

FlashTag™ Biotin

RNA Labeling Kit

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Introduction

Background Information

The FlashTag™ kit will label any RNA sample, including total RNA, senseRNA, severely degraded RNA, plant miRNA, and low molecular weight RNA. This protocol describes labeling low molecular weight (LMW) RNA for analysis by microarrays and ELOSA. Other methods can also be used to analyze the biotinylated LMW RNA***.

LMW RNA molecules (snRNA, hnRNA, piRNA, miRNA, etc.) have recently been shown to be involved in important biological processes such as mRNA degradation, transcriptional gene silencing (TGS) and translational repression.¹⁻⁶ As a result, these newly discovered biomolecules are gaining the interest of the scientific community as possible new drug targets and for use in diagnostics. FlashTag provides the necessary tools to identify such targets.

FlashTag labeling is simple, accurate and highly sensitive. Starting with approximately 1µg of total RNA (or LMW RNA enriched from 1µg of total), the process begins with a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. The labeling process is complete in less than one hour. The labeled RNA is ready for use in microarray hybridizations, ELOSA assays, or other platforms.

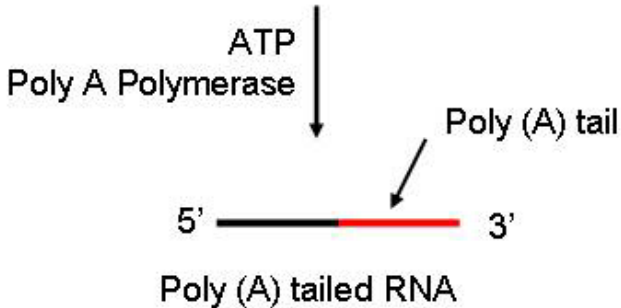
The high sensitivity of FlashTag is due to Genisphere's proprietary 3DNA dendrimer signal amplification technology. The 3DNA dendrimer is a branched structure of single and double stranded DNA conjugated with numerous labels.⁷⁻⁸ Whereas other labeling strategies typically target a single biotin to the sample, FlashTag's 3DNA molecule delivers approximately 15 biotins to the sample (see page 3).

***Marligen Biosciences, Inc. is the exclusive licensee for the research use of this labeling technology in microRNA Luminex® bead-based assays. Please refer to www.marligen.com to purchase the Vantage™ microRNA labeling kit for bead-based assays. This protocol, and the FlashTag Biotin kit, are not optimized for Luminex bead-based assays.

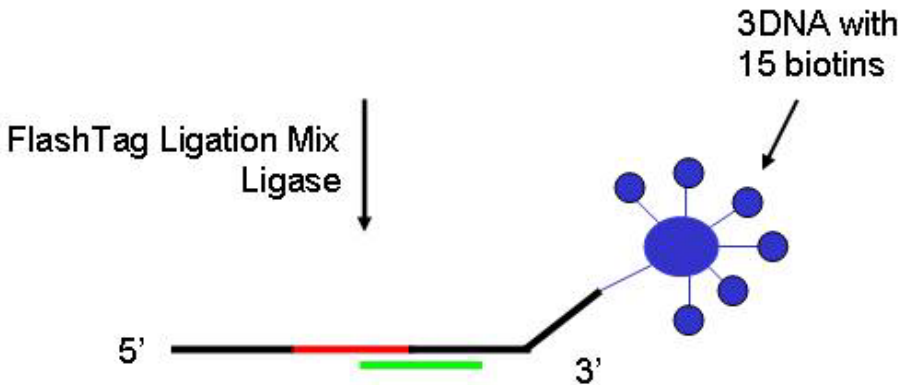
FlashTag: Procedure Overview

5' ——— 3'
RNA molecule

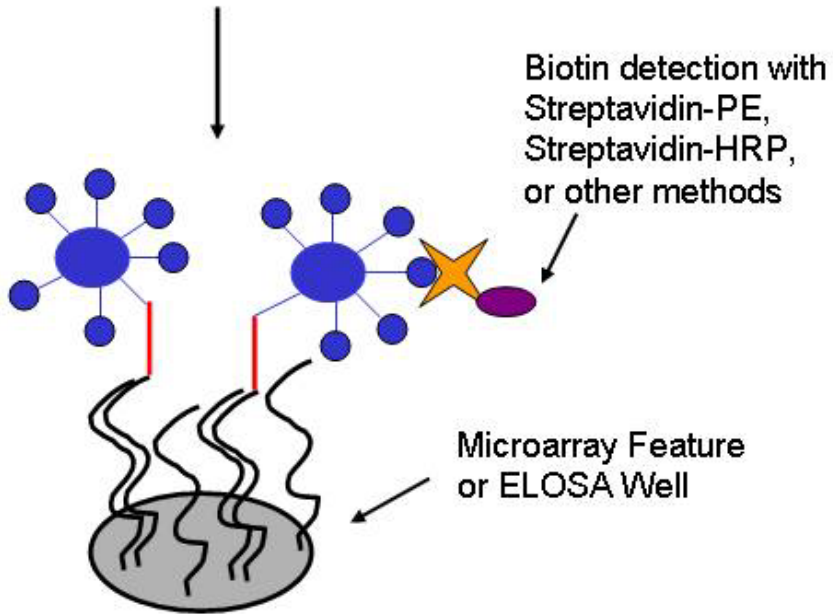
1 Poly (A) Tailing
(15 minutes)



2 Ligation
(30 minutes)



3 Analysis



Components, Storage, and Handling

FlashTag RNA Labeling Kit: **Store all components at -20°C**

Vial 1	10X Reaction Buffer
Vial 2	25mM MnCl ₂
Vial 3	ATP Mix
Vial 4	PAP Enzyme
Vial 5	5X FlashTag Ligation Mix Biotin
Vial 6	T4 DNA Ligase
Vial 7	Stop Solution
Vial 8	2X Enhanced Hybridization Buffer
Vial 9	2X SDS-Based Hybridization Buffer
Vial 10	10% BSA

Handling Kit Contents

Vials 1, 2, 5 and 7: Thaw at room temperature, vortex, and briefly microfuge.

Vials 3, 4, 6, and 10: Thaw on ice, microfuge if necessary, and keep on ice at all times. Do not vortex.

Vials 8 and 9: Heat to 70-80°C for 10 minutes, vortex, and repeat as necessary to resuspend the buffer. Microfuge for 1 minute.

Other Required Materials

- RNA sample containing low molecular weight (LMW) RNA (see "RNA Sample and Quantitation" on page 5)
- Recommended: Microcon[®] YM-100 Centrifugal Filter Devices (Millipore[®] cat. no. 42413)
- Optional: Microcon[®] YM-3 Centrifugal Filter Devices (Millipore[®] cat. no. 42404)
- Optional: Quant-iT[™] RiboGreen[®] RNA Assay Kit (Invitrogen cat. no. R11490)
- Nuclease-Free water (Ambion cat. no. AM9934 or equivalent)
- 1mM Tris pH 8.0
- 10mM Tris pH 8.0
- Reagents for analysis by microarray, Luminex, or ELOSA: Refer to pages 8-15

RNA Sample and Quantitation

FlashTag can accommodate total RNA, enriched RNA, and senseRNA that has been amplified from LMW RNA using the SenseAMP™ Plus LMW RNA Amplification Kit⁹ or the NCode™ miRNA Amplification System. Some applications may require enrichment for optimal profiling. For example, to distinguish mature and precursor miRNAs, enrichment may be necessary. In addition, degraded total RNA samples should be enriched prior to FlashTag labeling. A procedure for enrichment by Microcon YM-100 columns is provided on the next page. Other methods and kits may also be used to enrich total RNA for LMW RNA.

To accurately determine the concentration of the enriched miRNA sample, Genisphere recommends the use of the Quant-iT™ RiboGreen RNA Assay Kit (Invitrogen cat. no. R11490) or the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). If the enriched sample is not quantitated, use LMW RNA enriched from between 0.5 to 3µg of total RNA. Refer to the table below for RNA input recommendations for FlashTag labeling.

RNA Sample	Input for FlashTag Labeling
Total RNA containing LMW RNA	0.5 - 3µg*
Enriched LMW RNA	Enriched from 0.5 - 3µg* total RNA
senseRNA** amplified by Genisphere's SenseAMP Plus LMW RNA Amplification Kit (cat. no. RAMP110MIR or RAMP120MIR)	125 - 750ng

*Please contact Genisphere Technical Support if it is necessary to label more than 3µg Total RNA.

If labeling **senseRNA, the "Poly (A) Tailing" reaction is not required. Adjust the volume to 15µl with Nuclease-Free water and proceed directly to "FlashTag Ligation" on page 7.

Optional: Enrichment of LMW RNA using Microcon YM-100 columns (Millipore cat. no. 42413)

1. Dilute the total RNA sample to 100 μ l with 10mM Tris pH 8.0.
2. Heat to 80°C for 3 minutes, then immediately cool on ice for 3 minutes.
3. While the sample is cooling on ice, add 50 μ l of 10mM Tris pH 8.0 to the Microcon column, and spin for 3 minutes at 13,000g.
4. Discard the flow-through and the collection tube. Place the column into a new collection tube.
5. Add the 100 μ l of RNA to the Microcon column, and centrifuge for 7 minutes at 13,000g.
6. Save the eluate (~95 μ l) in the collection tube. This is the enriched LMW RNA. The LMW RNA can be quantitated with the Quant-iT RiboGreen RNA Assay Kit or the NanoDrop ND-1000 Spectrophotometer. Proceed to Concentration, below, or “FlashTag RNA Labeling Procedure” on page 7.

Note: To collect the high molecular weight RNA, add 5 μ l of 10mM Tris pH 8.0 to the Microcon column and gently mix by tapping the side. Carefully place the sample reservoir **upside down** in a **new collection tube** and centrifuge for 3 minutes at 13,000g.

Optional: Concentration of LMW RNA using Microcon YM-3 columns (Millipore cat. no. 42404)

1. Add the LMW RNA from step 6 above (~95 μ l flow-through of YM-100) to the YM-3 sample reservoir. Do not touch the membrane with the pipette tip. Secure the tube cap and centrifuge for 30 minutes at 13,000g.
2. Check the volume of the flow-through and continue the centrifugation if necessary. For adequate concentration, the flow-through volume should be equal to the loaded volume minus 5 μ l. For example, if 95 μ l was loaded, then the flow-through volume should measure 90 μ l.
3. Add 5 μ l of 10mM Tris-HCl, pH 8.0 to the sample reservoir and gently mix by tapping the side.
4. Carefully place the sample reservoir **upside down** in a **new collection tube**. Centrifuge for 3 minutes at 13,000g to collect the concentrated LMW RNA (~5-10 μ l). The LMW RNA can be quantitated with the Quant-iT RiboGreen RNA Assay Kit or the NanoDrop ND-1000 Spectrophotometer. Proceed to “FlashTag RNA Labeling Procedure” on page 7.

FlashTag RNA Labeling Procedure

FlashTag will label LMW RNA from total, enriched or amplified senseRNA. For RNA input requirements, see “RNA Sample and Quantitation” on page 5. Spike-in controls, such as those recommended manufacturers of microarrays, may also be added to the RNA sample prior to FlashTag labeling.

Note: If labeling **senseRNA**, the “Poly (A) Tailing” reaction is not required. Adjust the volume of senseRNA to 15µl with nuclease-free water and proceed directly to “FlashTag Ligation” below.

Poly (A) Tailing

1. Adjust the volume of RNA, including any spike-in controls, to 10µl with nuclease-free water.
2. Dilute the ATP mix (Vial 3) in 1mM Tris (pH 8.0) as follows:

For **total RNA samples**, dilute the ATP Mix 1:500.

For **enriched, quantitated samples**, calculate the dilution factor according to the following formula:

$$5000 \div \text{ng input LMW RNA}$$

Example: If using 100ng of enriched LMW RNA, the dilution factor is $5000 \div 100 = 50$.
Dilute the ATP Mix 1:50.

For **enriched samples that are not quantitated**, calculate the dilution factor according to the following formula:

$$1000 \div \mu\text{g input total RNA}$$

Example: If the sample was enriched from 2µg total RNA, the dilution factor is $1000 \div 2 = 500$.
Dilute the ATP Mix 1:500.

3. Add the following components to the 10µl RNA, for a volume of 15µl:
 - 1.5µl 10X Reaction Buffer (Vial 1)
 - 1.5µl 25mM MnCl₂ (Vial 2)
 - 1µl diluted ATP Mix (Vial 3 dilution from step 2)
 - 1µl PAP Enzyme (Vial 4)
4. Mix gently (do not vortex) and microfuge.
5. Incubate in a 37°C heat block for 15 minutes. Discard any unused, diluted ATP Mix from step 2.

FlashTag Ligation

1. Briefly microfuge the 15µl of tailed RNA and place on ice. If labeling **senseRNA**, adjust the volume to 15µl with nuclease-free water and proceed to step 2.
2. Add 4µl 5X FlashTag Ligation Mix Biotin (Vial 5).
3. Add 2µl of T4 DNA Ligase (Vial 6).
4. Mix gently and microfuge.
5. Incubate at room temperature for 30 minutes.
6. Stop the reaction by adding 2.5µl Stop Solution (Vial 7). Mix and microfuge the 23.5µl of ligated sample. The samples are now ready for analysis.

Analysis

Printed Microarray

Note: This procedure is written for arrays printed with T_m-optimized 20-22mers¹⁰ on epoxide. For other array types, including LNA™ oligos and other array surfaces, please contact Genisphere Technical Support.

Additional required materials:

- Glass microarray printed with miRNA sequences in antisense orientation
- Glass coverslips (Corning, VWR or other manufacturer)
- Wash buffers: 2X SSC/0.2% SDS, 2X SSC, 0.2X SSC, 1X PBS / 0.02% Tween-20, 1X PBS, 0.1X PBS
- Hybridization ovens set to 52°C and 37°C
- Dye Conjugate and Buffer: choose **one** of the following:
 1. Streptavidin-dye conjugate (various vendors) and 1X PBS + 2% BSA

OR

 2. UltraAmp anti-biotin reagent (Genisphere) and 1X PBS + 2.5% BSA + 2% dextran sulfate (UltraAmp catalog numbers: AB0550, AB3550, AB0560, or AB3560)

Array Hybridization

1. Resuspend the 2X Hybridization Buffer. Vial 8, 2X Enhanced Hybridization Buffer, is recommended. Other hybridization buffers, including Vial 9, 2X SDS-based Hybridization Buffer, can be used. Heat the hybridization buffer to 70-80°C for 10 minutes, vortex, and repeat as necessary to resuspend the buffer. Microfuge for 1 minute.
2. Add 5µl of 10% BSA (Vial 10) and 28.5µl of 2X hybridization buffer to the ligated sample. The **Hybridization Mix** is 57µl. For larger volumes, add equal parts 2X hybridization buffer and water.
3. Heat the **Hybridization Mix** to 65°C for 10 minutes. Gently vortex and briefly microfuge.
4. Apply some or all of the **Hybridization Mix** to a microarray, and cover with a glass coverslip (see table below). Other hybridization chambers can also be used. **Note:** 2X Enhanced Hybridization Buffer (Vial 8) requires higher hybridization volumes due to increased viscosity.

	<u>24x40mm</u>	<u>24x50mm</u>	<u>24x60mm</u>
Vial 8, 2X Enhanced Buffer	43µl	53µl	63µl
Vial 9, 2X SDS Buffer	37µl	45µl	53µl

5. Incubate the array 6 hours to overnight (6-20 hours) in a humidified chamber at 52°C.

Array Washing

1. Prewarm the 2X SSC/0.2%SDS wash buffer to 52°C.
2. Remove the coverslip by washing the array in 52°C 2X SSC/0.2% SDS for 2 minutes or until the coverslip floats off.
3. Wash for 15 minutes in 52°C 2X SSC/0.2%SDS.
4. Wash for 15 minutes in 2X SSC at room temperature.
5. Wash for 15 minutes in 0.2X SSC at room temperature.
6. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Optional: Array Blocking to reduce background

1. Apply 1X PBS + 2% BSA to the array and cover with a glass coverslip.
2. Incubate the array at room temperature in a humidified chamber for 10-15 minutes.
3. Wash with fresh 1X PBS for 1 minute.
4. Wash with fresh 1X PBS for 1 minute.
5. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface

Signal Development: Choose One of the Following:

Note: Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

Streptavidin-dye conjugate

1. Dilute the Streptavidin-dye conjugate in 1X PBS + 2% BSA. Use the dilution factor recommended by the manufacturer, or, use a final concentration of 0.25-1µg/mL. Competitor DNA may also be added (optional).
2. Add the hybridization mix to the array, and cover with a new glass coverslip.
3. Incubate in a dark, humidified chamber at room temperature or 37°C for 20-30 minutes.

UltraAmp anti-biotin reagent with 50 or 350 fluorescent dyes

1. Dilute the UltraAmp reagent to 0.25-1µg/mL in 1X PBS + 2.5% BSA + 2% dextran sulfate. Competitor DNA may also be added (optional).
2. Add at least 100µL of the hybridization mix to the array, and cover with a new glass coverslip.
3. Incubate in a dark, humidified chamber at 37°C for 1 hour.

Array Washing

Note: Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

1. Wash for 2-5 minutes in fresh 1X PBS/0.02% Tween-20 at room temperature.
2. Wash for 2-5 minutes in fresh 1X PBS/0.02% Tween-20 at room temperature.
3. Wash for 2-5 minutes in fresh 1X PBS/0.02% Tween-20 at room temperature.
4. Wash for 2-5 minutes in 1X PBS at room temperature.
5. Wash for 2-5 minutes in 0.1X PBS at room temperature.
6. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Signal Detection

Scan the array according to the manufacturer's recommendations.

Geniom® Biochip (procedure developed by febit GmbH)

Additional required materials:

- Blocking Buffer: 100mM 2-[N-morpholino]ethanesulfonic acid (Sigma, M2933), 0.9M NaCl, 20mM Na₂EDTA, 0.01% (v/v) Tween-20, 1% BSA (Sigma, B4287)
- Hybridization Buffer: 6 x SSPE, 10% formamide, 0.1 x TE, 0.5 mg/ml BSA, 0.1 mg/mL herring sperm DNA (Invitrogen, 15634-017), 0.033 µl/µl febit control oligos (buffer must be prepared freshly and should not be stored)
- 0.5 x SSPE (20 x stock solution: 0.2M sodium phosphate pH 7.4, 2.98M NaCl, 0.02M EDTA; Sigma, 85637)
- 6 x SSPE
- SA-PE Solution: 5 µg/mL streptavidin-phycoerythrin (SA-PE; Invitrogen, S866) in 6 x SSPE
- Antibody Solution (optional): 3 µg/mL biotinylated anti-streptavidin antibody (Vector Laboratories, BA-0500), 100mM 2-[N-morpholino]ethanesulfonic acid, 0.9M NaCl, 0.05% Tween-20, 2 mg/mL BSA, 0.1 mg/mL goat IgG (Sigma, I5256)

Biochip Hybridization

1. Insert the Biochip into the Geniom Instrument and start the Program “Hyb_Denaturing” to eliminate secondary structures within capture probes.
2. Transfer Biochip to the Geniom External Hybridization Chamber and proceed with blocking of free binding capacities. Fill the channels of each array with 14 µl Blocking Buffer and incubate for 15 minutes at room temperature.
3. In the meantime, dry labeled samples in a speed-vac.
4. Resuspend samples in 18µl freshly prepared Hybridization Buffer.
5. Denature samples at 94°C for 3 min.
6. Remove Blocking Buffer from Biochip and fill all arrays with target.
7. Hybridize over night at 42°C with active mixing to increase sensitivity.

Biochip Washing

1. Remove target from all arrays.
2. Transfer Biochip into Geniom instrument.
3. Make sure that all ports for washing buffers and solutions have been refilled (Buffer 1 = 0.5 x SSPE, Buffer 2 = 6 x SSPE, Pre-Buffer: SA-PE solution, Water = H₂O).
4. Start washing program “febit miRNA standard” to proceed with the fully automated washing procedure (approximate duration: 45 minutes).

Biochip Detection

1. Use the Cy™3 filter set for the detection of biotin/SA-PE. febit recommends using the “autoexposure time” modus of your instrument or the standard setting of any external scanner.
2. Save data to database and export it for external analysis.

Optional: Consecutive Signal Enhancement (CSE) For Increased Sensitivity

1. Add Antibody Solution to port Buffer 3.
2. Start washing program “febit signal amplification (CSE)”; approximate duration: 1 hour.
3. Proceed with Biochip Detection as described above.

Luminex (Bead Flow Cytometry)

Marligen Biosciences, Inc. is the exclusive licensee for the research use of this labeling technology in microRNA Luminex® bead-based assays. Please refer to www.marligen.com to purchase the Vantage™ microRNA labeling kit for bead-based assays.

ELOSA

The following procedure is an adaptation of the article: Enzymatic microRNA detection in microtiter plates with DNA dendrimers¹². Further optimization will be necessary depending on the ELOSA application.

Additional required materials:

- DNA oligonucleotides specific for miRNA¹⁰
- **CoStar Plates (Corning cat. no. 2592)**
- Microplate Press-on sealers (Perkin Elmer cat. no. 6005185)
- 1X PBS
- 1X PBS, 0.05% Tween-20
- 4% BSA in 1X PBS (BSA: Equitech-Bio cat. no. BAH65-0100)
- Deionized formamide (EMD cat. no. 4650)
- 2X SSC / 0.2% SDS
- 2X SSC
- 0.2X SSC
- Streptavidin-HRP (R&D Systems cat. no. 890803)
- TMB Substrate (Pierce cat. no. N301)
- Stop Buffer (Biosource cat. no. SS02)
- TMAC Solution (4.5M TMAC, 75mM Tris pH 8, 0.15% Sarkosyl, 6mM EDTA)

TMAC Solution: Make 20mL as needed. Store at room temp up to 8 weeks.

18 mL	5M TMAC (Sigma cat. no. T-3411)
1.5 mL	1M Tris pH 8
0.26 mL	12% Sarkosyl (Sarkosyl: Sigma cat. no. L-5777)
0.24 mL	0.5M EDTA pH 8 (Ambion cat. no. AM9262)

Procedural Notes:

During all incubation steps, cover the plate with a plate sealer.

Wash steps may be performed with the appropriate buffers in bottles that have screw caps with stem nozzles. A multichannel pipette is recommended, but not required.

Plate Coating

1. To each well, add 100 μ L of microRNA oligo¹⁰ at 1 μ g/mL in 1X PBS.
2. Incubate overnight at room temp.
3. Wash 2 times with 1X PBS, 0.05% Tween-20, blot dry.

Plate Blocking and MicroRNA Labeling

1. To each well, add 200 μ L of 4%BSA in 1X PBS.
2. Incubate for 1-2 hours at room temp.
3. During this incubation time, follow the FlashTag protocol to generate 23.5 μ L of biotinylated RNA.
4. Wash 2 times with 1X PBS, 0.05% Tween-20, blot dry.

Sample Hybridization

1. To the 23.5 μ l of biotinylated RNA, add:
 - 19 μ l TMAC Solution
 - 26 μ l Deionized formamide
 - 5 μ l 10% BSA (Vial 10)
 - 1.5 μ l Nuclease-free water
2. Gently mix and microfuge. Hybridize 70-75 μ l of the sample for 3-4 hours at room temp.
3. During this incubation time, pre-warm 2X SSC / 0.2% SDS to 52°C.
4. Wash 2 times with 52°C 2X SSC / 0.2% SDS.
5. Wash 2 times with 2X SSC at room temp.
6. Wash 2 times with 0.2X SSC at room temp.

Streptavidin-HRP Hybridization

1. Dilute SA-HRP in 4% BSA (in 1X PBS).
2. To each well, add 50 μ l of diluted SA-HRP.
3. Incubate for 1 hour at room temp, with gentle shaking.
4. Wash 2-4 times with 1X PBS, 0.05% Tween-20, blot dry.

Signal Development

1. To each well, add 100 μ l TMB Substrate.
2. Incubate at room temp. for 1 to 15 minutes.
3. To each well, add 100 μ l Stop Buffer.
4. Read the absorbance at 450.

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