



EAGLE
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2-Naphthol ELISA Assay Kit

Catalog Number:

NAP31-K01 (1 x 96 wells)

For Research Use Only. Not for use in diagnostic procedures.

v. 1.0

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INTENDED USE

The Eagle Biosciences 2-Naphthol ELISA Assay Kit (enzyme-linked immunoassay kit) is intended for the quantitative determination of 2-Naphthol levels in biological samples. The Eagle Biosciences 2-Naphthol ELISA Assay Kit is for research use only and not to be used in diagnostic procedures.

INTRODUCTION

Each kit for triplicate analyses of up to 24 samples contains one 96-well plate, one tube of 2-naphthol standard, one tube of 2-naphthol-conjugated HRP, and buffers for sample and HRP dilutions, and plate.

The 2-naphthol level in urine or cell culture media can be measured using the 2-naphthol ELISA, without ethyl acetate extraction after 4 to 10-fold dilution of the sample. This kit can be used for the determination of 2-naphthol in serum, plasma, cells, and tissues following proper isolation and purification.

STORAGE AND STABILITY

This kit will obtain optimal results if all of the components are stored at the proper temperature prior to use. Items should be stored at the designated temperatures upon receipt of this kit. All components are stored below -20°C and should not be re-frozen and thawed more than necessary.

MATERIALS PROVIDED

Part Number	Item	Description	Quantity
1	2-naphthol ELISA Plate	Solid 96-well plate coated with anti-2-naphthol antibody in each well	1
2	2-naphthol standard (10 ng in 2 µL)	Stock standard at a concentration of 5 µg/mL	1
3	2-naphthol HRP Conjugates (12 µL)	1000 X concentrated solution	1
4	Sample Dilution Buffer (25 mL)	10 X solution of Tris-buffered saline with preservatives	1
5	HRP Buffer (15 mL)	1 X solution of Tris-buffered saline with preservatives	1
6	Wash Buffer Solution (25 mL)	10 X solution of Tris-buffered saline with detergents and preservatives	1
7	TMB Substrate (24 mL)	A solution of TMB (tetra methyl benzidine)	1



ADDITIONAL REQUIRED MATERIALS (NOT PROVIDED)

- Plate reader with a 450 nm filter
- An 8-channel adjustable pipettor and an adjustable pipettor
- Storage bottles
- Costar cluster tubes (1.2 mL) and microcentrifuge tubes
- Speed-Vac (optional) or argon or nitrogen gas

PRECAUTIONS

- Please read all instructions carefully before beginning the assay
- The reagents in this kit have been tested and formulated to perform optimally. This kit may not perform correctly if any of the reagents are replaced or any of the procedures are modified.
- This kit is intended for research use only and is not to be used as a diagnostic.

PROCEDURAL NOTES

- Remove all of the reagents required, including the TMB, and allow them to equilibrate to room temperature before proceeding with the assay.
- It is necessary to thoroughly mix the concentrated buffer solutions. A stir bar is contained within each buffer solution.

SAMPLE PREPARATIONS

There are different protocols for isolating and purifying 2-naphthol depending on the medium it is in. Listed below are the different protocols. For optimal results follow the appropriate protocol based on the biological sample present.

2-Naphthol measurement in urine

1. Dilute urine 4- to 10-fold with sample dilution buffer.
2. Perform the ELISA for 2-naphthol (according to the instructions of the manufacturer).

2-Naphthol measurement in cells

1. Collect and homogenize and/or sonicate the cells.
2. Extraction with ethyl acetate. Add an equal volume of ethyl acetate to the homogenized cells and vortex very well. Place the upper organic phase into a fresh clean tube after centrifugation. Then add another equal volume of ethyl acetate to the homogenized cells to start the second-time extraction. It is strongly recommended that extraction is performed three times.
3. Evaporate the pooled ethyl acetate from the extractions until all has dried up under argon or nitrogen gas.
4. Add 10 μ L to 20 μ L ethanol, or N, N-dimethyl-formamide (DMF), to dissolve the dried-up residue from above step #4. Add 0.5 mL of 1x Sample Dilution Buffer (provided in kit). Load 100 μ L in each well, in triplicates, on the ELISA plate. (Note: We recommend measuring a different dilution of sample in attempt to fit the results to the standard curve. e.g., add 3 wells with 50 μ L of the rest of sample plus 50 μ L 1x Sample Dilution Buffer and 3 wells of 10 μ L of the rest of sample and 90 μ L of 1x Sample Dilution Buffer.)
5. Perform the ELISA for 2-naphthol (according to the instructions of the manufacturer).

2-Naphthol measurement in tissues

1. Homogenize 1 g of tissue in 4 mL of H₂O.
2. Extract with an equal amount of ethyl acetate, vortex thoroughly, spin down, and collect the organic phase. Repeat this extraction twice more and combine all of the organic phases.



3. Dry the organic substance with argon or nitrogen gas.
4. Dissolve the dried residue from above step #4 with ethanol or DMF. (Add approximately 20 μ L of ethanol or DMF to reconstitute the dried-up residue.)
5. Dilute further with 1x Sample Dilution Buffer. Add approximately 0.5 mL of 1x Sample Dilution Buffer and centrifuge at 10,000 rpm for five minutes at room temperature. The supernatant will be used for ELISA measuring.
6. Perform the ELISA for 2-naphthol (according to the instructions of the manufacturer).

2-Naphthol measurement in plasma or serum

1. Combine 1.8 mL of plasma and 1.8 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:
 - i. Upper organic phase – ethyl acetate phase
 - ii. Interphase – proteins
 - iii. Lower phase – aqueous phase
2. Collect the upper organic phase (a) and set aside.
3. Discard the interphase. Transfer the lower phase with a glass pipette to a new tube and repeat the ethyl acetate extraction step 2 more times.
4. Evaporation of pooled organic phase: There should be approximately 5-6 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speedvac or under argon or nitrogen gas to get the extracted sediment (b).
5. Store the sediment (e) at -20°C. For ELISA assay, dissolve the sediment (e) in 20 μ L of ethanol, then add 130 μ L of 1x Sample Dilution Buffer.
6. For the competitive 2-naphthol ELISA, the above 150 μ L sample needs to be further diluted: Dilute 1:4 (e.g., 80 μ L sample + 320 μ L Sample Dilution Buffer).

When calculating the concentration, consider the dilution factor. In this case, 150 μ L total sample volume from 1.8 mL plasma (12-fold concentration) and then, 80 μ L sample in 400 μ L SDB (5-fold dilution). Since, the samples are concentrated 2.4-fold; to get the actual concentration, you must divide by 2.4.
7. Perform the ELISA for 2-naphthol (according to the instructions of the manufacturer).

ASSAY PREPARATIONS

The solid 96-well plate and TMB solution are provided ready to use. The preparations of other assay reagents are detailed below.

Wash Buffer: Mix the solution with a stir bar, applying low heat until a clear colorless solution is obtained. Dilute the entire contents of the Wash Buffer Concentrate (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of 1 X Wash Buffer. This can then be refrigerated for the entire life of the kit.

HRP Conjugate: Dilute 1 vial of the 2-naphthol -HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

Standards: Label 5 microtubes as Standard 1 through Standard 5. Dilute the entire contents of Sample Dilution Stock buffer (25 mL) with 225 mL deionized water to yield a final volume of 250 mL of 1 X Sample Dilution Buffer. Add 0.9 mL of the Sample Dilution Buffer to the microtubes for Standards 1 to 5. Spin down the enclosed 2-naphthol standard vial (2 μ L, filled with inert gas) and add 1.998 mL of Sample Dilution Buffer to obtain 2 mL of solution. Label this Standard 6. Add 0.1 mL of the Standard 6 to the microtube labeled Standard 5 and mix thoroughly. Next,

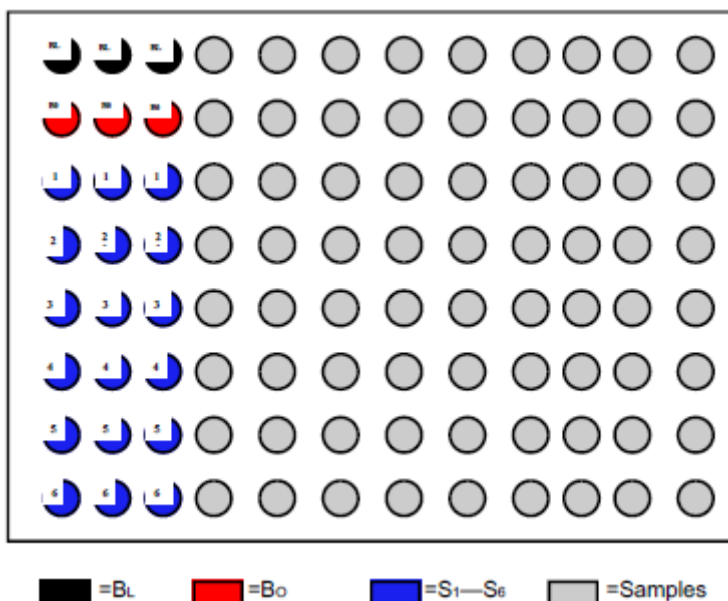


add 0.1 mL of Standard 5 into the microtube labeled Standard 4 and mix thoroughly. Continue to serially dilute the standards using 1:10 dilutions for the remaining standards.

Samples: Samples can be directly diluted into the 1 X Sample Dilution Buffer if it is in solution. For extracted and dried samples, it is recommended to dissolve the dried-up samples with a minimal amount of ethanol or N, N-dimethyl-formamide (DMF, 10 μ L to 20 μ L) and vortex well. Before ELISA assay, add 100 μ L of 1 X Sample Dilution Buffer to make the stock sample solution ready for quantification with ELISA. The stock sample solution can be further diluted to a proper range of concentration for ELISA test.

PERFORMING THE ASSAY

Plate Setup: Each plate must contain a minimum of three blank wells (BL), three maximum binding wells (BO), and a six-point standard curve (S1-S6). Each sample should be assayed in triplicate. A suggested plate format is shown below:



Standard Dilutions Table

Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
No. 6	1,000,000	1.998	2 μ L of stock solution
No. 5	100,000	0.9	Add 0.1 mL of No. 6
No. 4	10,000	0.9	Add 0.1 mL of No. 5
No. 3	1,000	0.9	Add 0.1 mL of No. 4
No. 2	100	0.9	Add 0.1 mL of No. 3
No. 1	10	0.9	Add 0.1 mL of No. 2

Assay Procedure

Step 1: Load 200 microliters of Sample Dilution Buffer into the blank (BL) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (BO) wells.

Step 2: Load 100 microliters of each of the standards into the appropriate wells.



Step 3: Load 100 microliters of each of the samples into the appropriate wells.

Step 4: Load 100 microliters of the diluted 2-naphthol-HRP conjugate in the BO wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the BL wells.

Step 5: Incubate the plate at room temperature for two hours.

Step 6: Wash the plate three times with 400 microliters of the diluted Wash Buffer per well.

Step 7: After the last of the three wash cycles pat the plate dry onto some paper toweling.

Step 8: Add 200 microliters of the TMB substrate to all of the wells (including BL wells).

Step 9: Incubate the plate at room temperature for 15-30 minutes.

Step 10: Add 50 microliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

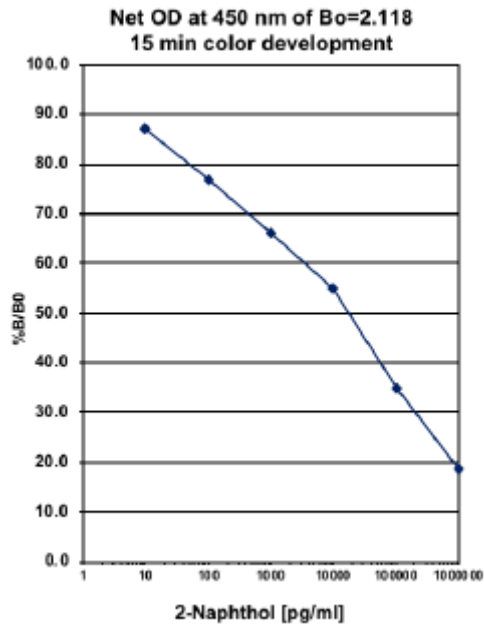
CALCULATING THE RESULTS

Most plate readers provide data reduction software that can be used to plot the standard curve and determine the sample concentrations. If your plate reader does not have this option, then a data reduction program can be used (4 parameter of log-log curve fit). If you do not have these options, the results can be obtained manually as follows:

1. Average the absorbance readings from the blanks and subtract that value from each well of the plate to obtain the corrected readings. (Note: Some plate readers do this automatically. Consult the user manual of your plate reader.)
2. Average the corrected absorbance readings from the BO wells. This is your maximum binding.
3. Calculate the %B/BO for Standard 1 by averaging the corrected absorbance of the two S1 wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.
4. Plot the %B/BO versus the concentration of 2-naphthol; from the standards using semi-log paper.
5. Calculate the %B/BO for the samples and determine the concentrations, utilizing the standard curve.
6. Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.



TYPICAL RESULTS



The data shown here is an example of typical results obtained using the 2-naphthol ELISA kit. These results are only a guideline, and should not be used to determine values from your samples. The user must run their own standard curve every time.

Mean OD for BL wells = 0.049

Mean OD for BO wells = 2.167

Standard	Concentration	O.D.	%B/Bo
No. 1	10 pg/mL	1.845	87.1
No. 2	100 pg/mL	1.623	76.6
No. 3	1,000 pg/mL	1.401	66.
No. 4	10,000 pg/mL	1.167	55.1
No. 5	100,000 pg/mL	0.739	34.9
No. 6	1,000,000 pg/ mL	0.400	18.9

SPECIFICITY OF anti-2-naphthol IgG

The specificity of the 2-naphthol ELISA was investigated using authentic 2-naphthol and a panel of eicosanoids structurally similar to the 2-naphthol.

2-naphthol	100.00 %
8-amino-2-naphthol	< 0.01 %
BPA	< 0.01 %
BPS	< 0.01 %



TROUBLESHOOTING

No color present in standard wells.

- The HRP conjugate was not added. Redo the assay and add the conjugate at the proper step.
- The HRP conjugate was not incubated for the proper time. Redo the assay and incubate for the proper time.

No color in any wells, including the TA wells.

- The TMB substrate was not added. Add substrate.
- The TMB substrate was not incubated for the proper time. Continue incubation until desired color is reached.

The color is faint.

- One or all of the incubation times were cut short. Redo the assay with the proper incubation times.
- The TMB substrate was not warmed up to room temperature. Redo the assay making sure all reagents are at room temperature.
- The lab is too cold. Be sure the lab temperature is between 21-27°C and redo the assay.

The background color is very high.

- The TMB substrate has been contaminated. Redo the assay with a fresh bottle of substrate.

Scattered O.D. obtained from the sample.

- Redo assay using an 8-channel pipetman making sure that 8 channels are equal volume while loading.



Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.