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Labeling Protocol for myTags Immortal Libraries

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Reagents and Equipment

Please check all kit instructions for protocol updates.

Part 1. PCR Amplification

KAPA HiFi HotStart ReadyMix (KAPA Biosystems; #KK2601)

Part 2. *In vitro* Transcription

MEGAshortscript™ T7 Kit (Invitrogen; #AM1354)

Part 3. Reverse Transcription

Superscript II (Invitrogen; #18064014)

10mM deoxynucleotide triphosphate (dNTP) mix (New England Biolabs; #N0447S)

SUPERase• In™ RNase Inhibitor (Invitrogen; #M2696)

Exonuclease I with 10X buffer (New England Biolabs; #M0293S)

0.5M EDTA, pH 8.0 (Sigma; #03690)

Labeled primer (/5' Dye/ CGTGGTCGCGTCTCA)

Use dual-HPLC purification for fluorophore-labeled primers. Please check with primer vendor (IDT-DNA, Bio-synthesis, Operon, etc) for dye quenching

Part 4. RNA Digestion

RNase H with 10X buffer (New England Biolabs; #M0297S)

RNase A (Thermo Scientific; #EN0531)

Purification Kits

QIAquick PCR Purification Kit (Qiagen; #28104)

RNeasy Mini Kit (Qiagen; #74104)

Quick-RNA MiniPrep (Zymo; #R1054S).

Molecular-biology grade ethanol (96-100%)

Nuclease-free water

Equipment

Vortex

Mini-centrifuge *(for all "quick spin down" steps)*

Micro-centrifuge *(for all "centrifuge" steps)*

Thermo cycler

Agarose and polyacrylamide gel electrophoresis setup

Materials provided by Arbor Biosciences

Store at -20°C.

myTags PCR primer mix, 50 reactions

myTags immortal library

Resuspend at 1 ng/µl in 10 mM Tris-HCl pH 7.5 or nuclease-free water. Prepare working stock aliquots (0.07 ng/µl) by diluting 2 µl of immortal library in 28 µl nuclease-free water.

Labeling Protocol

Generating labeled probes from an immortal myTags probe library.

Part 1. PCR Amplification

1A. Debubbling PCR Amplification

1. Assemble the master mix and debubbling mix in separate 0.2 ml PCR reaction tubes as indicated in the table.
2. Vortex for 5 sec and quick spin down in mini-centrifuge for 5 sec.
3. Set the debubbling mix aside on ice or at 4°C.
4. Pipet 5 µl of the master mix into a third PCR tube and set aside as a negative control.
5. Add 2.5 µl of the working stock myTags immortal library (0.07 ng/µl) to 45 µl of the master mix and mix by pipetting.
6. Perform PCR Cycling Conditions (Program 1) on both the negative control and template master mix in thermo cycler.
7. At step 10 of the PCR Cycling Conditions, leave the negative control in the thermocycler at 24°C and remove the template master mix. Add 20 µl debubbling mix to the template master mix and mix by pipetting. Return the template master mix + debubbling mix to the thermocycler and continue with Program 2 for both the template master mix + debubbling mix (hereafter "template PCR sample") and the negative control.
8. At the completion of the PCR Cycling Conditions, store both the negative control and the template PCR sample at -20°C or proceed to step 9.

Components	Master Mix (50 µl)	Debubbling Mix (20µl)
KAPA HiFi Hotstart Readymix	25 µl	10 µl
myTags PCR Primer Mix	0.25 µl	1.2 µl
Nuclease-free water	24.75 µl	8.8 µl

PCR Cycling Conditions *(hot lid on)*		
Program 1	1	95°C - 3 min
	2	98°C - 20 sec
	3	54°C - 15 sec
	4	72°C - 30 sec
	5	Repeat steps 2-4, 4 times
	6	98°C - 20 sec
	7	56°C - 15 sec
	8	72°C - 30 sec
	9	Repeat steps 6-8, 14 times
	10	Hold at 24°C
Program 2	11	95°C - 3 min
	12	98°C - 20 sec
	13	54°C - 15 sec
	14	72°C - 30 sec
	15	Repeat steps 12-14, 1 time
	16	Hold at 24°C to end program

1B. Agarose Gel Electrophoresis

9. After PCR cycling is complete, mix 2 µl of a non-denaturing gel loading buffer (such as 5x green GoTaq, Promega M8911) with 5 µl of the template PCR sample, bring volume to 10µl with nuclease-free water to make the temple PCR gel sample.
10. Add 1 µl of the non-denaturing gel loading buffer to the negative control gel sample.
11. Load the template PCR gel sample, negative control gel sample, and a low molecular weight ladder in separate wells on a 2.5% agarose gel to verify the length of the PCR product. The expected band size of the desired PCR product for the template PCR gel sample is 99bp (for probe length of 45nt). The negative control sample should show no bands at 99bp, however you may see a primer band around 40-50 bp.
12. After verifying the PCR product length of the template PCR gel sample at 99bp and the absence of a 99bp band in the negative control gel sample, continue to step 13.

1C. DNA Purification - QIAquick PCR Purification Kit

13. Prepare a QIAquick spin column and 2mL collection tube.
14. In a 1.5 ml tube, combine 5 µl of 3M sodium acetate solution and the template PCR sample. Add nuclease-free water (35 µl) to bring the volume to 100 µl.
15. Add 500 µl of Qiagen Buffer PB to the sample and vortex for 5 sec.
16. Apply the entire sample to the QIAquick column and centrifuge for 60 sec at 13,000 rpm (17,900 x g).
17. Discard flow-through, return the QIAquick column to the collection tube.
18. Add 700 µl of Qiagen Buffer PE to the QIAquick column and centrifuge for 60 sec at 13,000 rpm.
19. Discard flow-through, return the QIAquick column to the collection tube and centrifuge for an additional 2 min at 13,000 rpm.
20. Place QIAquick column in a 1.5 ml microcentrifuge tube and add 30 µl of nuclease-free water (or Qiagen Buffer EB) to the center of the QIAquick membrane. Let the column stand for 1 min then centrifuge for 1 min at 13,000 rpm.
21. Quantify the purified dsDNA using Nanodrop or other method. A minimum yield of 480 ng is required for *in vitro* transcription.
22. Store at -20°C or proceed to Part 2: *In Vitro* Transcription

Part 2. *In vitro* Transcription

2A. In vitro Transcription – MEGAshortscript™ T7 Kit

Please check all kit instructions for protocol updates. Pool 52 µl of each rNTP in one tube labeled "rNTP mix".

1. In a 0.2 ml tube, assemble the *in vitro* transcription (IVT) mix on ice.

Components	*In vitro* Transcription Mix (40 µl)
480 ng DNA, add nuclease-free water to volume	16 µl
T7 10X Reaction Buffer	4 µl
Four rNTP pool	16 µl
T7 Enzyme Mix	4 µl

2. Vortex for 5 sec and quick spin down in a mini-centrifuge for 5 sec.
3. Incubate in thermocycler at 37°C for 4 hr (with hot lid on, set to 42°C).
4. Store at -80°C, or proceed to step 5.

2B. RNA Purification – RNeasy Mini Kit

5. Prepare 2 RNeasy spin columns in 2 ml collection tubes.
6. In a 1.5 ml tube, combine 160 µl of RNase-free water with the IVT sample, vortex 5 sec.
7. Add 700 µl of Qiagen Buffer RLT and vortex 5 sec.
8. Add 500 µl of ethanol and vortex 5 sec. Proceed immediately to step 9.
9. Transfer 700 µl of sample to each RNeasy spin column. Centrifuge for 60 sec at 12,000 rpm (>= 8,000 x g) , discard flow-through.
10. Add 500 µl of Qiagen Buffer RPE to each RNeasy spin column. Centrifuge for 60 sec at 12,000 rpm, discard flow-through.
11. Repeat step 10 once.
12. Place each RNeasy spin column in a new 2 ml collection tube. Centrifuge at 12,000 rpm for 3 min.
13. Place each RNeasy spin column in a new 1.5 ml tube for elution.
14. Add 50 µl nuclease-free H_2O directly to the membrane. Let RNeasy spin column stand for 1 min then centrifuge for 1 min at 12,000 rpm to elute the RNA sample.
15. Repeat step 14 once.
16. Pool the elutions and quantify the RNA using Nanodrop. A minimum yield of 42 µg of RNA is required to proceed to reverse transcription. RNA can be dried in a speed vacuum centrifuge on the lowest heat setting to achieve the desired concentration/volume for reverse transcription.
17. Store the RNA sample at -80°C or proceed to Part 3: Reverse Transcription.

Minimizing RNA storage by converting all the RNA into ssDNA probes as soon as possible prevents degradation.

Part 3. Reverse Transcription

3A. Reverse Transcription - SuperScript II Reverse Transcriptase

1. In a 0.2 ml tube, assemble mix 1 on ice.

Components	Mix 1 (60 µl)
42 µg RNA, add nuclease-free water to volume	41.6 µl
1 mM (1 nmol/ µl) labeled primer	2.4 µl
10 mM dNTP	15 µl
20 U/µl SUPERase-In	1 µl

2. Vortex for 5 seconds, quick spin down for 5 seconds.
3. Incubate in a thermocycler at 65°C (with hot lid on, set to 75°C) for 5 minutes, then chill on ice for 5 min. While waiting, proceed to step 4.
4. In a 0.2 ml tube, assemble mix 2 on ice.

Components	Mix 2 (35 µl)
Nuclease-free water	4 µl
5 X First-strand buffer	20 µl
0.1 M DTT	10 µl
20 U/ µl SUPERase-In	1 µl

5. Vortex for 5 sec, quick spin down for 5 sec, and store on ice while waiting to complete step 3.
6. Following the completion of steps 3-5, add Mix 2 to Mix 1 to assemble the reverse transcription (RT) mix. Vortex for 5 sec, quick spin down for 5 sec.
7. Incubate the reaction at 42°C (with hot lid on, set to 52°C) for 5 min.
8. Add 2.5 µl SuperScript II Reverse Transcriptase to RT mix, vortex for 5 sec, quick spin down for 5 sec.
9. Incubate the reaction at 42°C (with hot lid on, set to 52°C) for 2 hr.
10. Repeat steps 8-9. The total incubation time required to convert the RT mix into the RT sample is 4 hours at 42°C.
11. Store the RT sample at -20°C or proceed to step 12.

3A. Unincorporated Primer Digestion - Exonuclease I

Steps 12-17 are time-sensitive, after completing each step, proceed immediately to the next step.

12. Add 11 µl of Exonuclease I Buffer to RT sample.
13. Add 2 µl of Exonuclease I to RT sample. Vortex for 5 sec and quick spin down for 5 sec.
14. Incubate in thermo cycler at 37°C for 15 min.
15. Remove RT sample from the thermo cycler, then preheat thermo cycler to 80°C.
16. Add 12 µl 0.5 M EDTA to RT sample, vortex for 5 sec and quick spin down for 5 sec.
17. Incubate at 80°C in pre-heated thermo cycler for 20 min. Store on ice to cool.
18. Store at -20°C or proceed to step 19.

3B. ssDNA Purification - Zymo Quick-RNA

19. Prepare a Zymo-Spin™ IIICG spin column in a 2 ml collection tube.
20. In a 1.5 ml tube, mix 500 µl Zymo RNA Lysis Buffer and 125 µl RT sample. Vortex 5 sec.
21. Add 625 µl 100% ethanol. Vortex 5 sec.
22. Transfer 500 µl to the Zymo-Spin™ IIICG spin column and centrifuge for 30 sec at 13,000 rpm. Discard flow-through.

    The flow-through will have dye color which indicates successful removal of excess primer.
23. Repeat step 21 once to load the remainder of the sample on the Zymo-Spin™ IIICG spin column.
24. Add 400 µl Zymo RNA Prep Buffer to the Zymo-Spin™ IIICG spin column and centrifuge at 13,000 rpm for 30 sec. Discard flow-through.
25. Add 700 µl Zymo RNA Wash Buffer to the Zymo-Spin™ IIICG spin column and centrifuge at 13,000 rpm for 30 sec. Discard flow-through.
26. Add 400 µl Zymo RNA Wash Buffer to the Zymo-Spin™ IIICG spin column and centrifuge at 13,000 rpm for 30 sec. Discard flow-through.
27. Place the Zymo-Spin™ IIICG spin column back into collection tube and centrifuge at 13,000 rpm for 3 min. Discard collection tube with flow-through.
28. Place the Zymo-Spin™ IIICG spin column into a new 1.5 ml tube.
29. Add 42 µl room temperature nuclease-free water to the Zymo-Spin™ IIICG spin column matrix, wait 1 min, and then centrifuge at 13,000 rpm for 1 min to elute RNA:DNA hybrid and ssDNA.
30. Repeat step 29 once.
31. Store at -20°C or proceed to Part 4: RNA Digestion.

Part 4: RNA Digestion

DISCLAIMER: Unless extracted with phenol chloroform, probes are not guaranteed to be RNase free and should not be used for RNA FISH.

4A. RNA Digestion - RNase H and RNase A

1. In a 0.2 ml tube, assemble the RNA digestion mix on ice.

Components	RNA Digestion Mix (18 µl)
10X RNase H buffer	10 µl
5 U/ µl RNase H	4 µl
5 U/ µl RNase A	4 µl

2. Vortex for 5 sec and quick spin down for 5 sec.
3. In a 0.2 ml tube, combine 18 µl of RNase digestion mix with eluted RNA:DNA (~82-84 µl).
4. Vortex 5 sec and quick spin down for 5 sec.
5. Incubate in thermo cycler using the RNA Digestion Program.
6. Store at -20°C or proceed to step 7.

RNA Digestion Program *(hot lid on)*		
Step	Temperature	Time
1	37˚C	120 min
2	70˚C	20 min
3	50˚C	60 min
4	95˚C	5 min
5	Ramp down 95˚C to 50˚C	0.1 ˚C/sec
6	50˚C	60 min
7	4˚C	Hold

4B. ssDNA Purification - Zymo Quick-RNA

7. Preheat a minimum of 120 µl of nuclease-free water at 65˚C (for step 18, 19).
8. Prepare a Zymo-Spin™ IIICG spin column in a 2 ml collection tube.
9. In a 1.5 ml tube, mix 400 µl Zymo RNA Lysis Buffer and 100 µl RNA digestion sample. Vortex 5 sec.
10. Add 500 µl 100% ethanol. Vortex 5 sec.
11. Transfer 650 µl to Zymo-Spin™ IIICG column and centrifuge for 30 sec at 13,000 rpm. Discard flow-through.
12. Repeat step 12 once to load the remainder of the sample on the Zymo-Spin™ IIICG column.
13. Add 400 µl Zymo RNA Prep Buffer to the Zymo-Spin™ IIICG column and centrifuge at 13,000 rpm for 30 sec. Discard flow-through.
14. Add 700 µl Zymo RNA Wash Buffer to the Zymo-Spin™ IIICG column and centrifuge at 13,000 rpm for 30 sec. Discard flow-through.

15. Add 400 µl Zymo RNA Wash Buffer to the Zymo-Spin™ IIICG column and centrifuge at 13,000 rpm for 30 sec. Discard flow-through.
16. Place the Zymo-Spin™ IIICG column back into collection tube and centrifuge at 13,000 rpm for 3 min. Discard collection tube with flow-through.
17. Place the Zymo-Spin™ IIICG column in a new 1.5 ml tube.
18. Add 50 µl of pre-heated (65˚C) nuclease-free water to the Zymo-Spin™ IIICG column matrix, wait 1 min, and then centrifuge at 13,000 rpm for 1 min to elute the labeled ssDNA.
19. Repeat step 18.
20. Using Nanodrop, measure concentration of dye (pmol/µl) and single-stranded nucleic acid (ng/µl) from the labeled ssDNA under the Microarray setting.
21. Recover the labeled ssDNA sample from Nanodrop to analyze on 7% denaturing PAGE gel if desired. The expected band size for RNA and labeled ssDNA is 66nt.
22. To determine dye labeling efficiency, convert the nucleic acid reading in ng/µl to pmol/ µl and compare with dye pmol/µl. If the ratio for pmol/ µl dye : pmol/ µl ssDNA is not greater than 0.9, repeat Part 4: RNase Digestion.

    Labeled ssDNA size: 66 nt. 1 µg = 50 pmol.

Adapted from:

Murgha YE, Rouillard J-M, Gulari E (2014) Methods for the Preparation of Large Quantities of Complex Single-Stranded Oligonucleotide Libraries. PLoS ONE 9(4): e94752. DOI:10.1371/journal.pone.0094752

Murgha, Y, Beliveau B, Semrau K, Schwartz D, Wu C-T, Gulari E, Rouillard J-M (2015) Combined in vitro transcription and reverse transcription to amplify and label complex synthetic oligonucleotide probe libraries. BioTechniques 58:301-307. DOI: 10.2144/000114298