5840 Interface Drive, Suite 101
Ann Arbor, MI 48103
+1 (734) 998 0751
techsupport@arbor.daicel.com

Labeling Protocol for myTags Immortal Libraries

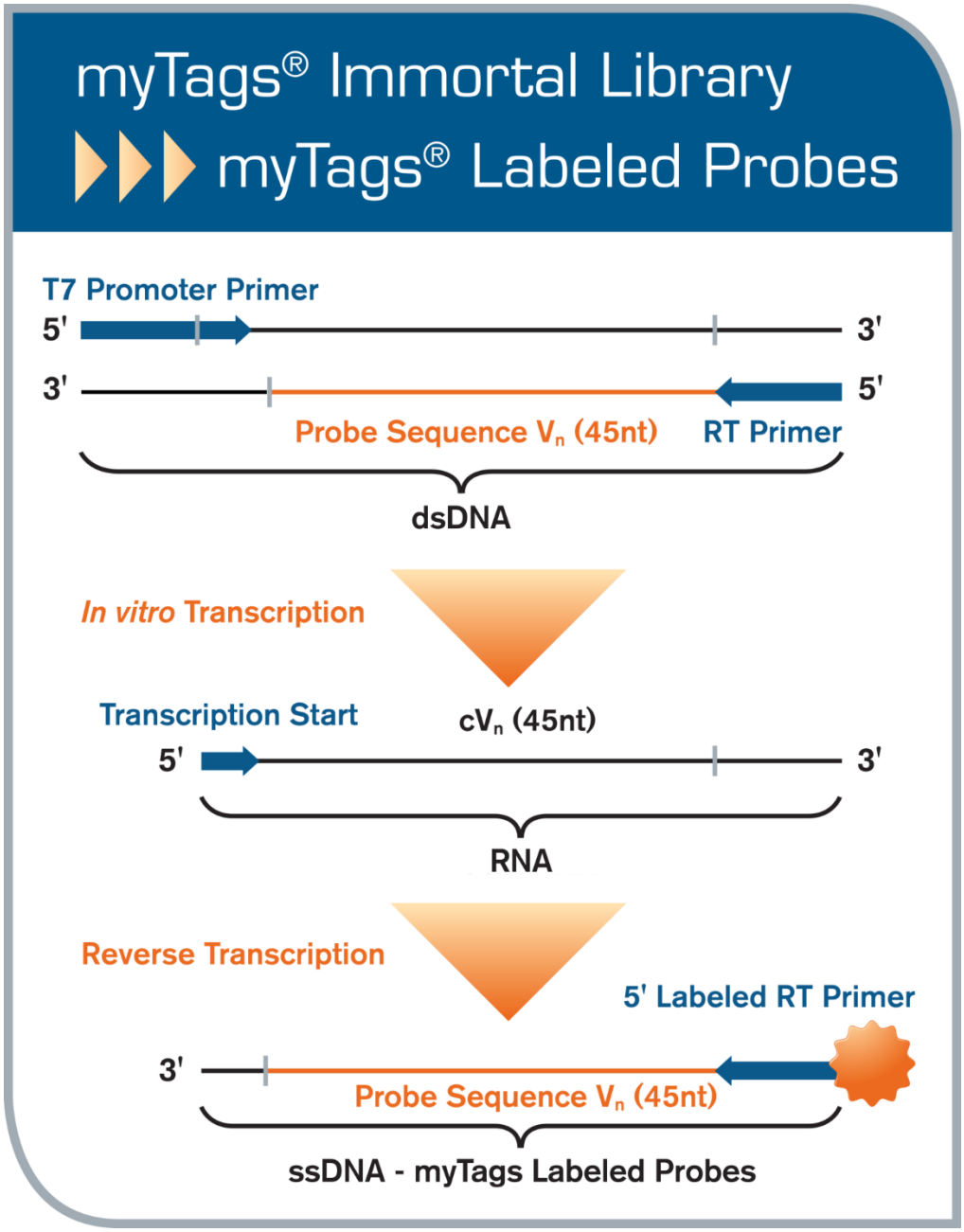
April 2022

Version 2.2

Contents

Purpose

The purpose of this procedure is to produce custom labeled single-stranded DNA libraries starting from a double-stranded myTags Immortal DNA library.



Materials, Equipment, and Reagents Provided by End User

Please check all kit instructions for protocol updates.

Materials

0.2 ml PCR tubes in strips with caps (VWR® Cat No. 93001-118)
1.5 ml Microcentrifuge Tubes (VWR Cat. No. 802050-504)
10 µl Pipette Filter Tips (VWR Cat. No. 89168-750)
20 µl Pipette Filter Tips (VWR Cat. No. 89140-932)
200 µl Pipette Filter Tips (VWR Cat. No. 89140-936)
1250 µl Pipette Filter Tips (VWR Cat. No. 89168-754)
Qiagen® QIAquick® PCR Purification Kit (Qiagen; Cat. No. #28104)
Macherey-Nagel® NucleoSpin® RNA Clean-up Kit (Macherey-Nagel; Cat. No. 740948.10/50/250)
Zymo® Quick-RNA MiniPrep Kit (Zymo; Cat. No. #R1054S)
Molecular biology grade ethanol (96-100%)
Low Molecular Weight DNA Ladder (New England Biolabs®; Cat. No.N3233S)
Nuclease-free water

Equipment

Vortex
Minicentrifuge (for 0.2 ml PCR tubes)
Microcentrifuge (for 1.5 ml tubes and column purifications)
Thermocycler (with a thermoblock for 0.2 ml PCR tubes)
Agarose gel electrophoresis setup
Instrument capable of UV absorbance-based quantification of nucleic acids and fluorophores (e.g., Nanodrop® from Thermo Fisher Scientific®; Cat. No. ND-2000)
Optional: Speed vacuum centrifuge, polyacrylamide gel electrophoresis setup

Reagents

Part 1. PCR Amplification

KAPA® HiFi HotStart ReadyMix (Roche Cat. No. KK2601)

Part 2. *In vitro* Transcription

MEGAshortscript™ T7 Kit (Thermo Fisher Sci.; Cat. No. AM1354)

Part 3. Reverse Transcription

Invitrogen® Superscript® IV Reverse Transcriptase (Thermo Fisher Sci.; Cat. No. 18090010)
10 mM deoxyribonucleotide triphosphate (dNTP) mix (New England Biolabs; Cat. No. N0447S or N0447L)
Invitrogen® SUPERase• In™ RNase Inhibitor (Thermo Fisher Sci.; Cat. No. AM2696)
Exonuclease I with 10X buffer (New England Biolabs; Cat. No. M0293S or M0293L)
0.5M EDTA, pH 8.0 (Millipore Sigma®; Cat. No. 03690-100 ML)

Labeled primer (/5' Dye/Spacer/CGTGGTCGCGTCTCA 3') – see appendix

Use HPLC purification for fluorophore-labeled primers. Please check with primer vendor (e.g. IDT®, Bio-Synthesis, Inc., Eurofins®, etc.) for dye quenching. Spacer can be triethylene glycol (iSp9 for IDT) or similar.

Part 4. RNA Digestion

RNase H with 10X buffer (New England Biolabs; Cat. No. M0297S or M0297L)

RNase A (Thermo Fisher Sci.; Cat. No. EN0531)

Materials, Equipment, and Reagents Provided by Daicel Arbor Biosciences

myTags® PCR Primer Mix, 7.5 nmoles delivered dry, 50 reactions

myTags® Custom immortal DNA library (200 ng delivered dry)

Store all at -20°C.

Labeling Protocol

Generating labeled probes from a myTags immortal library.

Part 1. PCR Amplification of Immortal Library

1A. PCR Amplification and Debubbling

1. Briefly centrifuge myTags PCR Primer Mix tube and resuspend primers in 75 µl nuclease-free water. Final concentration will be 100 µM in 10 mM Tris-HCl and 0.1 mM EDTA pH 8.0.
2. Briefly centrifuge myTags Immortal DNA Library tube and resuspend myTags Immortal Library at 1 ng/µl by adding 200 µl 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 26 µl nuclease-free water.
3. Assemble the master mix and debubbling mix in separate 0.2 ml PCR reaction tubes as indicated in the table. Scale up according to the desired number of reactions and use 1.5 ml tubes if necessary.

Components (1 reaction)	**Master Mix**	**Debubbling Mix**
2X 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
Total	50 µl	20 µl

4. Mix by pipetting and spin down in minicentrifuge for 5 sec.
5. Set the debubbling mix aside on ice or at 4°C.
6. Pipet 5 µl of the master mix into a third 0.2 ml PCR tube as a negative control (optional).
7. Assemble the template master mix by adding 2.5 µl of the working stock myTags immortal library template (0.07 ng/µl) to the remaining 45 µl of master mix and mix by pipetting. Spin down 5 seconds in a minicentrifuge.
8. Perform PCR Cycle Program 1 on both the negative control and template master mix in thermocycler. Use hot lid (+5°C).

PCR Cycle					
Program 1	1	95°C 3 min	**Program 2**	7	95°C 3 min
	2	98°C 20 sec		8	98°C 20 sec
	3	56°C 15 sec		9	56°C 15 sec
	4	72°C 30 sec		10	72°C 30 sec
	5	Go to step 2 19 more times		11	Go to step 8 1 more time
	6	Hold at 24°C		12	Hold at 24°C

9. To visualize PCR product: Before PCR cycle is complete, pour a 1.8% agarose 1X TAE or TBE gel with sufficient wells for a DNA ladder and your PCR samples including the negative control.
10. At step 6 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, mix by pipetting and briefly spin in a minicentrifuge. Return sample to the thermocycler and continue with Program 2 for both the template master mix + debubbling mix (hereafter "PCR sample") and the negative control.
11. At the completion of the PCR, store both the negative control and the PCR sample at -20°C or proceed to step 12.

1B. Agarose Gel Electrophoresis

12. After PCR cycling is complete, mix 2 µl of a non-denaturing gel loading buffer (such as 6X NEB Loading buffer included with the low molecular weight DNA ladder) with 5 µl of the PCR sample. Adjust volume to 12 µl with nuclease-free water to make the PCR gel sample.
13. Add 1 µl of the non-denaturing gel loading buffer to the 5 µl negative control gel sample.
14. Load the library PCR gel sample, negative control gel sample, and the NEB low molecular weight ladder in separate wells on a 1.8% agarose 1X TAE or TBE gel to verify the length of the PCR product. The expected band size of the desired PCR product for the PCR sample is 99 bp (for probe length of 45 nt). The negative control sample should show no bands at 99 bp, however you may see a primer band around 40-50 bp.
15. After verifying the PCR product length of 99 bp* and the absence of a 99 bp band in the negative control gel sample, continue to step 16.

**** Note: Immortal libraries made using our Indexing Service are 115 bp in length, not 99 bp***

1C. DNA Purification

16. Place a QIAquick spin column in a 2 ml collection tube (from Qiagen QIAquick PCR purification kit)
17. Transfer PCR sample to a 1.5 ml tube and bring the volume up to 100µl with 35 µl nuclease-free water.
18. Add 500 µl of Qiagen Buffer PB to the sample and vortex for 5 sec.
19. Apply the entire 600 µl sample to the QIAquick column and spin for 1 min at 17,900 RCF in a microcentrifuge.
20. Discard flow-through, return the QIAquick column to the collection tube.
21. Add 700 µl of Qiagen Buffer PE (with ethanol added) to the QIAquick column and spin for 1 min at 17,900 RCF.
22. Discard flow-through, return the QIAquick column to the collection tube and spin for an additional 3 min at 17,900 RCF.
23. Discard collection tube. Place QIAquick column in a labeled 1.5 ml microcentrifuge tube and add 30 µl of nuclease-free water to the center of the QIAquick matrix. Let the column stand for 1 min then spin for 1 min at 17,900 RCF in a microcentrifuge to elute the dsDNA.
24. Quantify the purified dsDNA using a Nanodrop or another absorbance-based method. Record dsDNA concentration, A260/280 and A260/230 values. A260/280 value should be between 1.8-2.0 and A260/230 value should be 1.8-2.3. Significant deviation from these values could signal contaminants

that may compromise the *in vitro* T7 Transcription step (likely due to salt or ethanol). A minimum yield of 480 ng dsDNA is required for *in vitro* transcription (Part 2). If the concentration is less than 30 ng/µl, drying down the dsDNA template will be necessary to fit an appropriate volume in the T7 transcription reaction.

25. Store dsDNA at -20°C or proceed to Part 2: *In Vitro* Transcription.

Part 2. *In vitro* Transcription

2A. *In vitro* Transcription

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

1. Follow Thermo Fisher Sci. MEGAshortscript T7 kit instructions with the following modifications to the initial reaction mix. Thaw 10X reaction buffer and rNTPs at room temperature, place kit components on ice immediately after use. Vortex 10X reaction buffer briefly prior to use. Inspect 10X reaction buffer for precipitate, if present heat at 37°C 10 minutes and vortex to dissolve precipitate.
   In a 0.2 ml tube, assemble the *in vitro* transcription (IVT) mix at room temperature in the order given below.

Components	***In vitro* Transcription Mix**
Nuclease-free water (16 uL-volume of DNA)	__ µl
T7 10X Reaction Buffer	4 µl
rNTP Mix	16 µl
480 ng dsDNA Template	__ µl
T7 Enzyme Mix	4 µl
Total	40 µl

2. Mix by pipetting and quick spin down in a minicentrifuge for 5 sec.
3. Incubate at 37°C for 4 hr (with hot lid on, set to 42°C) to generate the IVT sample. It is not necessary to treat the RNA with DNase.
4. Store transcription reaction containing RNA at -80°C or proceed to step 5.

2B. RNA Purification

5. This section uses the Macherey-Nagel RNA Clean-Up Mini Kit. Before starting this purification, be sure to check if the appropriate amount of 100% ethanol was added to Wash Buffer RA3 for your chosen kit size (see kit instructions).
6. Assemble as many Nucleospin RNA clean-up columns and collection tubes as needed (one per transcription reaction). Also, obtain one 1.5 ml tube per reaction and label all tubes, columns, and collection tubes accordingly.
7. Add 60 µl nuclease-free water to the transcription reaction from Part 2A for a total volume of 100 µl.
8. Mix 300 µl Buffer RA1 with 300 µl 100% ethanol for each transcription reaction in a 1.5 ml tube.

9. Add the 100 µl transcription reaction from step 6 to the prepared RA1/Ethanol mix from step 8. Mix the solution with a pipette and transfer the entire volume to a NucleoSpin RNA clean-up column. Centrifuge 1 min. at 8000 RCF. Discard flow-through from collection tube.
10. 1st Wash: Add 700 µl Buffer RA3 to the Nucleospin column and centrifuge 1 min. at 8000 RCF. Discard flow-through from collection tube.
11. 2nd Wash: Add 350 µl Buffer RA3 to the Nucleospin column and centrifuge 2 mins. at 8000 RCF. Discard flow-through from collection tube.
12. Place column into a new, labeled, 1.5 ml tube. Open column lid and let air dry 3 min.
13. Add 50 µl RNase-free water directly to the center of the column, let sit 1 min. and centrifuge 2 mins. at 8000 RCF.
14. Remove column and quantify the RNA using a Nanodrop or similar UV absorbance-based method. Concentration should be 1 µg/µl or greater. If the concentration is under 1 µg/µl drying down the RNA and resuspending in a smaller volume may be required prior to Part 3.
15. 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

Fluorophores are light-sensitive. Minimize light exposure of the labeled primer stock and the sample.

3A. Reverse Transcription

1. In a 0.2 ml tube, assemble Mix 1 at room temperature. Mix by pipetting, spin down for 5 seconds in a minicentrifuge.

Components	Mix 1
52 µg RNA, add nuclease-free water to volume*	42 µl
Labeled primer 1 mM (1 nmol/ µl)	2.0 µl
dNTPs 10 mM	15 µl
SUPERase-In 20 U/µl	1 µl
Total	60 µl

*If more volume is needed for RNA, use up to 48.5 µl volume here and leave out water in Mix 2 of step 3.

2. 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 3.
3. In a 0.2 ml PCR tube, assemble Mix 2 at room temp. Thaw 5X buffer and DTT at room temperature. Vortex 5X buffer and check for precipitate. If present, heat at 37°C for 5 minutes and vortex buffer (repeat until most or all precipitate is dissolved). A master mix can be prepared for Mix 2 in a 1.5 ml tube to save time.

Components	Mix 2
Nuclease-free water	6.5 µl
5 X RT buffer	20 µl
0.1 M DTT	10 µl
20 U/ µl SUPERase-In	1 µl
SuperScript IV Reverse Transcriptase	2.5 µl
Total	40 µl

4. Mix by pipetting, spin down for 5 sec in a minicentrifuge and store at room temperature while waiting for step 2 to complete.
5. Following the completion of steps 1-4, add Mix 2 to Mix 1 to assemble the Reverse Transcription (RT) Mix. Mix by pipetting and spin down for 5 sec in a minicentrifuge.
6. Incubate the reaction at 50°C (with hot lid on, set to 55°C) for 2 hr.
7. Store the RT sample at -20°C or proceed to step 8.

3B. Unincorporated Primer Digestion

Steps 8-15 are time-sensitive, after completing each step, proceed immediately to the next step.

8. Thaw Exonuclease I buffer at room temperature. Add 11 µl of Exonuclease I Buffer to RT sample.
9. Add 2 µl of Exonuclease I to RT sample.
10. Mix by pipetting and spin down for 5 sec in a minicentrifuge.
11. Incubate in thermocycler at 37°C for 15 min.
12. Remove RT sample from the thermocycler, place on ice, and preheat thermocycler to 80°C.
13. Add 12 µl 0.5 M EDTA pH 8.0 to RT sample, mix by pipetting and spin for 5 sec in a minicentrifuge.
14. Incubate at 80°C in pre-heated thermocycler for 20 min. Place on ice to cool for 5 min.
15. Store the resulting RNA:DNA hybrid product at -20°C or proceed to step 16.

3C. RNA:DNA Hybrid Purification

16. Place a Zymo-Spin™ IIICG spin column in a 2 ml collection tube (from Zymo Quick-RNA Miniprep Kit)
17. In a 1.5 ml tube, mix 500 µl Zymo RNA Lysis Buffer and 125 µl RT sample. Mix by pipetting.
18. Add 625 µl 96-100% ethanol. Mix by pipetting.
19. Transfer 625 µl to the Zymo-Spin IIICG spin column and centrifuge for 30 sec at 11,000 RCF. Discard flow-through.

    Note: *When a fluorescent primer is used, the flow-through will likely appear colored which indicates successful removal of excess primer. Typically, the color is also visible in the column matrix due to the binding of the RNA:DNA hybrid that is also labeled (note not all fluorescent dyes can be easily seen in the gel matrix, Atto488 for example can be hard to see).*
20. Repeat step 19 once to load the remainder of the sample on the Zymo-Spin IIICG spin column.
21. Add 400 µl Zymo RNA Prep Buffer to the spin column and centrifuge at 11,000 RCF for 30 sec. Discard flow-through.

22. Add 700 µl Zymo RNA Wash Buffer to the spin column and centrifuge at 11,000 RCF for 30 sec. Discard flow-through.
23. Add 400 µl Zymo RNA Wash Buffer to the spin column and centrifuge at 11,000 RCF for 30 sec. Discard flow-through.
24. Place the spin column back into collection tube and centrifuge at 11,000 RCF for 3 min. Discard collection tube.
25. Place the spin column into a new 1.5 ml tube.
26. Add 42 µl room temperature nuclease-free water to the spin column matrix, wait 1 min, and then centrifuge at 11,000 RCF for 1 min to elute RNA:DNA hybrid.
27. Repeat step 26.
28. Store eluate at -20°C or proceed to Part 4: RNA Digestion.

Part 4: RNA Digestion of RNA:DNA Hybrid

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

1. Thaw the 10X RNase H buffer at room temperature and vortex. If precipitate is present in the buffer, heat at 37°C for 10 minutes and vortex to dissolve the precipitate.
2. In a 0.2 ml tube, assemble the RNA digestion mix at room temperature. A master mix can also be made and then aliquoted into individual 0.2 ml tubes.

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
Total	18 µl

RNA Digestion Program		
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

3. Mix by pipetting and spin down in a minicentrifuge for 5 sec.
4. Add all eluted RNA:DNA hybrid (~82-84 µl) from Part 3C to the 18 µl of RNase digestion mix in a 0.2 ml tube.
5. Mix by pipetting and spin down in a minicentrifuge for 5 sec.
6. Incubate in thermocycler using the RNA Digestion Program with the hot lid on.
7. Store at -20°C or proceed to step 8.

4B. ssDNA Purification

8. Preheat a minimum of 120 µl of nuclease-free water per reaction in 0.2 ml tubes at 65˚C on a thermocycler (for step 20-21).
9. Place a Zymo-Spin IIICG spin column in a 2 ml collection tube (from Zymo Quick-RNA Miniprep Kit).
10. In a 1.5 ml tube, mix 400 µl Zymo RNA Lysis Buffer and 100 µl RNA digestion sample. Mix by pipetting.
11. Add 500 µl 100% ethanol. Mix by pipetting.
12. Transfer 500 µl to the Zymo-Spin IIICG column and centrifuge for 30 sec at 11,000 RCF. Discard flow-through.
13. Repeat step 12 once to load the remainder of the sample on the column.

14. Add 400 µl Zymo RNA Prep Buffer to the column and centrifuge at 11,000 RCF for 30 sec. Discard flow-through.
15. Add 700 µl Zymo RNA Wash Buffer to the column and invert 3 times to remove salt from entire column. Centrifuge at 11,000 RCF for 30 sec. Discard flow-through.
16. Repeat step 15.
17. Add 400 µl Zymo RNA Wash Buffer to the column and centrifuge at 11,000 RCF for 30 sec. Discard flow-through.
18. Place the column back into collection tube and centrifuge at 11,000 RCF for 3 min. Discard collection tube with flow-through.
19. Place the column in a new labeled 1.5 ml tube.
20. 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 11,000 RCF for 1 min to elute the labeled ssDNA.
21. Repeat step 20 for a total of ~100 µL eluted ssDNA.
22. Using a Nanodrop, measure the concentration of dye (pmol/µl) and single-stranded nucleic acid (ng/µl) of the labeled ssDNA under the Microarray setting with ssDNA selected from the dropdown menu. Select the dye from the Dye 1 dropdown menu prior to loading your sample. You may need to enter the appropriate molar extinction coefficient for your dye of choice using the dye/chromophore edit option on the Nanodrop software main page. Record both the ssDNA concentration and the pmol/µl dye concentration.
23. Recover the labeled ssDNA sample from the Nanodrop to analyze on a 7% denaturing Urea PAGE gel if desired. Alternatively run the ssDNA on a 1.8% agarose 1X TBE gel with samples and a low molecular weight DNA ladder heated at 95°C in a formamide containing loading buffer (such as from the Ambion® T7 transcription kit) for 5 minutes and put on ice. The expected band size for labeled ssDNA is 66 nt*. Note that the 5′ label may impact the mobility of the ssDNA.
24. To determine dye labeling efficiency, convert the ssDNA concentration in ng/µl to pmol/µl and compare with dye pmol/µl. If the ratio for pmol/ µl dye : pmol/ µl ssDNA is not 0.9 or higher, repeat Part 4: RNase Digestion. Note: Biotin or DIG labeling efficiency cannot be calculated in this manner.

    Labeled ssDNA size=66 nt, assuming a 45mer variable region. 1 µg = 47 pmol. (47pmol/1000 ng=0.047 pmol/ng)

    Example Calculation: 175 ng/µl ssDNA X 0.047 pmol/ng=8.2 pmol/µl ssDNA

    8.2 pmol/µl l X 95 µl = 779 pmol ssDNA

    If 7.9 pmol/µl is the label concentration, then divide that by 8.2 pmol/µl ssDNA to get labeling efficiency (0.96 in this case). For triple-labeled probes (3 label groups per ssDNA molecule), the ratio is generally 2.0-2.7 but can vary by label type.

    ****Note: For Immortal Libraries made with our Indexing Service the ssDNA is 82 bases long and 1 ug = 38 pmol (0.038 pmol/ng). Use this value to do the ng/µl to pmol/µl conversion above for ssDNA in place of 0.047 pmol/ng.***

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

Appendix

Half Reaction Recipe for Reverse Transcription

Alternative half reaction setup for Reverse Transcription is below for Mix 1 and 2. Replaces recipes in Part 3. Exonuclease and RNase recipes should also be cut in half. Return reaction volumes to full volume of normal protocol prior to column purifications (or reduce RNA Lysis buffer and 100% ethanol volumes by half to save reagents).

Components	**Mix 1**
26 µg RNA, add nuclease-free water to volume*	21 µl
Labeled primer 1 mM (1 nmol/ µl)	1.0 µl
dNTPs 10 mM	7.5 µl
SUPERase-In 20 U/µl	0.5 µl
Total	30 µl

Components	**Mix 2**
Nuclease-free water	3.25 µl
5 X First-strand buffer	10 µl
0.1 M DTT	5 µl
20 U/ µl SUPERase-In	0.5 µl
Superscript IV Reverse Transcriptase	1.25 µl
Total	20 µl

Alternative RNA Purification Kits

The same Zymo RNA Miniprep kit Cat. No. R1054/R1055 used for ssDNA purification in this protocol can also be used for purification of RNA from the T7 Transcription. Follow kit protocol for Liquids/Reaction Clean-Up and proceed with RNA Purification that starts with adding 1 volume 100% ethanol to the sample in RNA lysis buffer. DNase treatment is not needed. If possible, it is recommended to use a separate set of pipettors for all RNA handling prior to the RNase step as the pipettors used in the RNase step may get RNase on them.

Alternative T7 Transcription Kit

New England Biolabs HiScribe® High Yield RNA Synthesis Kit (E2040S) following the protocol for Short RNA Transcripts can also be used for T7 transcription, use 480 ng of template and double the reaction volume of the reaction to 40 uL

Information for myTags Immortal Libraries Labeling Primers

The generation of labeled myTags *in situ* hybridization probes from immortal libraries requires the acquisition of labeled primers. There are many vendors that carry modified oligonucleotides (IDT, Bio-Synthesis, Inc., Eurofins Genomics, etc).

Labeled Primer Design for 1X Tagged Fluorescent Probes

5' Dye/Spacer/CGTGGTCGCGTCTCA 3'

Spacer can be triethylene glycol (iSp9 for IDT) or similar. 3 dT nucleotides in place of the spacer will also work. Use single or dual HPLC purification for fluorophore labeled primers (single HPLC is sufficient for IDT, but Bio-Synthesis requires dual HPLC). Please check with the vendor for dye quenching and purification recommendations. A 1 OD scale synthesis yields about 50 nmoles primer when single HPLC is used from IDT

Labeled Primer Design for 3X Tagged Fluorescent Probes

5' Dye/AmC6-Glen/Spacer18-/Dye/Amino C6 dT/-Spacer18-/Amino C6 dT/TGGTCGCGTCTCA 3'

Preferred vendor for 3X labeled primers is Bio-Synthesis. Spacer is hexaethylene glycol. Shorter spacers or string of T's may also work if the vendor cannot synthesize the above oligo design. Order at 10 OD scale with Triple HPLC purification from Bio-Synthesis. The yield from a 10 OD order is typically 40-50 nmoles, enough for 20+ labeling reactions. We recommend 3X dyes only for fluorescent labeled primers.

Labeled Primer Design for 1X Tagged Digoxigenin or Biotin Probes

5' Hapten/CGTGGTCGCGTCTCA 3'

Use single or dual HPLC purification for labeled primers (single HPLC is sufficient for IDT, but Bio-Synthesis requires dual HPLC). Spacer is not required for non-fluorescent tags. A 1 OD scale synthesis yields about 50 nmoles primer when single HPLC is used.

Storage and Use of Labeled Primers

Upon arrival all primers should be resuspended at 1mM concentration in the appropriate amount of nuclease-free water or 10 mM Tris-HCl pH 8.0 with 0.1 mM EDTA. For example, 50 nmoles of dried primer should be resuspended in 50 µl to give a 1 mM solution.

Troubleshooting

1. Low RNA yield from transcription reaction. Generally, there are 3 reasons for this:
    a. Low purity/integrity dsDNA template. Check 260/280 and 260/230 ratios on a Nanodrop or similar instrument. They should be in the ranges of 1.8-2 and 2-2.3 respectively. If these ratios are off, redo the PCR and possibly add an extra wash step to the dsDNA purification step. After all washes are complete, it can also help to do a final 3 min. spin.
    b. Expired or old T7 transcription kit, possibly with obvious precipitate in 10X buffer. First try to heat the 10X buffer to remove precipitate as mentioned in the protocol, then redo the transcription using the positive control in the kit. If this does not fix the problem, a new kit is the best solution.
    c. RNase in one or more of the components of the transcription reaction or in the reagents used to purify and elute the RNA. Components can be tested individually by incubation of a small volume with an RNA sample known to be RNase-free followed by an agarose gel analysis of each test. RNase containing reagents should be discarded and replaced.
2. Low ssDNA yield can be caused at different stages of the preparation.
    a. Reverse transcription may be inefficient due to poor RNA quality or a compromised reverse transcription kit.
        i. A260/280 and A260/230 values for RNA should be 2-2.2 and >2.2, respectively. Significant deviation from these values (generally lower) may indicate contamination of the RNA with ethanol or wash buffer reagents from the kit.
        ii. Check 5X SSIV RT buffer for precipitate, vortex and heat at 37C until it is dissolved. Spin down any precipitate that cannot go into solution. If this does not help, it may be time to order a new kit.
        iii. Be sure dNTPs and DTT are not expired. DTT should have a strong sulfur smell upon opening the tube. It should be stored at -20C and less than 6 months old.
        iv. Confirm all components of the RT reaction are free of RNase.
    b. Exonuclease I may have been ineffective (leftover primer) or incompletely inactivated (leading to ssDNA degradation). Test activity and effectiveness of heat inactivation by incubation of 200 ng of two different non-fluorescent oligos with or without Exo I as in the protocol, followed by EDTA addition and heat inactivation. Do a control without any oligo also. Heat inactivated reaction should no longer have active Exo I, mix 200 ng non-fluorescent oligo with a portion of the heat inactivated reaction that did not have any oligo in it originally. This test should not show any degradation of the newly added oligo, if it does the heat inactivation is ineffective. This may be remedied by extending the inactivation time at 80C from 20 to 30 minutes and/or confirming your thermocycler is reaching 80C. Oligo integrity can be analyzed on an agarose gel similar to that described in Part 4.
        i. To be sure ssDNA is not degraded by Exo I, after addition of EDTA and mixing, reactions should be transferred immediately to a thermocycler block already at 80C.

c. Buffers in the Zymo kit used to purify the RNA:DNA hybrid and ssDNA can lose their ability to perform well over time. This is sometimes evident during the RNA:DNA purification where the colored probe appears to wash off the column after the RNA Prep wash step. The best solution is to either order a new kit or try to replace the individual buffers by ordering them separately (note that the Lysis buffer is not available for individual purchase).
d. Low ratio of pmol fluor to pmol ssDNA may be due to inefficiently labeled primer or the fluor is pH-sensitive and not being measure accurately in nuclease-free water which is often at a pH around 4 (especially true of 6-FAM). Ratio of fluor to ssDNA of original starting primer can be measured on a Nanodrop similar to how the labeled ssDNA probes are measured at the end of Part 4. If you suspect the fluor may be pH-sensitive, dilute the final ssDNA 1:2 in 2X TE Buffer (1X final). Be sure to blank the Nanodrop with 1X TE, then redo the Nanodrop measurement.