Preparation of Encoding Probes SOP005.v1.2 (PCR, In-vitro Transcription, Reverse Transcription and USER ENZYME Digest) V.2

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Human Cell Atlas Method Development Community

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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.

Preparation of Encoding Probes SOP005.v1.2.pdf

Rory Kruithoff, Douglas Shepherd 2021. Preparation of Encoding Probes SOP005.v1.2 (PCR, In-vitro Transcription, Reverse Transcription and USER ENZYME Digest). **protocols.io** https://protocols.io/view/preparation-of-encoding-probes-sop005-v1-2-pcr-in-byx3pxqn Rory Kruithoff

protocol

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

PCR, amplification, qPCR, In-vitro transcription, probe, USER enzyme, Uracil, specific, excision, reagent, alkaline hydrolysis, DNA, oligo, libary, encoding probe, reverse transcription

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☒20X

EvaGreen **Biotium Catalog #31000**

☒Phusion Hot Start Flex 2X Master Mix - 100 rxns **New England Biolabs Catalog #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)

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

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)

v1.2 revision notes

1. *Updated document summary.*
2. *Removed primer selection from part 1 which is more applicable to probe design than preparation.*

Part 1 - PCR Amplification - Step 1: Prepare the PCR reaction

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 **50 µL volumes** into 16 PCR tubes.

Part 1 - PCR Amplification - Step 2: Amplify the template 3m 35s

3m 35s

3 Run the following protocol on a qPCR machine:
1) **98 °C** for **00:03:00** ;
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 30s

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.

9 30s

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** .

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

Part 1 - PCR Amplification - Step 4: (Optional) Quality control for template reaction.

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 **0.01 µg/µL** to **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 which amplifies each PCR product multiple times while converting the product to RNA complements.

Part 2 – In-Vitro Transcription - Step 1: In-vitro transcription. 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 16h

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 **06:00:00** - **08:00:00** hours, it is convenient to leave this reaction **Overnight** . Remove **20 µL** for quality control.

Part 2 – In-Vitro Transcription - Step 2: (Optional) Quality control for the in vitro transcription. 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 30s

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

18 30s

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

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

20 If successful, the concentration of the in vitro transcription should be between **0.5 µg/µL** to **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, our RNA molecules are used as Reverse Transcription templates to convert the large quantities of RNA produced by the high yield in vitro transcription to single-stranded DNA. 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. The RNA template is then removed via alkaline hydrolysis, and the final encoding probes are purified and concentrated.

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

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.

Part 4 – USER Enzyme Digest & Purification of Encoding Probes - Step 1: USER Enzyme Digest 1d

25 1d

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.

Part 4 – USER Enzyme Digest & Purification of Encoding Probes - Step 2: Alkaline Hydrolysis 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  15m

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

Part 4 – USER Enzyme Digest & Purification of Encoding Probes - Step 3: Purification of ssDNA Product 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 Probes - Step 4: Concentration of Probe 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 Probes - Step 5: (Optional) Quality control of in vitro template, RNA, and probe.
1d

37 We recommend 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.