Preparing Single-Stranded Labeled Probes from a MYtags Immortal Library

Version 1.4 - May 6th, 2014

Adapted from Murgha et al, 2014

Reagents and Equipment

Material provided by MYcroarray (Store at -20°C upon arrival)

MYtags immortal oligonucleotide library (shipped dry, resuspend at 1ng/ul in nuclease-free H₂O or 10mM Tris-HCl pH 7.5) Store at -20°C.

MYtags PCR primer mix (50 reactions). Store at -20°C.

Reagents

Oil phase

ABIL EM 90, a surfactant from Evonik (US distributor: innovadex.com; Request a sample)
Triton X-100, a surfactant (molecular biology grade; Sigma-Aldrich; Cat no. T8787)
Mineral oil (for molecular biology, light oil; Sigma-Aldrich; Cat no. M5904)

Emulsion breaking

Diethyl ether (ACS reagent, ≥99.8%; Sigma-Aldrich; Cat no. 32203)
Ethyl acetate (ACS reagent, ≥99.5%; Sigma-Aldrich; Cat no. 319902)
5x Green GoTaq® Flexi Reaction Buffer, Promega M8911

Aqueous phase (PCR)

Phusion HF polymerase (New England Biolabs; Cat no. M0530S)
Bovine serum albumin (BSA) (molecular biology, powder; Sigma-Aldrich; Cat no. B6917)
Deoxynucleotide triphosphate (dNTP) mix (10mM; New England Biolabs; Cat no. N0447S)

In vitro transcription

MEGAshortscript™ T7 Kit (Invitrogen; Cat no. AM1354)

Reverse transcription

Superscript II (Invitrogen; Cat no. 18064014)
SUPERase• In™ RNase Inhibitor (Invitrogen; Cat no. AM2696)
Dye-labeled RT primer /5’ Dye/ CGTGGTCGCGTCTCA resuspended at 1 mM

RNA removal

RNase H (New England Biolabs; Cat no. M0297S)
RNase A (Thermo Scientific; Cat no. EN0531)

Purification kits

Qiaquick PCR purification kit (spin columns; Qiagen; Cat no. 28104)
RNeasy Mini Kit (spin columns; Qiagen; Cat no. 74104)
Quick-RNA MiniPrep (spin columns; Zymo; Cat no. R1054S)

Note. Please check kit instructions for protocol updates. If any, please contact us for recommendation.

Equipment

Magnetic stirrer with speed controller (Fisher Scientific, cat no. 11-675-924Q)
Pivot stir bar (3x8mm; Sigma-Aldrich; Cat no. Z329061)
Glass vials (1/2 dr. (1.8mL); O.D. x L: 12 x 35mm; Fisher Scientific; Cat no. 03-339-25A)
Thermo cycler
Mini-centrifuge and Micro-centrifuge
Vortex
Agarose gel and PAGE electrophoresis setup
EMULSION PCR – First stage amplification

Before starting, prepare water-saturated diethyl ether and ethyl acetate in a fume hood. Mix 3 volumes of diethyl ether with 1 volume of molecular biology-grade water in a glass bottle and shake thoroughly for 30s. Mix 3 volumes of ethyl acetate with 1 volume of molecular biology-grade water in a glass bottle and shake thoroughly for 30s. Allow the phases to separate before use. The top phase is diethyl ether/ethyl acetate, the bottom phase is water. The water saturated solvents can be stored at room temperature for 3 months.

Step 1. Prepare a working solution of MYtags immortal oligonucleotide library by mixing 2 ul of the stock solution (1ng/ul) received from MYcroarray with 26 ul of water. Label as working solution and store at -20°C.

Step 2. Heat ABIL EM90 and Triton X-100 between 60°C – 75°C to ease pipetting of the viscous surfactants.

Step 3. Cut the last 5 mm of a 200 ul pipette tip to ease aspiration of ABIL EM90.

Step 4. Prepare oil surfactant mixture by mixing the following components. Vortex for at least 30 seconds, spin down for 15 seconds and put on ice for 5 minutes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral Oil</td>
<td>2000 ul</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1 ul</td>
</tr>
<tr>
<td>ABIL EM90</td>
<td>80 ul</td>
</tr>
</tbody>
</table>

**Notes:** Once you become experienced with performing emulsion PCR, make 10 mL batches of oil-surfactant formulation and store at 4°C. Prior to use, vortex the formulation for 30 s and sit on ice for at least 5 min.

Step 5. Prepare aqueous phase (No template master mix) of the emulsion (on ice) by mixing the following components in order. Vortex for 5 seconds and spin down for 5 seconds. Store on ice. **Use filter tips.**

**Note.** Prepare only 4 tubes at a time to minimize cross-contamination risk

<table>
<thead>
<tr>
<th>No template master mix</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclease free water</td>
<td>99.5 ul</td>
<td>182 ul</td>
<td>264.5 ul</td>
<td>347 ul</td>
</tr>
<tr>
<td>5 X HF buffer</td>
<td>30 ul</td>
<td>55 ul</td>
<td>80 ul</td>
<td>105 ul</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>3 ul</td>
<td>5.5 ul</td>
<td>8 ul</td>
<td>10.5 ul</td>
</tr>
<tr>
<td>MYtags PCR Primer mix</td>
<td>1.5 ul</td>
<td>2.75 ul</td>
<td>4 ul</td>
<td>5.25 ul</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>3 ul</td>
<td>5.5 ul</td>
<td>8 ul</td>
<td>10.5 ul</td>
</tr>
<tr>
<td>10 ug/ul BSA (High Q)</td>
<td>7.5 ul</td>
<td>13.75 ul</td>
<td>20 ul</td>
<td>26.25 ul</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>144.5 ul</strong></td>
<td><strong>264.5 ul</strong></td>
<td><strong>384.5 ul</strong></td>
<td><strong>504.5 ul</strong></td>
</tr>
</tbody>
</table>

Step 6. Transfer 420 ul of oil surfactant mixture to glass vial and add a 3x8mm pivot stir bar. Begin stirring at 1000 rpm on the magnetic stirrer.

**Note.** Place the tube at the center of the magnetic stir in a container (we use a plastic petri dish filled with ice. Please see figure at the end of this document). It is critical to maintain the tube in a melting ice bath during emulsification to ensure proper droplet size. See Appendix B for a procedure to cleanup and reuse stir bars.
Step 7. Add according to table below 2 U/ul Phusion HF polymerase to the “no template master mix” prepared in Step 5 above. Vortex for 5 seconds and spin down for 5 seconds. Store on ice.

<table>
<thead>
<tr>
<th>Number of Libraries. Vol.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2U/ul Phusion polymerase</td>
<td>3 ul</td>
<td>5.5 ul</td>
<td>8 ul</td>
<td>10.5 ul</td>
</tr>
</tbody>
</table>

Step 8. Prepare aqueous phase (PCR Master mix) of the emulsion (on ice) by mixing the following components in order. Vortex PCR master mix tube (10 s) and spin down contents (10 s). Use filter tips.

<table>
<thead>
<tr>
<th>PCR Master mix</th>
<th>Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYtags immortal library working solution</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>No template master mix</td>
<td>122.5 ul</td>
</tr>
<tr>
<td>Total Volume</td>
<td>125 ul</td>
</tr>
</tbody>
</table>

Note. If agarose gel image shows two bands, then reduce MYtags input volume from 2.5 ul to 1.75 ul and add 0.75 ul of water to master mix.

Step 9. Aspirate 100 ul of PCR Master mix and add drop-wise (10 ul) to the glass vial prepared in Step 6 while stirring. Wait 1 second in-between drops.

Step 10. After addition is complete continue stirring for 15 min to create a uniform Master mix/oil emulsion. Important: Make sure that the glass vial is held in place, not rotating in the ice during the 15 min stirring. It is critical to maintain the tube in a melting ice bath during emulsification to ensure proper droplet size.

Step 11. Transfer 20 ul volume of “PCR Master mix” and “No template master mix” to PCR tubes. They will serve as non-emulsified controls. Store on ice.

Step 12. Cut the last 5 mm of a 200 ul pipette tip to ease aspiration of the emulsion. Dispense 60 ul of emulsion into 0.2 ml PCR strip-tube. Keep using the same tip until all the emulsion has been transferred to PCR tubes. Due to the viscous nature of the emulsion, PCR tubes will contain from 40 to 60 ul of emulsion. Such variation is acceptable.

Step 13. Subject the PCR tubes (emulsion and the 2 non-emulsion controls prepared in Step 11) to the following program of temperature – cycling.

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98°C – 2 min</td>
<td>56°C – 30 sec</td>
<td>72°C – 30 sec</td>
</tr>
<tr>
<td>30</td>
<td>98°C – 15 sec</td>
<td>56°C – 30 sec</td>
<td>72°C – 5 min</td>
</tr>
<tr>
<td>1</td>
<td>End step: Keep tubes at 10°C till Step 14. The reaction can be stored overnight</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Annealing temperature has been optimized for using the Q5 HF polymerase in the Q5 buffer.

Step 14. Pool the emulsified PCR reactions in a 1.6ml Axygen microcentrifuge tubes. Add 5 ul of any non-denaturing gel loading Buffer (like 5x Green GoTaq® Flexi Reaction Buffer, Promega M8911 to help identify water droplet in organic solvents) and 1 ml of mineral oil. Vortex 60 seconds and centrifuge at 13,000g for 15 min at room temperature. Dispose of the upper (oil) phase.

Note: The next 4 steps should be performed in a fume hood because of organic solvent handling.
Step 15. Add 1 ml of water-saturated diethyl ether, vortex 30 seconds and spin 15 seconds. Dispose of the upper solvent phase.  

**Note**: Do not remove the ‘gel-like’ interphase as it contains PCR products.

Step 16. Add 1 ml of water-saturated ethyl acetate, vortex 30 seconds and spin 15 seconds. Dispose of the upper solvent phase.

Step 17. Add 1 ml of water-saturated diethyl ether, vortex 30 seconds and spin 15 seconds. Dispose of the upper solvent phase.

Step 18. Let the remaining traces of solvent evaporate in the fume hood for 5 minutes at 37°C

Step 19. Add nuclease free water to bring emulsion PCR volume to 100 ul and proceed to purification using the Qiagen Qiaquick PCR purification kit.

Step 20. Add 5 ul of 3 M sodium acetate solution.

Step 21. Add 500 ul of Qiagen Buffer PB to 1 volume of the PCR sample and vortex for 5 seconds.

Step 22. Place a QIAquick spin column in a provided 2 ml collection tube.

Step 23. Apply the entire sample to the QIAquick column and centrifuge for 60 seconds at 13,000 rpm.

Step 24. Discard flow-through. Place the QIAquick column back into the same tube.

Step 25. Add 700 ul of Qiagen Buffer PE to the QIAquick column and centrifuge for 60 seconds at 13,000 rpm.

Step 26. Discard flow-through and place the QIAquick column back in the same tube.

Step 27. Centrifuge the column for an additional 2 minutes at 13,000 rpm.

Step 28. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

Step 29. To elute DNA, add 30 ul of Qiagen Buffer EB to the center of the QIAquick membrane, let the column stand for 1 minute and centrifuge the column for 1 minute at 13,000 rpm.

Step 30. Quantify the PCR product using picogreen assay or gel quantification. Spectrophotometric assays can give inaccurate quantification. Expected yield is 400 – 800 ng of DNA. **A minimum of 480 ng is required for subsequent steps.**  

**Note**: It is very important to accurately quantify DNA to ensure maximum yield in the subsequent process.

Step 31. Analyze 30 ng to 50 ng of PCR product on a 2.5% agarose gel along with 10 ul of non-emulsion controls. Expected PCR product size is 99 bp. If a higher molecular weight band is visible, repeat following recommendations given in Step 78.  

**Note**: if necessary, it is safe to freeze (-20°C) the sample at this step.
IN VITRO TRANSCRIPTION – Second stage amplification

Note. MEGAscript™ T7 Kit (Invitrogen; Cat no. AM1354). Read manufacturers guidelines for working with RNA and potential updates in protocol.

Note. Before starting pool 52 ul of the stock rNTPs. This is suitable for 12.5 reactions of 40 μl each.

Step 32. Assemble the in vitro transcription reaction mix on ice as follows. Vortex for 10 seconds to mix and spin down for 10 seconds.

<table>
<thead>
<tr>
<th>Components</th>
<th>Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclease free water</td>
<td>___ ul</td>
</tr>
<tr>
<td>Template DNA (480 ng)</td>
<td>___ ul</td>
</tr>
<tr>
<td>10x T7 reaction buffer</td>
<td>4 ul</td>
</tr>
<tr>
<td>Four rNTP pool</td>
<td>16 ul</td>
</tr>
<tr>
<td>T7 enzyme mix</td>
<td>4 ul</td>
</tr>
<tr>
<td><strong>Reaction Volume</strong></td>
<td><strong>40 ul</strong></td>
</tr>
</tbody>
</table>

Step 33. Incubate the reaction at 37°C for 4 h (Hot lid: 42°C). If necessary, store at -80°C overnight prior to RNeasy purification.

Step 34. Add 260 ul of RNase-free water, mix and split into three 100 ul aliquots. Proceed with Qiagen RNeasy purification for each aliquot.

Step 35. Add 350 ul of Qiagen Buffer RLT and vortex 5 seconds.

Step 36. Add 250 ul of ethanol and vortex 5 seconds. Do not centrifuge. Proceed immediately to Step 37.

Step 37. Transfer the sample (700 ul) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied in Qiagen kit). Centrifuge for 60 seconds at 12,000 rpm. Discard the flow-through.

Step 38. Add 500 ul of Qiagen Buffer RPE to the RNeasy spin column. Centrifuge for 60 seconds at 12,000 rpm to wash the spin column membrane. Discard the flow-through.

Step 39. Repeat Step 38 once.

Step 40. Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Centrifuge at 12,000 rpm for 3 min.

Step 41. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 50 ul RNase-free water directly to the spin column membrane. Let column stand for 1 min. Centrifuge for 1 min at 12,000 rpm to elute the RNA.

Step 42. Repeat Step 41 using 50 ul RNase-free water once. Reuse the collection tube from Step 41.

Step 43. Pool the three spin column elutes and then measure RNA concentration. The expected RNA yield from a 40 ul reaction is >80 ug. Store RNA at -80°C overnight if necessary or on ice if proceeding to reverse transcription within 1-2 hr.

Step 44. Analyze RNA sample on Bioanalyzer or 7 % denaturing PAGE gel (more accurate to detect RNA degradation)

Note. RNA size: 66 bases. 1 ug = 47.3 picomole (Amount required for 100-ul RT reaction = 2000 pmol or 42 ug)

Note: It is safe to freeze (-80°C) the sample overnight at this step. However, we recommend minimizing RNA storage and converting all the RNA into more stable single-stranded DNA probes as soon as possible (next day). We also recommend minimizing the number of freeze/thaw cycle the RNA sample will experience.
Step 45. Prepare the following reaction on ice (0.2 ml PCR tube): Vortex for 5 seconds and spin 5 for 5 seconds

<table>
<thead>
<tr>
<th>Components</th>
<th>Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add nuclease free water up to reaction volume</td>
<td>___ul</td>
</tr>
<tr>
<td>RNA (42 ug)</td>
<td>___ul</td>
</tr>
<tr>
<td>1 mM (1 nmol/ul) Dye-labeled RT primer*</td>
<td>2.4 ul</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>15 ul</td>
</tr>
<tr>
<td>20 U/µl SUPERase-In</td>
<td>1 ul</td>
</tr>
</tbody>
</table>

**Reaction Volume** 60

**Note.** Expected yield of MYtags (cDNA) = 400–700 pmol

*Dye-labeled RT primer /5’ Dye/ CGTGGTCGCGTCTCA resuspended at 1 mM

**Note.** Order dye-labeled primers from commercial oligo companies such as IDT-DNA, Operon, Trilink Biotechnologies. Some dyes are quenched when adjacent to ‘G’ DNA base. Please check with primer vendor for dye quenching by adjacent ‘C’ DNA base and add extra base / spacer between 5’ dye and ‘C’ DNA base.

Step 46. Vortex for 5 seconds, spin for 5 seconds and incubate the tube in a thermocycler at 65°C with hot lid set to 75°C for 5 minutes. Quick chill on ice for 5 min.

Step 47. Prepare the following components on ice and add 35 ul to the tube containing the RNA and primer:

<table>
<thead>
<tr>
<th>Components</th>
<th>Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclease free water</td>
<td>4 ul</td>
</tr>
<tr>
<td>5 X First-strand buffer</td>
<td>20 ul</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>10 ul</td>
</tr>
<tr>
<td>20 U/µl SUPERase-In</td>
<td>1 ul</td>
</tr>
</tbody>
</table>

**total volume** 35 ul

Step 48. Vortex for 5 seconds, spin for 5 seconds and incubate the reaction at 42°C (hot lid: 52°C) for 5 minutes.

Step 49. Add 2.5 µl 200 U/µl SuperScript II Reverse Transcriptase

Step 50. Vortex for 5 seconds, spin for 5 seconds and incubate the reaction at 42°C (hot lid: 52°C) for 2 hours.

Step 51. Add 2.5 µl 200 U/µl SuperScript II Reverse Transcriptase (125 U/25 ul).

Step 52. Vortex for 5 seconds, spin for 5 seconds and incubate the reaction at 42°C (hot lid: 52°C) for 2 hours.

Step 53. Add 11 ul of exonuclease I buffer

Step 54. Add 2 ul of exonuclease I enzyme. Vortex for 5 seconds and spin for 5 seconds.

Step 55. Incubate at 37°C for 15 min. This step will remove unincorporated reverse-transcription primers

Step 56. Add 12 ul of 0.5 M EDTA pH 8.0. Vortex for 5 seconds and spin for 5 seconds.
Step 57. Put the tube directly in thermocycler set at 80°C for 20 min (No temperature ramp-up). Transfer tube to ice immediately.

**Important.** Perform Step 58 to Step 86 only for Enzymatic RNA removal else go to Step 87

Step 58. Clean up the reaction (100 ul; RNA:DNA hybrids) with the Zymo Quick-RNA MiniPrep kit

Step 59. Add 500 ul of Zymo RNA lysis buffer. Vortex for 5 seconds.

Step 60. Add 625 ul of 100% ethanol. Vortex for 5 seconds.

Step 61. Transfer 500 ul of the mixture to a Zymo-Spin™ IIICG Column in a collection tube and centrifuge for 30 seconds at 13,000 rpm. Discard the flow-through.

**Note.** The flow-through will have dye color which indicates removal of excess primers. This is expected in the first Zymo Quick-RNA cleanup.

Step 62. Repeat Step 61 once such that the entire sample has been loaded into the column.

Step 63. Add 400 µl of Zymo RNA Prep Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 64. Add 700 µl of Zymo RNA Wash Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 65. Add 400 µl of Zymo RNA Wash Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 66. Place the Zymo spin column back into collection tube and centrifuge at 13,000 rpm for 3 minutes to ensure complete removal of the wash buffer. Discard the collection tube with the flow-through.

Step 67. Place the column into a new RNase-free tube. Add 42 µl of room temperature nuclease-free water to the column matrix, wait 1 min, and then centrifuge at 13,000 rpm for 1 min.

Step 68. Repeat Step 67 using 42 µl of room temperature nuclease-free water. Reuse the collection tube from Step 67.

**Note.** There is a loss of ~2-4ul of water on the spin column.

**Note:** if necessary, It is safe to freeze (-80°C) the sample overnight at this step.
Removal of RNA

**Note.** Certain 5’ end labels (especially NHS ester dyes and moieties e.g. DIG (NHS ester), ATTO NHS ester, AlexaFluor NHS ester) are susceptible to alkaline hydrolytic damage. For these dyes use the enzymatic RNA removal protocol. For other alkaline-resistant labels, such as biotin, either enzymatic or chemical RNA removal protocol can be used. The chemical RNA removal protocol is cheaper and faster.

**A. Enzymatic RNA removal**

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

Step 69. Add the following components to a tube on ice (enzyme mix):

<table>
<thead>
<tr>
<th>Components</th>
<th>Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclease free water</td>
<td>6 ul</td>
</tr>
<tr>
<td>10x RNase H buffer</td>
<td>10 ul</td>
</tr>
<tr>
<td>5 U/ul RNase H</td>
<td>4 ul</td>
</tr>
</tbody>
</table>

**Reaction Volume** 20 ul

Step 70. Add 20 ul of enzyme mix to 80 ul RNA:DNA hybrids. Vortex gently for 5 seconds and spin for 5 seconds.

Step 71. Incubate at 37°C for 2 h.

Step 72. Add 4 ul of RNase A. Vortex gently for 5 seconds and spin for 5 seconds.

Step 73. Put tube back in thermocycler programmed as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>60 min</td>
</tr>
<tr>
<td>70°C</td>
<td>20 min</td>
</tr>
<tr>
<td>50°C</td>
<td>60 min</td>
</tr>
<tr>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Ramp down 95°C to 50°C</td>
<td>0.1 °C/sec</td>
</tr>
<tr>
<td>50°C</td>
<td>60 min</td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Step 74. Cleanup reaction (100 ul) with the Zymo Quick-RNA MiniPrep kit

Step 75. Add 400 ul of Zymo RNA lysis buffer. Vortex for 5 seconds.

Step 76. Add 500 ul of 100% ethanol. Vortex for 5 seconds.

Step 77. Transfer 650 ul of sample to a Zymo-Spin™ IIICG Column in a collection tube and centrifuge for 30 seconds at 13,000 rpm. Discard the flow-through.

Step 78. Repeat Step 77 once such that the entire sample has been loaded into the column.

Step 79. Add 400 µl of Zymo RNA Prep Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.
Step 80. Add 700 µl of Zymo RNA Wash Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 81. Add 400 µl of Zymo RNA Wash Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 82. Place the Zymo spin column back into collection tube and centrifuge at 13,000 rpm for 3 minutes to ensure complete removal of the wash buffer. Discard the collection tube with the flow-through.

Step 83. Place the column into a new RNase-free tube. Add 50 µl of warm (65°C) nuclease-free water to the column matrix, wait 1 min, and then centrifuge at 13,000 rpm for 1 min.

Step 84. Repeat the previous Step 83 using 50 ul of warm (65°C) nuclease-free water, for a total of 100 ul.

Step 85. Measure concentration of dye (pmol/ul) and single-stranded nucleic acid (ng/ul) using ‘microarray setting’ on a nanodrop spectrophotometer. Recover the sample from Nanodrop to analyze on 7 % denaturing PAGE gel.

Step 86. Calculate dye efficiency – compare dye (pmol/ul) and nucleic acid (ng/ul converted to pmol/ul) readouts.

   **Note.** cDNA size: 66 bases. 1 ug = 50 pmol

B. Chemical RNA removal

Caution: Applicable for phosphoramidite dyes/moieties such as Cy3, Cy5, 6-FAM, Biotin. NOT applicable for NHS ester dyes

Step 87. Put the tube directly in thermocycler set at 95°C for 5 min.

Step 88. Quick chill on ice for 5 min.

Step 89. Add 2.5 ul of fresh 10 M NaOH, vortex for 5 seconds, spin for 5 seconds and incubate at 65°C for 15 minutes. Transfer tube to ice immediately.

Step 90. Add 2 ul of concentrated HCl (~36%) and 2.5 ul of 1M Tris-HCl (pH 8.0) to neutralize the solution.

Step 91. Cleanup reaction (132 ul) with the Zymo Quick-RNA MiniPrep kit

Step 92. Add 530 ul of Zymo RNA lysis buffer. Vortex for 5 seconds.

Step 93. Add 660 ul of 100% ethanol. Vortex for 5 seconds.

Step 94. Transfer 500 ul of sample to a Zymo-Spin™ IIICG Column in a collection tube and centrifuge for 30 seconds at 13,000 rpm. Discard the flow-through.

Step 95. Repeat Step 94 twice more (each time load 500 ul) until the entire sample has been loaded into the column.

Step 96. Add 400 µl of Zymo RNA Prep Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 97. Add 700 µl of Zymo RNA Wash Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 98. Add 400 µl of Zymo RNA Wash Buffer to the column and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through.

Step 99. Place the Zymo spin column back into collection tube and centrifuge at 13,000 rpm for 3 minutes to ensure complete removal of the wash buffer. Discard the collection tube with the flow-through.

Step 100. Place the column into a new RNase-free tube. Add 50 µl of warm (65°C) nuclease-free water to the column matrix, wait 1 min, and then centrifuge at 13,000 rpm for 1 min.

Step 101. Repeat the previous Step 100 using 50 ul of warm (65°C) nuclease-free water, for a total of 100 ul.
Step 102. Measure concentration of dye (pmol/ul) and single-stranded nucleic acid (ng/ul) using ‘microarray setting’ on nanodrop spectrophotometer. Recover the sample from Nanodrop to analyze on 7 % denaturing PAGE gel.

Step 103. Calculate dye efficiency – compare dye (pmol/ul) and nucleic acid (ng/ul converted to pmol/ul) readouts.

Note. cDNA size: 66 bases. 1 ug = 50 pmol

Note. Making working aliquots of fluorescent labeled ssDNA – MYtags and store at -20°C in the dark.
APPENDIX A: Ice container to create water–in–oil emulsion

Cap a glass vial (Fisher Scientific; Cat no. 03-339-25A) with parafilm (to be removed just before usage). Place the vial at the center of a 4” plastic petri dish and fill with about 30ml of water. Place in a -20°C freezer for several hours. Do not freeze at -80°C as this would freeze the oil formulation. It may be necessary to place a weight on top of the tube to prevent the ice pushing the tube up.

During emulsion formation, it may be necessary to use either a weight on top of the tube or tweezers as shown on the picture below to prevent the tube from rotating.

MYcroarray is currently designing a low-cost plastic container with integrated tube holder.
APPENDIX B: Procedure to clean stir bars

Caution: Review Safety Procedures before using Concentrated HCl

1. Fill 5mL of 10% soap solution in 15mL falcon tube. Soak used stir bars for > 10 min (Process all spin bars together).
2. Wash stir bars with deionized water to completely remove soap solution (no soap bubbles on shaking).
3. Change gloves.
4. In fume hood, add conc. HCl to cover stir bars. Close lid, shake and let stand for 10 min.
5. In fume hood, Open cap and discard conc. HCl into Acid Waste container (Contact OSEH for acid disposable procedure). Close cap.
6. Change gloves.
7. Wash stir bars thoroughly with deionized water to completely remove any residual HCl.
8. In fume hood (with UV light ON), spread stir bars on Kimwipes and let air dry (~ 10 min).
9. Change gloves
10. Store stir bars in 15mL falcon tubes