2-Naphthol (2-NAP) ELISA kit

Catalog Number: NAP1

Introduction

This competitive ELISA kit is for determination of 2-naphthol (2-NAP) levels in biological samples.

The 2-NAP and metabolites were detected in urine samples (n=54) from male (43%) and female (57%) and sewage water samples collected for 5 days from 3 counties of Metro Detroit using the Detroit R&D 2-NAP ELISA[1,2].

Burning fossil fuels and woods produce toxic PM2.5 and PM10 mixed with polycyclic aromatic hydrocarbons (PAHs)[3,4] which increase risk of cardiovascular diseases and lung cancer[8-11]. Naphthalene is one of the major PAHs in the PMs (4.5%-13.5%)[5]. Inhaled PAHs through ambient air or occupationally exposed are biologically oxidized and excreted primarily by urine[2,3,6]. Among various PAH metabolites, the level of urinary 2-NAP was significantly correlated with PM2.5 or PM10 exposure[3,4,7], especially after wood smoke exposure[7]. Burn pit smoke exposure may correlate to various health problems of soldiers. It was found that asthma among children was significantly associated with urinary 2-NAP levels[12].

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

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

Instructions are provided as to the proper isolation and purification in the following pages.

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 (22 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-NAP 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 2-(NAP) measurement in urine

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

2-NAP 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 µL to 20 µL 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 µL 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 µL of the rest of sample plus 50 µL 1x Sample Dilution Buffer and 3 wells of 10 µL of the rest of sample and 90 µL of 1x Sample Dilution Buffer.)
5. Perform the ELISA for 2-naphthol (according to the instructions of the manufacturer).

2-NAP measurement in tissues

1. Homogenize 1 g of tissue in 4 mL of H_2O.

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 µL 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-NAP (according to the instructions of the manufacturer).

2-NAP measurement in plasma or serum

3. 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
4. Collect the upper organic phase (a) and set aside.
5. 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.
6. 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).
7. Store the sediment (e) at -20°C. For ELISA assay, dissolve the sediment (e) in 20 µL of ethanol, then add 130 µL of 1x Sample Dilution Buffer.
8. For the competitive 2-naphthol ELISA, the above 150 µL sample needs to be further diluted: Dilute 1:4 (e.g., 80 µL sample + 320 µL Sample Dilution Buffer).

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

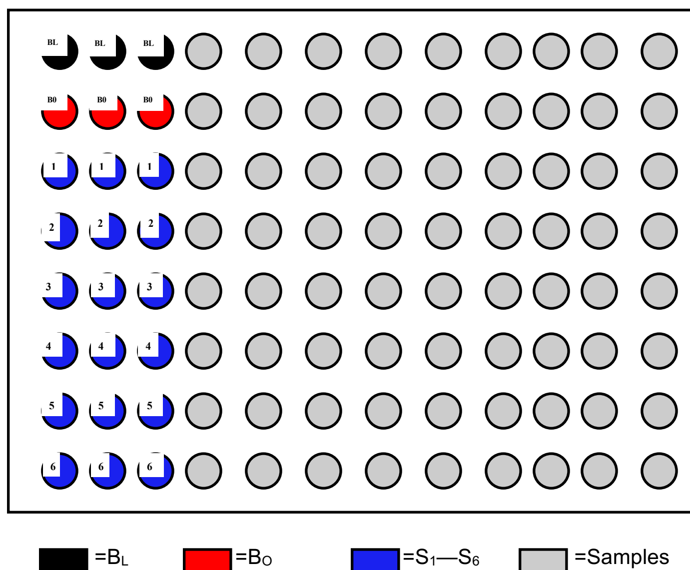
HRP Conjugate: Dilute 1 vial of the 2-naphthol -HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

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 µL, 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 of N, N-dimethyl-formanmide (DMF, 10 µL to 20 µL) and vortex well. Before ELISA assay, add 100 µL 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 (B_L), three maximum binding wells (B_O), and a six-point standard curve (S_1-S_6). 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 µL 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 (B_L) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (B_O) 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 B_O wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the B_L 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 B_L 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.

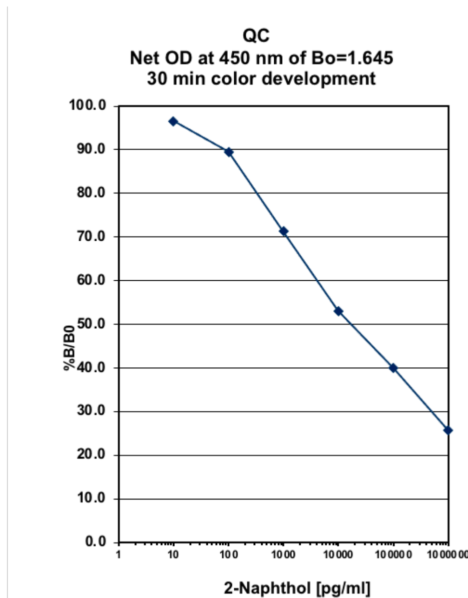
Calculating the Results

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 B_O wells. This is your maximum binding.
3. Calculate the %B/B_O for Standard 1 by averaging the corrected absorbance of the two S_1 wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.
4. Plot the %B/B_O versus the concentration of 2-naphthol; from the standards using semi-log paper.
5. Calculate the %B/B_O 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 Detroit R & D 2-naphthol (2-NAP) 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 B_L wells = 0.063
Mean OD for B_O wells = 1.708

Standard	Concentration	O.D.	%B/B_O
No. 1	10 pg/mL	1.588	96.5
No. 2	100 pg/mL	1.472	89.5
No. 3	1,000 pg/mL	1.173	71.3
No. 4	10,000 pg/mL	0.873	53.1
No. 5	100,000 pg/mL	0.656	39.9
No. 6	1,000,000 pg/mL	0.423	25.7

Specificity of anti-2-naphthol (2-NAP) 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

Detroit R&D, Inc., makes no warranty of any kind expressed, or implied, including, but not limited to the warranties of fitness for a particular purpose and merchantability.

References

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