Preparation of Encoding Probes SOP005.v1.5 (PCR, In-vitro Transcription, Reverse Transcription and USER ENZYME Digest) V.3

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Parts 1-3: Moffitt, J. R., & Zhuang, X. (2016). RNA imaging with multiplexed error-robust fluorescence in situ hybridization (MERFISH). In Methods in enzymology (Vol. 572, pp. 1-49). Academic Press. doi: 10.1016/bs.mie.2016.03.020.
Part 4: Wang, G., Moffitt, J. R., & Zhuang, X. (2018). Multiplexed imaging of high-density libraries of RNAs with MERFISH and expansion microscopy. Scientific reports, 8(1), 1-13. doi 10.1038/s41598-018-22297-7

ABSTRACT

Document Summary: This document, Preparation of Encoding Probes (SOP005), describes the procedure used to produce the final encoding probes used in multiplexed iterative FISH experiments, from commercially-derived, low-yield yet affordable oligo libraries. To prepare the ordered oligo pool into the final encoding probe set, oligos are amplified using limited-cycle PCR, then amplified again and shortened during in-vitro transcription. We follow the amplification steps with reverse transcription to convert our product back into the intended DNA-based, mRNA target complements and then digest away RNA using Uracil-Specific Excision Reagent (USER) enzyme digest to shorten the probes and alkaline hydrolysis to remove RNA nucleotides leaving single-stranded DNA-based encoding probes. Column purification is used to clean the products at various intermediate steps of the protocol and the final product is concentrated using ethanol precipitation prior to use in experiments. This protocol is *strongly* derived from Moffitt 2016 (https://doi.org/10.1016/bs.mie.2016.03.020) with some modifications with credit for a majority of this protocol due to Moffitt et al 2016.

ATTACHMENTS

SOP005.v1.5 Preparation of Encoding Probes - USER Enzyme Method.pdf

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MATERIALS

- 20X EvaGreen **Biotium Catalog #31000**
- Phusion Hot Start Flex 2X Master Mix - 100 rxns **New England Biolabs Catalo #M0536S**
- Tris-EDTA (TE) pH 8 buffer **Ambion Catalog #AM9849**
- DNA binding buffer **Zymo Research Catalog #D4004-1-L**
- DNA wash buffer **Zymo Research Catalog #C1016-50**
- Oligo binding buffer **Zymo Research Catalog #D4060-1-40**
- 100-µg capacity silicon columns (Spin-V) **Zymo Research Catalog #D4003-2- 48**
- RNA binding buffer (Optional) **Zymo Research Catalog #R1013-2-100**
- RNA prep buffer (Optional) **Zymo Research Catalog #R1060-2-100**
- RNA wash buffer (Optional) **Zymo Research Catalog #R1003-3-24**
- HiScribe T7 Quick High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2050S**
- RNasin plus **Promega Catalog #N2611**
- Maxima H- reverse transcriptase **Thermo Scientific Catalog #EP0751**
- Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447S**
- 0.5 M EDTA **Ambion Catalog #AM9261**
- 1 N NaOH **Vwr Catalog #JT5635-2**
- Nuclease-free water **Ambion Catalog #AM9932**
- 100% Ethanol (KOPTEC) **Vwr Catalog #89125-186**
- D/RNAaseFree **Vwr Catalog #47751-044**
- 1.5 mL LoBind tubes **Eppendorf Catalog #022431021**
- PCR tubes **Contributed by users**
- USER Enzyme - 250 units **New England Biolabs Catalog #M5505L**

Required Equipment

The following protocols will require the following 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)

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE START INSTRUCTIONS

Quick Overview:

Part 1 - PCR Amplification
Step 1 -Prepare the PCR reaction
Step 2- Amplify the template
Step 3 -Purify the template
Step 4–Quality control for template reaction (optional)

Part 2 - In-vitro Transcription
Step 1 – In-vitro Transcription
Step 2 – Quality control of in-vitro transcription (optional)

Part 3 - Reverse Transcription of RNA to DNA
Step 1 – Reverse transcription of mRNA with Uracil Modified Primer.

Part 4 – USER Enzyme Digest
Step 1 – USER Enzyme Digest
Step 2 – Alkaline Hydrolysis
Step 3 – Purification of ssDNA product.
Step 4 – Concentration of Probe.
Step 5 - Quality control of USER enzyme digest product (optional)

v1.5 revision notes
1. *Updated final elution volume to 150µL per column per manufacturer.*

Part 1 - PCR Amplification - Step 1: Prepare the PCR reactio..

1 In a 1.7 mL Eppendorf tube, mix the following:
- 40 µL 20X Eva Green ;
- 2 µL 200 µM forward primer ;
- 2 µL 200 µM reverse primer ;
- 1 µL of 80 ng/µL complex oligopool ;
- 355 µL nuclease free water ;
- 400 µL 2X Phusion hot start polymerase master mix .

2 Aliquot 25 µL volumes into 32 PCR tubes.

Part 1 - PCR Amplification - Step 2: Amplify the template

3m 35s

3 Run the following protocol on a qPCR machine: 1m 5s

1) 98 °C for 00:00:30 ;
2) 98 °C for 00:00:10 ;
3) * 63 °C for 00:00:10 ;
4) 72 °C for 00:00:15 ;
5) Measure the fluorescence of each sample.
*Adjust temp for the specific primer pair.

4 Repeat **cycle steps 2** through **5** from **step 3** until the rate at which the sample amplification decreases and starts to reach a plateau.

5 Remove samples during the 72 °C elongation step. Since the oligo pool consists of a complex number of sequences, samples removed outside of the elongation step will likely lead to hybridization with complement mismatches. The simplest method would be to run 10-14 rounds of PCR amplification and allow the instrument to complete the final round. If it is uncertain how many rounds you need to run for a particular oligo pool, you can set the instrument for an estimated number of rounds and then stop the round at any 72°C elongation step.

Part 1 - PCR Amplification - Step 3: Purify the template.

1m

6 Column purify to remove enzyme, nucleotides, and primers.
In a 15 mL Falcon tube, mix the following:
800 µL of the PCR reaction generated in **Step 5**;
4 mL of DNA binding buffer .

7 Run this mixture across a 100-µg capacity column using either a vacuum manifold.

8 Wash the column twice with 300 µL DNA wash buffer, spinning the column in a table top centrifuge at maximum speed for 00:00:30 each time. 30s

9 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 00:00:30. 30s

10 Set aside 10 µL of this reaction for quality control.

Part 1 - PCR Amplification - Step 4: (Optional) Quality contr..

11 Two quality control steps can be performed to verify the quality of your PCR product.
1. Using a spectrophotometer, such as the Nanodrop or similar, measure the concentration of dsDNA in your product. The concentration should be between [M] 0.01 µg/µL to [M] 0.05 µg/µL.
2. The second quality control step is gel electrophoresis and will be described in **part 4, step 5 (>>step 36 below).**

Part 2 – In-Vitro Transcription

12 The second step of this protocol is a high yield in vitro transcription reaction that further amplifies the template molecules created in Part 1 and converts them into RNA.

Part 2 – In-Vitro Transcription - Step 1: In-vitro transcriptio... 1d 18h

13 In a fresh 1.7 mL Eppendorf tube, mix the following:
160 µL of the in vitro template created in **Part 1**.
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
25 µL T7 polymerase (from the same HiScribe kit)

14 Incubate the reaction in a 37 °C incubator or dry bath for 12:00:00 – 16:00:00 hours. While the reaction is complete after 6-8 hours, it is convenient to leave this reaction overnight. Remove 20 µL for quality control. 1d 4h

Part 2 – In-Vitro Transcription - Step 2: (Optional) Quality c... 1m

15 To confirm that the in vitro transcription was successful, column purify the reaction then measure its concentration with a spectrophotometer.

To purify, mix the following:
- 20 µL of the in vitro reaction
- 30 µL nuclease-free water
- 100 µL RNA binding buffer
- 150 µL 100% ethanol .

16 Pass across a 100-ug-capacity spin column in a table-top centrifuge.

17 Wash this column once with 400 µL RNA prep buffer , centrifuge 00:00:30 top speed. 30s

18 Wash twice with 200 µL RNA wash buffer , centrifuge 00:00:30 top speed. 30s

19 Elute the RNA with 100 µL nuclease-free water .

20 If successful, the concentration of the in vitro transcription should be between [M] 0.5 µg/µL to [M] 2 µg/µL .

21 Purified RNA can also be run on a gel as described in **Part 4**, **Step 5 (>>step 36 below).**

Part 3 - Reverse Transcription RNA to DNA

22 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. To cleave off the 5' priming region and shorten the final probe, we use a uracil-modified reverse transcription primer for the reverse transcription reaction followed by use of a Uracil-Specfic Excision Reagent (USER Enzyme) to cleave the uracil nucleotide and remove the primer. This RNA templates are then removed via alkaline hydrolysis, and the final encoding probes are purified and concentrated.

Part 3 - Reverse Transcription RNA to DNA - Step 1: Revers... 1h

23 To the unpurified in vitro transcription created in **Part 2**, add the following and mix well:
- 200 µL 10 mM dNTP mix
- 120 µL 200 µM Reverse Primer with uracil modification.
- 240 µL 5X Maxima buffer
- 24 µL RNasin Plus
- 24 µL Maxima H- reverse transcriptase .

24 Incubate in a 50 °C water bath for 01:00:00 hour. It is important to use a water bath, not an air incubator, to ensure that the temperature of the sample rises to 50 °C quickly. 1h

Part 4 – USER Enzyme Digest & Purification of Encoding Pro... 1d

25 To digest, use a 1:20 (vol/vol) and incubate at 37 °C for 24:00:00 to cleave off the priming region at the site of the uracil. 1d

Part 4 – USER Enzyme Digest & Purification of Encoding Pro.. 1d

26 Split the above reaction into two 1.7-mL Eppendorf tubes and add the following to each:
- 300 µL 0.5 M EDTA
- 300 µL 1 N NaOH

27 Incubate in a 95 °C water bath for 00:15:00. 15m

Part 4 – USER Enzyme Digest & Purification of Encoding Pro.. 1d

28 Combine the two aliquots above into a single 50 mL Falcon tube and add the following:
- 4.8 mL Oligo binding buffer
- 19.2 mL 100% ethanol .

29 Mix well and split equally between eight 100-µg capacity spin columns.

30 Pull the sample across the columns with a vacuum manifold or via centrifugation.

31 Wash the columns once with 750 µL DNA wash buffer. Centrifuge to remove all of the wash buffer. You may need to centrifuge this twice to fully remove all of the wash buffer.

32 Elute the columns using 100 µL of nuclease-free water .

33 Combine eluates and set aside 10 µL for quality control.

Part 4 – USER Enzyme Digest & Purification of Encoding Pro..

1d

34 Use a vacuum concentrator to dry the samples. This process could take several hours. Re-suspend the dried pellet in 24 µL nuclease-free water, or if desired, hybridization buffer. Store probe at -20 °C and avoid unnecessary Freeze-thaw cycles.

35 If vacuum concentrator isn't available, concentrate probe using ethanol precipitation (refer to SOP009).

36 Quality Control. Analyze the concentration and the purity of your finalized probe sample on a Nanodrop spectophotometer or similar. Your ssDNA concentration should be around 34µg/µL.

Part 4 – USER Enzyme Digest & Purification of Encoding Pro..

1d

37 We suggest running your PCR product, IVT product and your final probe product on a 15% TBE-urea polyacrylamide gel to review the size and quality of your products. Smearing can indicate degradation of your product, particularly for RNA molecules. You should also note the size of the bands your are expecting to see and compare each band to one another. IVT will shorten your product by 20nt removing the 3' primer as the reaction only occurs after the T7 promoter region at the 3' end of the probe. Additionally, the USER enzyme digested product should be approximately 20nt shorter as this removes the 5' priming region. These changes in the product lengths should be visible when running your gel.