverse Transcription and Purification of Encoding Probes

Step 20.

Elute the columns using 100 µL of nuclease-free water. Combine eluates and set aside 10 µL for quality control.

AMOUNT
100 µl Additional info: Nuclease-free water

Reverse Transcription and Purification of Encoding Probes

Step 21.

Concentration of probe. Use a vacuum concentrator to dry the samples.

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This process could take several hours.

Reverse Transcription and Purification of Encoding Probes

Step 22.

Resuspend the dried pellet in 24 µL of nuclease-free water, or if desired, the hybridization buffer described in the attached full manuscript (Section 5.1).

AMOUNT

24 µl Additional info: Nuclease-free water

Reverse Transcription and Purification of Encoding Probes

Step 23.

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.

TEMPERATURE

-20 °C Additional info: Storage

Reverse Transcription and Purification of Encoding Probes

Step 24.

(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.