Supporting Information for

A Click Chemistry-Based microRNA Maturation Assay Optimized for High-Throughput Screening

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A. General Materials and Methods

General chemistry methods. Reactions were carried out under a nitrogen atmosphere with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25-mm SiliCycle silica gel plates (60F-254) using UV-light (254 nm), ninhydrin staining or submersion in aqueous potassium permanganate followed by brief heating on a hot plate. RP-HPLC was performed using binary gradients of solvents A and B, where A is 0.1% HCO$_2$H in water and B is 0.1% HCO$_2$H in acetonitrile. Analytical RP-HPLC was performed using an Agilent 1260 Infinity HPLC and a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm; 5 µm) at a flow rate of 1 mL/min, with detection at 214 or 254 nm. Preparative RP-HPLC was performed using an Agilent 1260 Infinity HPLC and a PrepHT XDB-C18 column (21.2 × 150 mm; 5 µm) at a flow rate of 15 mL/min, with detection at 214 or 254 nm. In all cases, fractions were analyzed off-line using a Micromass LCT Time-of-Flight mass spectrometer with Electrospray and APCI. Other mass analyses were carried out using an Agilent Q-TOF HPLC-MS or Bruker AutoFlex Speed MALDI-TOF.

General molecular biology and assay methods. Gels were imaged on a ProteinSimple Fluorchem M Gel Imager. Fluorescence and chemiluminescence data was collected on either a BioTek Cytation3 or PHERAstar FS plate reader. Gel densitometry measurements were done using Image J.

Data analysis. All data was analyzed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, www.graphpad.com).

Materials. Chemically synthesized pre-miR-21 and pre-let-7d (deprotected, desalted and HPLC purified), containing biotin and aminoallyl uridine modifications and an 18-atom spacer, was purchased from Thermo Fisher Scientific Biosciences and used as received. Lissamine rhodamine was purchased from Acros. Methyltetrazine (mTet)-NHS (cat #1128) and trans-cyclooctene (TCO)-PEG4-NHS (cat #A137) were purchased from Click Chemistry Tools. Horseradish peroxidase (HRP), streptavidin-coated 384-well plates (cat #15407), and SuperSignal West Pico Chemiluminescent substrate kit were purchased from Pierce. Azide-Fluor 488 and N-Boc-ethylenediamine were purchased from Sigma-Aldrich. RNaseOUT™ Recombinant Ribonuclease Inhibitor, and SYBR® Gold were purchased from LifeTechnologies. RNA ladders were purchased from New England Biolabs. All reagents were used as received without further purification. The plasmid for human Dicer (His6-tev-hDicer) was received from the laboratory of Jennifer Doudna (UC Berkeley).
B. Synthetic and Bioconjugation Methods

pre-miR-21 RNA Sequence:
5’-Biotin-(18-atom spacer; hexaethylene glycol)-UAGCUUAUCAGACUGAUGUUUGACUGUUGAA-(5-aminoallyl uridine)-CUCAUGGCAACACCAGUGCAUGGCGUGUC-3’

pre-let-7d RNA Sequence:
5’-Biotin-(18-atom spacer; hexaethylene glycol)-AGAGGUAGGUUGCAUAGUUUAGGCGAGGA-(5-aminoallyl uridine)-UUUGCCCAAGGAGGAACUAUACGACCUGCUGCCUUUCU-3’

Preparation of RNA-Alkyne:
RNA-Alkyne was prepared as previously described.2

Preparation of RNA-TCO:
pre-miRNA (1.0 mM in 100 mM phosphate buffer, pH 8.0) was mixed with an equivalent volume of TCO-PEG4-NHS (10 mM in DMSO). The reaction was then allowed to proceed at 25 °C for 1 h. RNA-TCO was precipitated by the addition of 1.1× volume of 3.0 M sodium acetate (pH 5.2) and 40 volume equivalents of cold ethanol, and pelleted at 20,000 × g for 40 min at 4 °C. The pellet was then suspended in 100 mM phosphate buffer (pH 8.0) at a concentration of 1.0 mM and stored at -80 °C.

Preparation of RNA-mTet:
RNA-mTet was prepared in the same way as RNA-TCO but mTet-NHS was used in place of TCO-PEG4-NHS.

Preparation of HRP-N3:
HRP-N3 was prepared following an established procedure and stored at 4 °C (100 mM phosphate buffer, pH 7.0).3 Q-TOF HPLC-MS confirmed the coupling of 4 azides per molecule of HRP. HRP mass: 43261.6294, HRP-N3 mass: 43365.1259

Preparation of HRP-TCO and HRP-mTet:
2.5 mg HRP was dissolved in 185.8 μL PBS (100 mM phosphate buffer, pH 7.0, 150 mM NaCl) and mixed with 14.2 μL 100 mM TCO-PEG4-NHS or mTet-NHS dissolved in DMSO. The
mixture was gently shaken at room temperature for 3 h then exchanged using a microcentrifuge concentrator into PBS to remove unreacted NHS esters and DMSO. HRP-TCO and HRP-mTet were stored at 4 °C.

**Rhodamine-amine (RHOD-NH$_2$):**

Lissamine rhodamine (0.25 mmol) was dissolved in 10 mL anhydrous DMF under N$_2$ followed by the addition of N-Boc-ethylenediamine (0.375 mmol) and triethylamine (1.25 mmol). The reaction was stirred at 25 °C overnight. The mixture was extracted with ethyl acetate, washed with saturated sodium bicarbonate, and the organic layer was dried in vacuo overnight. The resulting crude residue was purified by HPLC. The Boc group was removed by addition of 80% trifluoroacetic acid in dichloromethane for 1 h at 25 °C. The final product was concentrated in vacuo and dissolved in DMSO. RHOD-NH$_2$ m/z calc. [M+H]$^+$ 601.2149, found 601.2145.

**Rhodamine-TCO (RHOD-TCO) and Rhodamine-mTet (RHOD-mTet):**

RHOD-NH$_2$ (1.66 μmol) was mixed with either TCO-PEG4-NHS or mTet-NHS (1.66 μmol) in DMSO followed by the addition of triethylamine (2 μL). The reaction was allowed to proceed overnight at 25 °C. Products were confirmed by analytical HPLC and mass spectroscopy and used as is. RHOD-TCO m/z calc. [M+H]$^+$ 1000.4406, found 1000.4384; RHOD-mTet m/z calc. [M+H]$^+$ 813.2847, found 813.2844.

**RNA IEDDA click reaction:**

RNA-X (500 nM final) was mixed with L-Y (1.0 μM final) in phosphate buffer (100 mM, pH 7.0). The substrates were then incubated for 2 h at 25 °C.

**Dicer Purification:**

Dicer was prepared as reported; however, the enzyme was instead dialyzed overnight and stored at -20 °C in 20 mM Tris pH 7.5, 100 mM NaCl, 1.0 mM MgCl$_2$, 50% glycerol, and 0.1% Triton X-100.

**Dicer Digestion:**

Solution digests were carried out in 10-μL volume. RNA-X (500 nM final) was treated with Dicer (1.0 μL, 1.3mg/ml) in buffer (20 mM Tris-HCl, pH 7.4, 12 mM NaCl, 2.5 mM MgCl$_2$, 40 U/mL RNase Out, 1.0 mM fresh DTT) at 37 °C for 3 h. Digests were analyzed using a 12.5% TBE-Urea gel and visualized using SYBR® Gold.
C-1. Coolest miRNA Assay Ever Protocol (384-well format) - IEDDA

**Buffer A:** 100 mM Phosphate Buffer (pH 7.0)

**Buffer B:** 20 mM Tris-HCl, pH 7.4, 12 mM NaCl, 2.5 mM MgCl$_2$, RNase Out (1.0 μL of a 40 U/mL solution), fresh 1.0 mM DTT

**Buffer C:** 2 mM Imidazole, 260 mM NaCl, 0.5 mM EDTA, 0.1% Tween-20, pH 7.0

1. Wash the wells with Buffer A (2× 50 μL)
2. Immobilization of **RNA-TCO or RNA-mTet** (10 μL of 500 nM in Buffer A):
   a. Overnight (4 °C)
   b. Gently agitate the plate covering the wells with plate-sealing tape
3. Wash the wells with Buffer A (2× 50 μL)
4. Dicer digestion:
   a. Incubate at 37 °C for 5 h
   b. Dicer (1 μL, 1.3mg/ml) in Buffer B (10 μL)
      i. If using 5% DMSO, add to Buffer B
   c. Denatured Dicer: Dicer (1 μL, 1.3mg/ml), Buffer B (10 μL), EDTA (0.5 μL, 500 mM; 25 mM final); heat to 95 °C prior to assay
   d. Compound incubation:
      i. **RNA-TCO or RNA-mTet** were pre-incubated with compounds and Buffer B (5.0 μL) for 5 min at 23 °C; more Buffer B (4 μL) and Dicer (1 μL, 1.3mg/ml) were then added and the assay proceeded as in step 4.
5. Wash the wells with Buffer A (2× 50 μL)
6. Click chemistry with HRP-TCO or HRP-mTet:
   a. Conditions: HRP (1.0 μM final), Buffer A for total volume = 10 μL /well.
   b. Incubate at 25 °C for 2 h, covering the wells with plate-sealing tape
7. Wash the wells with Buffer C (3× 50 μL)
   a. Incubate for 5 min between each wash
8. Wash the wells with Buffer A (3× 50 μL)
   a. Incubate for 5 min between each wash
9. For chemiluminescence detection:
   a. Add 50 μL SuperSignal West Pico Chemiluminescent Substrate (prepared following kit instructions)
C-2. Coolest miRNA Assay Ever Protocol (384-well format) – IEDDA-HTS variant
(Changes highlighted in red)

Buffer A: 100 mM Phosphate Buffer (pH 7.0)
Buffer B: 20 mM Tris-HCl, pH 7.4, 12 mM NaCl, 2.5 mM MgCl₂, fresh 1.0 mM DTT
Buffer C: 2 mM Imidazole, 260 mM NaCl, 0.5 mM EDTA, 0.1% Tween-20, pH 7.0

1. Wash the wells with Buffer A (2× 50 μL)
2. Immobilization of RNA-TCO (5 μL of 500 nM in Buffer A):
   a. Either overnight (4 °C)
   b. Gently agitate the plate covering the wells with plate-sealing tape
3. Wash the wells with Buffer A (2× 50 μL)
4. Dicer digestion:
   a. Incubate at 37 °C for 5 h
   b. Dicer (1 μL, 1.3mg/ml) in Buffer B (10 μL)
      i. If using 5% DMSO, add to Buffer B
   c. Denatured Dicer: Dicer (1 μL, 1.3mg/ml), Buffer B (10 μL), EDTA (0.5 μL, 500 mM; 25 mM final); heat to 95 °C prior to assay
   d. Compound incubation:
      i. 50 nL was pintoole from 2 mM stocks
      ii. RNA-TCO was pre-incubated with compound and Buffer B (5.0 μL) for 10 min at 23 °C; more Buffer B (4 μL) and Dicer (1 μL, 1.3mg/ml) were then added and the assay proceeded as in step 4.
5. Wash the wells with Buffer A (2× 50 μL)
6. Click chemistry with HRP-mTet:
   a. Conditions: HRP (750 nM final), Buffer A for total volume = 10 μL /well.
   b. Incubate at 25 °C for 2 h, covering the wells with plate-sealing tape
7. Wash the wells with Buffer C (3× 50 μL)
   a. Incubate for 5 min between each wash
8. Wash the wells with Buffer A (3× 50 μL)
   a. Incubate for 5 min between each wash
9. For chemiluminescence detection:
   a. Add 50 μL SuperSignal West Pico Chemiluminescent Substrate (prepared following kit instructions)
D. Supplemental Figures

**Figure S1. Quantification of pre-miR21-HRP Click Efficiency.** Image J was used to calculate band intensities from Fig. 3b. Percent RNA-HRP conjugate was determined by the following equation: (RNA-HRP conjugate band intensity)/(total RNA intensity)*100.

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Z' = 1 - \left[ \frac{3SD_+ + 3SD_-}{(Avg_+ - Avg_-)} \right]
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**Figure S2. Z’ factor.** To evaluate the signal window and signal-to-noise ratio of the assay, we performed a test for Z’ factor calculation. The Z’ factor is a quantitative method of scoring assay performance.\(^6\) Individual Z’ factors were calculated using the formula shown above (SD+ = standard deviation of positive controls; SD- = standard deviation of negative controls; Avg+ = average signal of positive controls; Avg- = average signal of negative controls). The reported Z’ of 0.69 was calculated by averaging the Z’ factor from 6 plates run in 384-well format that contained 32 positive controls (i.e. reactions without Dicer) and either 32 (5) or 352 (1) negative controls (i.e. reactions with Dicer).
E. References


