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# A HABA dye based colorimetric assay to detect unoccupied biotin binding sites in a fusion protein containing avidin

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**We use this protocol and it's working**

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## Abstract

HABA (4'-hydroxyazobenzene-2-carboxylic acid) dye is an anionic dye which is used to assess the biotin binding sites in avidin. Herein we describe an assay protocol to utilize the avidin binding property of HABA to assess the number of available biotin binding sites in an avidin containing HSP70 fusion protein. This approach reduces the technical and instrumentation requirements as compared to fluorescence-based assays to evaluate biotin binding. We have also miniaturized the assay using a Nanodrop detector for the readout, thereby sparing reagents.

## Attachments

PDF

**HABA dye assay narra...**

221KB

## Materials

**Reagents:**

**4'-hydroxyazobenzene-2-carboxylic acid (HABA) dye solution stock (10 mM)**

| | A | B |
|---|---|---|
| | HABA (Fisher Thermo Scientific Inc, Catalog number: 28010) | 24.2 mg |
| | Dulbecco's phosphate-buffered saline (Sigma-Aldrich, Catalog number: D8 537) | 9.8 mL |
| | 1N NaOH | 0.2 mL |

HABA (4'-hydroxyazobenzene-2-carboxylic acid) **Thermo Fisher Catalog #**28010

Dulbecco's Phosphate Buffered Saline **Sigma Aldrich Catalog #**D8537

**4mM D-biotin stock solution (244.3 g/mol)**

| | A | B |
|---|---|---|
| | D-Biotin | 9.8 mg |
| | Ultrapure water | Made up to 10 mL |
| | 4 mM (4 mM = 0.997 mg/mL) | |

**20 µM Avidin (66,000 g/mol)**

| | A | B |
|---|---|---|
| | Avidin | 5.3 mg |
| | Ultrapure water | Made up to 4 mL |

Avidin from egg white **Sigma Aldrich Catalog #**A9275-10MG

- The Mtb HSP70-avidin fusion protein designed by Leblanc et al. (2014; Hum Vaccin Immunother 10:3022) was produced by WuXi Biologics Shanghai, China from a pool of stably transfected CHO3 cells (Ye et al., 2010; Biotechnology Progress 26:1431).
- Four biotinylated peptides were synthesized and HPLC purified to >90% purity by 21st Century Biochemicals, Inc. Each peptide was composed of two MHC class 1 epitopes concatemerized with an MHC class II epitopes. The peptides were designed and biotinylated as described by Leblanc et al. (2014).

Equipment

| | |
|---|---|
| **NanoDrop Spectrophotometer** | NAME |
| 2000/2000c Spectrophotometers | TYPE |
| NanoDrop | BRAND |
| ND2000CLAPTOP | SKU |
| https://www.thermofisher.com/order/catalog/product/ND2000CLAPTOP | LINK |

## Before start

Wipe the work station with 70% Ethanol. Wear gloves before handling the proteins and dyes.

## Impact of biotin concentration on the displacement of HABA from Avidin in a colorimetric assay

1 Prepare Avidin-HABA complex:
Add 100 µL of a [M] 12 micromolar (µM) Avidin stock solution to a 1.7 mL eppendorf tube containing 10.4 µL DPBS.

2 Add 9.6 µL of a [M] 1 millimolar (mM) HABA stock solution to obtain a final concentration of [M] 80 micromolar (µM) HABA dye.

3 Prepare a control avidin tube lacking HABA by adding [M] 10 micromolar (µM) avidin in a final volume of 120 µL DPBS.

4 Prepare a control HABA tube by adding 9.6 µL of [M] 1 millimolar (mM) HABA to 110.4 µL of DPBS only.

5 Measure the absorbance at a wavelength of 500 nm of the HABA-Avidin complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000) by pipetting 2 µL solution onto the nanodrop pedestal.

6 Prior to sample measurement, determine the spectrophotometer baseline reading using a blank solution of 2 µL DPBS.

7 Add an aliquot of 10 µL of HABA-Avidin complex to each of five 0.5 mL PCR tubes.

8 Add DPBS to the five tubes adequately so that after addition of D-biotin the final volume would be 15 µL .

9 Add D-biotin to generate a range of D-biotin concentrations as indicated in Table 1.

**Table 1**: Volumes of D-biotin and DPBS added to HABA-Avidin from stock solutions to titrate out HABA in the table below. The formula C1V1=C2V2 was used to determine the volumes of

D-biotin (V1) required from the stock solution (concentration C1) to achieve the final concentration (C2) in a total volume of 15 µL solution (V2).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **D-biotin stock concentration (µM) C1** | **Final D-biotin concentration (µM) C2** | **D-biotin volume (µL) V1** | **HABA- Avidin complex volume (µL)** | **DPBS (µL)** |
| | C1 | 4 | 0.75 | 10 | 4.3 |
| | 80 | 8 | 1.5 | 10 | 3.5 |
| | 80 | 16 | 3 | 10 | 2 |
| | 500 | 32 | 1 | 10 | 4 |
| | 500 | 64 | 2 | 10 | 3 |

Measure the absorbance at a wavelength of 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer.

## Displacement of HABA from biotin binding pockets of a MtbHSP70-avidin fusion protein using different concentrations of free biotin, measured using nanodrop based detection

10 Prepare MtbHsp70-avidin-HABA complex:
Add 54 µL of [M] 1.85 mg/mL a stock solution of a MtbHsp70-avidin (MAV) fusion protein to a 1.5 mL eppendorf tube containing 56.4 µL DPBS.

11 Add 9.6 µL of a [M] 1 millimolar (mM) HABA stock solution for a final concentration of [M] 80 micromolar (µM) HABA dye and [M] 10 micromolar (µM) concentration MAV in a final volume of 120 µL .

12 Add 9.6 µL of [M] 1 millimolar (mM) HABA to 110.4 µL of DPBS only as a control.

13 Measure the absorbance at a wavelength of 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer.

14 Add an aliquot of 10 µL of HABA-MAV complex each of to six 0.5 mL PCR tubes.

15 Add DPBS to the six tubes adequately so that after addition of biotin the total volume would be 15 µL .

16 Add D-biotin to the tubes to generate a range of D-biotin concentrations as indicated in Table 2.

**Table 2**: Volumes of D-biotin and DPBS added to HABA-MAV from stock solutions to titrate out HABA in the table below. The formula C1V1=C2V2 was used to determine the volumes of D-biotin (V1) required from the stock solution (concentration C1) to achieve the final concentration (C2) in a total volume of 15 µL solution (V2).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **D-biotin stock concentration (µM) C1** | **Final D-biotin concentration (µM) C2** | **D-biotin volume (µL) V1** | **HABA- Avidin complex volume (µL)** | **DPBS (µL)** |
| | 40 | 2 | 0.8 | 10 | 4.2 |
| | 40 | 4 | 1.5 | 10 | 3.5 |
| | 80 | 8 | 1.5 | 10 | 3.5 |
| | 80 | 16 | 3 | 10 | 2 |
| | 200 | 32 | 2.4 | 10 | 2.6 |
| | 200 | 64 | 4.8 | 10 | 0.2 |

Measure the absorbance at a wavelength of 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer.

## Competitive displacement of HABA by PEG4-biotinylated peptides measured in colorimetric assay

17 Predict the water solubility of the peptides using Pepcalc (https://pepcalc.com).

18 Dissolve the peptides to a concentration of [M] 5 mg/mL in ultrapure water with 0.5% DPBS.

> **Note**
>
> The HABA-MAV complex was prepared as described above.

19 Measure the absorbance at 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer at 500 nm wavelength.

20 Add an aliquot of 10 µL of HABA-MAV complex each of to six 0.5 mL PCR tubes.

21 Add DPBS to the six tubes for each peptide appropriately so that after addition of peptide the total volume would be 15 µL .

22 Add biotinylated peptide to the tubes to generate a range of biotinylated peptide concentrations as indicated in Table 3.

**Table 3**: Volumes of biotinylated peptide and DPBS added to HABA-MAV from stock solutions to titrate out HABA in the table below. The formula C1V1=C2V2 was used to determine the volumes of biotinylated peptide (V1) required from the stock solution (concentration C1) to achieve the final concentration (C2) in a total volume of 15 µL solution (V2).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **Biotinylated peptide stock concentration (µM) C1** | **Final peptide concentration (µM) C2** | **Biotinylated peptide stock volume (µL) V1** | **HABA- Avidin complex volume (µL)** | **DPBS (µL)** |
| | 50 | 4 | 1.2 | 10 | 3.8 |
| | 50 | 8 | 2.4 | 10 | 2.6 |
| | 50 | 16 | 4.8 | 10 | 0.2 |
| | 400 | 32 | 1.2 | 10 | 3.8 |
| | 400 | 64 | 2.4 | 10 | 2.6 |
| | 400 | 128 | 4.8 | 10 | 0.2 |

Measure the absorbance at 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer at 500 nm wavelength.

## Expected results and Data Analysis

23 The calculation for this assay is based on Beer Lambert's law (Beer's law): $\Delta A = \varepsilon bC$

$\Delta A$ is the difference in absorbance at 500 nm after addition of Biotin or biotin derivatives to the sample.
$\varepsilon$ is the absorptivity or extinction coefficient at the wavelength ($\lambda$).
For HABA-Avidin samples at pH 7.0, the extinction coefficient at 500 nm is equal to 34,000 M-1 cm-1.
b is the cell path length expressed in centimeters (cm). A 10 mm-equivalent absorbance at 500 nm has a path length of 1.0 cm in Nanodrop 2000.
C is the concentration of biotin in the sample expressed in molarity (= mol/L = mmol/mL).

#Calculation 1
Absorbance at 500 nm for (HABA-avidin) reaction or (HABA-MAV) mixture= A1
Absorbance at 500 nm for (HABA-avidin) + (biotin) reaction mixture or (HABA-MAV) + (biotin) mixture= A2
ΔA=A1-A2
#Calculation 2
C (mmol/mL) = ΔA/εb

#Calculation 3
Ratio of biotin: protein
Molar concentration of bound biotin (C) / Molar concentration of original protein (MAV or Avidin)

For example,
If 10 µM Avidin is mixed with 80 µM HABA to obtain an absorbance at 500 nm, A1= 1.298, and an unknown concentration of biotin was added to the complex and absorbance at 500 nm was measured as, A2= 0.04
ΔA= (1.298-0.04)
ΔA=1.258
C (mmol/mL) = ΔA/εb
C= 1.258/(34000*1)
C= 0.000037 mmol/ml
C= (0.000037 x 1000000) µM = 37 µM
Ratio of µmoles of biotin per µmole of Avidin= 37 µM/10 µM Avidin
µmoles of biotin per µmole of Avidin= 3.7