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_2H in water and B is 0.1% HCO_2H 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.

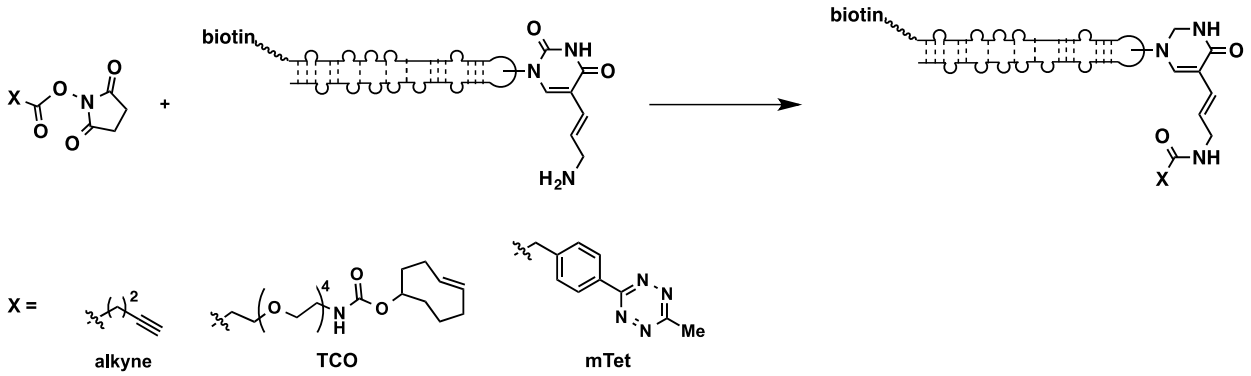
Data analysis. All data was analyzed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, www.graphpad.com).

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).[1]

B. Synthetic and Bioconjugation Methods

pre-miR-21 RNA Sequence:
5'-Biotin-(18-atom spacer; hexaethylene glycol)-
UAGCUUAUCAGACUGAUGUUGACUGUUGAA-(5-aminoallyl uridine)-
CUCAUGGCAACACCAGUCGAUGGGCUGUC-3'

pre-let-7d RNA Sequence:
5'-Biotin-(18-atom spacer; hexaethyleneglycol)-
AGAGGUAGUAGGUUGCAUAGUUUUAGGGCAGGGA-(5-aminoallyl uridine)-
UUUGCCCACAAGGAGGUAACUAUACGACCUGCUGCCUUUCU-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.

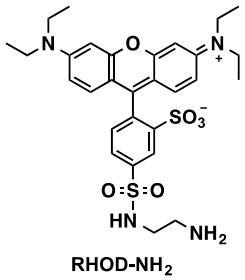
Preparation of RNA-mTet:
RNA-mTet was prepared in the same way as RNA-TCO but mTet-NHS was used in place of TCO-PEG4-NHS.

Preparation of HRP-N_3:
HRP-N_3 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-N_3 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):



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;[1] 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.[4]

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.[5] 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 $MgCl_2$, 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 pintooled 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

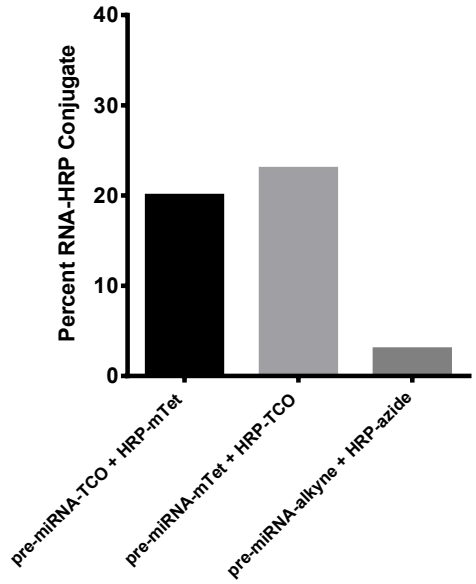


Figure S1. Quantification of pre-miR21-HRP Click Efficiency. Image J was used to calculate band intensities from Fig. 3b. Percent RNA-HRP conjugate was determined by the following equation: (RNA-HRP conjugate band intensity)/(total RNA intensity)*100.

$$Z' = 1 - \left[\frac{(3SD_+ + 3SD_-)}{(Avg_+ - Avg_-)} \right]$$

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

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