(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Catalog No: E-EL-0110

Product size: 96T/48T/24T/96T*5

Elabscience® BH4(Tetrahydrobiopterin) ELISA Kit

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Tel: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label outside of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Rev V5.0

Intended Use

Designed for the *in vitro* quantitative determination of Tetrahydrobiopterin (BH4) concentrations in serum, plasma and other biological fluids.

Performance Characteristics

Parameter	Specification
Sensitivity	18.75 pg/mL
Detection Range	31.25-2000 pg/mL
Specificity	Specific for BH4. No significant cross-reactivity or interference with related analogues was observed
Repeatability	Coefficient of variation is < 10%

Test Principle

This assay is based on the Competitive enzyme-linked immunosorbent assay (ELISA) technique. The micro ELISA plate provided in this kit has been pre-coated with BH4. During the reaction, BH4 in samples or Standard competes with a fixed amount of BH4 on the solid phase supporter for sites on the Biotinylated Detection Ab specific to BH4. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Upon reaction with a chromogenic substrate, the HRP enzyme catalyzes the development of a colored product. The resulting signal intensity, measured spectrophotometrically at 450 ± 2 nm, is inversely proportional to the concentration of BH4 in the sample. Quantitative determination is achieved by comparison with a standard curve generated from known concentrations.

Kit Components & Storage

An unopened kit should be stored at 2-8 °C for 12 months. After opening, components should be stored under the conditions specified below:

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	96T: 8 × 12 strips 48T: 8 × 6 strips 24T: 8 × 3 strips 96T*5: 96T × 5 plates	-20 °C, stable until expiry (12 months)
Reference Standard	96T: 2 vials 48T/24T: 1 vial 96T*5: 10 vials	
Concentrated Biotinylated Detection Ab(100×)	96T: 120 µL × 1 vial 48T/24T: 60 µL × 1 vial 96T*5: 120 µL × 5 vials	
Concentrated HRP Conjugate (100×)	96T: 120 µL × 1 vial 48T/24T: 60 µL × 1 vial 96T*5: 120 µL × 5 vials	
Reference Standard & Sample Diluent	96T/48T/24T: 20 mL × 2 vials 96T*5: 20 mL × 10 vials	2-8 °C, stable until expiry (12 months)
Biotinylated Detection Ab Diluent	96T/48T/24T: 14 mL × 1 vial 96T*5: 14 mL × 5 vials	
HRP Conjugate Diluent	96T/48T/24T: 14 mL × 1 vial 96T*5: 14 mL × 5 vials	
Concentrated Wash Buffer(25×)	96T/48T/24T: 30 mL × 1 vial 96T*5: 30 mL × 5 vials	
Substrate Reagent	96T/48T/24T: 10 mL × 1 vial 96T*5: 10 mL × 5 vials	
Stop Solution	96T/48T/24T: 10 mL × 1 vial 96T*5: 10 mL × 5 vials	
Plate Sealing Film	96T/48T/24T: 5 pieces 96T*5: 25 pieces	
Manual	1 copy	

Note

Reagents may contain slightly more volume than indicated on the label; mix and centrifuge the vial of reagents before use. Use precise measuring instruments rather than pouring directly from the vial.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- High-precision pipettes, EP tubes, and disposable pipette tips
- Incubator capable of maintaining 37 ℃
- Deionized or distilled water
- Absorbent paper
- Loading slot (Suction Tank)

Precautions

1. **For Research Use Only**
   - This kit is intended for research purposes and is not for diagnostic or therapeutic use.
2. **Laboratory Safety**
   - Always wear lab coats, safety goggles, and disposable gloves when handling reagents or biological samples.
   - Follow local and national biosafety regulations, especially when working with blood, plasma, or other bodily fluids.
3. **Reagent Handling**
   - Protect light-sensitive reagents such as **Concentrated HRP Conjugate (100×)** and **Substrate Reagent** from exposure to light.
   - Ensure all reagent caps are tightly closed to prevent evaporation and microbial contamination.
   - Do **not reuse** reconstituted standards, working solutions of detection antibody, or HRP conjugate. Stock solutions must be stored according to specified conditions.

- Do not mix reagents from different kit lots or sources.

4. **Pipetting and Cross-Contamination Prevention**
   - Use fresh tips for each standard, sample, and reagent addition.
   - Use separate reservoirs for each reagent to prevent cross-contamination.
5. **Kit Expiry**
   - Do not use the kit beyond the expiration date indicated on the label.

Sample Collection & Handling

Proper sample handling and collection are critical for the accurate measurement of BH4. The following guidelines are recommended:

General Guidelines

- Use disposable, endotoxin-free tubes for blood collection.
- Avoid samples with high hemolysis or excessive lipids, as they may interfere with the assay.
- Predict sample concentration beforehand to determine appropriate dilutions.

Storage and Stability

- For longer storage: -20 °C for up to 1 month, −80 °C for up to 3 months.
- Avoid repeated freeze-thaw cycles. Thaw frozen samples slowly and centrifuge to remove precipitates before assay.

Sample Preparation Considerations

- When using lysis buffers for tissue homogenates or cell lysates, note that chemical components may affect assay results.
- If the sample type is not listed in the manual, perform preliminary validation experiments.
- Some recombinant proteins may not be detected due to mismatches with coated or detection antibodies.

Sample Types and Preparation Guidelines

Serum

- Collect blood in pyrogen-free, endotoxin-free coagulation tubes or plain tubes.
- Allow clotting, at room temperature, for 2 hours, or overnight, at 2-8 °C.
- Centrifuge at 1,000 × g for 20 minutes. For hyperlipidemic samples, centrifuge at 10,000 × g at 2-8 °C for 10 minutes to separate serum and remove chylomicrons.
- Aliquot the supernatant (serum) into sterile cryopreservation tubes.

Plasma

- Use EDTA-K2/Na2 (1.5-2 mg/mL) as anticoagulant; sodium citrate can be used for coagulation-related targets.
- Gently invert tubes 5-8 times immediately after collection to prevent air bubbles and hemolysis.

- Centrifuge at 1,000 × g at 2-8 °C for 15 minutes, within 30 minutes after collection.
- Collect the plasma fraction after centrifugation.

Tissue Homogenate

- Rinse tissue 2-3 times with pre-cooled PBS (0.01 M, pH 7.2-7.4) to remove residual blood.
- Cut tissue into small pieces (2-3 mm^3) and add to a glass homogenizer with PBS containing protease inhibitor (add phosphatase inhibitor for phosphorylated targets; tissue-to-homogenate ratio 1:9).
- Homogenize on ice 30-50 times. For further lysis, repeat freeze-thaw cycles or use ultrasonic disruption.
- Centrifuge at 5,000 × g at 2–8 °C for 5–10 minutes and collect supernatant.
- Optional: Determine total protein concentration using a BCA Protein Quantification Kit.

Cell Lysate

- Gently rinse adherent cells with cold PBS; digest with trypsin if needed.
- Collect cells by centrifugation at 1,000 × g for 5 minutes; suspension cells can be collected directly.
- Wash cells 3 times with cold PBS and resuspend in 150-200 µL PBS per 1.0 × 10^6 cells (add protease inhibitor if required).

- Lyse cells using repeated freeze-thaw cycles or ultrasonic disruption.
- Centrifuge at 1,500 × g at 2-8 °C for 10 minutes and collect supernatant.

Cell Culture Supernatant

- Collect aseptically and centrifuge at 1,000 × g at 2-8 °C for 20 minutes to remove debris.
- Collect the clarified supernatant for ELISA measurement.

Urine

- Centrifuge at 1,500 × g at 2-8 °C for 15 minutes to remove particulate matter.
- Use supernatant immediately or aliquot and store at ≤ -20 °C.

Recommended Reagents for Sample Processing

- PMSF Protease Inhibitor (Cat No. E-EL-SR002)
- 0.25% Trypsin Solution (Cat No. E-EL-SR001)
- BCA Protein Quantification Kit (Cat No. E-BC-K318-M)

Dilution method

- Estimate the expected concentration of your samples before the assay.
- Please predict the concentration range of samples in advance, and determine the dilution ratio through preliminary experiments or technical support recommendations.

- Use the same diluent provided in the kit to maintain assay consistency.

Sample Dilution Protocol for Dilution Factors > 100-Fold

The following is the general procedure that can be adjusted based on sample volume and dilution factor:

General Principles

- Serial dilution is recommended; the dilution factor at each step should not exceed 100-fold.

  Pipetting volume at each step should be ≥ 3 µL. Mix thoroughly and avoid air bubbles.

Procedure Examples

100-fold dilution: One-step dilution. Add 5 µL sample to 495 µL sample diluent.

1,000-fold dilution: Two-step dilution. Add 5 µL sample to 95 µL sample (20-fold), then 5 µL 20-fold diluted sample to 245 µL sample diluent (final 1,000-fold).

10,000-fold dilution: Two-step dilution. Add 5 µL sample to 495 µL sample diluent (100-fold), then 5 µL 100-fold diluted sample to 495 µL sample diluent (final 10,000-fold).

100,000-fold dilution: Three-step dilution. Add 5 µL sample to 195 µL sample diluent (40-fold), then 5 µL 40-fold diluted sample to 245 µL sample diluent (50-fold dilution), and finally 5 µL 2,000-fold diluted sample to 245 µL sample diluent (final: 100,000-fold).

Higher dilution factors can be achieved by increasing the number of serial dilutions, following the same principles. Select the dilution strategy based on the sample volume and estimated concentration to ensure accuracy and repeatability.

Reagent preparation

1. General Handling

- Equilibrate all reagents to room temperature (18-25°C) before use.
- If the kit is to be used partially in a single assay, take only the strips and reagents required for the current assay, leaving the remainder under the conditions specified in the storage table.

2. **Wash Buffer**

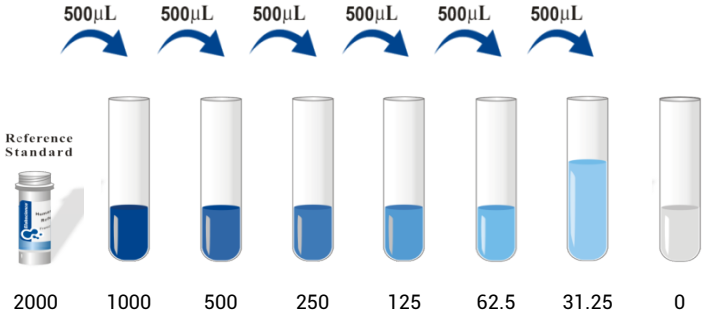
- Prepare the working Wash Buffer by diluting 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water yielding 750 mL.
- Note: If crystalline precipitates have formed in the concentrate, warm it gently in a 40 °C water bath and mix until fully dissolved. Use the prepared Wash Buffer on the same day only.

3. **Standard Working Solution**

- Centrifuge the standard at 10,000 × g for 1 minute.
- Reconstitute with 1 mL of the Standard & Sample Diluent, allow to stand for 10 minutes and gently invert several times until completely

dissolved. Alternatively, vortex briefly at low speed; remove bubbles by low-speed centrifugation if necessary.

- This produces a 2000 pg/mL stock solution. Prepare serial dilutions according to experimental requirements. Recommended dilution gradient: 2000, 1000, 500, 250, 125, 62.5, 31.25, 0 pg/mL.
- Serial Dilution Procedure:
    i. Dispense 500 µL of Standard & Sample Diluent into 7 EP tubes, respectively.
    ii. Transfer 500 µL of the 2000 pg/mL stock solution into the first tube to obtain 1000 pg/mL.
    iii. Sequentially transfer 500 µL from the preceding tube into the next tube for subsequent dilutions. The illustration on the next page is provided for reference. Designate the last tube as the blank; do not transfer any solution into it.
- Aliquot the 2000 pg/mL stock and store at −20 °C. Use within two weeks and avoid repeated freeze-thaw cycles.
- Serially diluted standards should be prepared immediately, prior to use.



4. **Biotinylated Detection Ab Working Solution**

- Calculate the required volume (50 μL/well) plus a slight excess.
- Centrifuge the Concentrated Biotinylated Detection Antibody (100×) at 800 × g for 1 minute.
- Dilute to 1× with Biotinylated Detection Ab Diluent at a ratio of 1:99.
- Prepare the working solution immediately, prior to use.

5. **HRP Conjugate Working solution:**

- Calculate the required volume (100 μL/well) plus a slight excess.
- Centrifuge the Concentrated HRP Conjugate (100×) at 800 × g for 1 minute.
- Dilute to 1× with HRP Conjugate Diluent at a ratio of 1:99.
- Prepare the working solution immediately, prior to use.

Assay procedure

1. **Sample、Standard Loading and Biotinylated Detection Antibody**
    - Assign wells for diluted standards, blanks, and samples.
    - Add 50 µL of each standard, blank, or sample to the designated wells. Analyze all samples and standards in duplicate. Determine sample dilution factors based on preliminary experiments or technical support recommendations.
    - Add 50 µL of Biotinylated Detection Ab Working Solution to each well immediately. Cover with a fresh sealer and incubate for 45 minutes at 37 °C.
    - Note: Dispense solutions gently to the bottom of the well, avoiding contact with the sidewalls and foaming.

2. **Washing Step 1**
    - Decant the solution and add 350 µL of Wash Buffer to each well. Soak for 1 minute, then aspirate or decant and blot dry with clean absorbent paper.
    - Repeat three times.
    - Note: A microplate washer may be used. Do not allow wells to dry. Proceed immediately with the next step.

3. **Addition of HRP Conjugate**
    - Add 100 µL of **HRP Conjugate Working Solution** to each well. Cover the plate with a new sealer. Incubate for 30 min at 37 °C.

4. **Washing Step 2**

- Repeat the wash procedure, described in Step 2, five times.

5. **Substrate Reaction**

- Add 90 µL of **Substrate Reagent** to each well. Cover and incubate for approximately 15 min at 37°C. Protect the plate from light.
- Note: Reaction time may be adjusted based on observed color development but should not exceed 30 minutes. Switch on the Microplate Reader for 15 min prior to OD measurement.

6. **Stopping the Reaction**

- Add 50 µL of **Stop Solution** to each well in the same order as the substrate addition.

7. **Optical Density Measurement**

- Measure the optical density (OD value) at 450 ± 2 nm using a micro-plate reader immediately after adding Stop Solution .

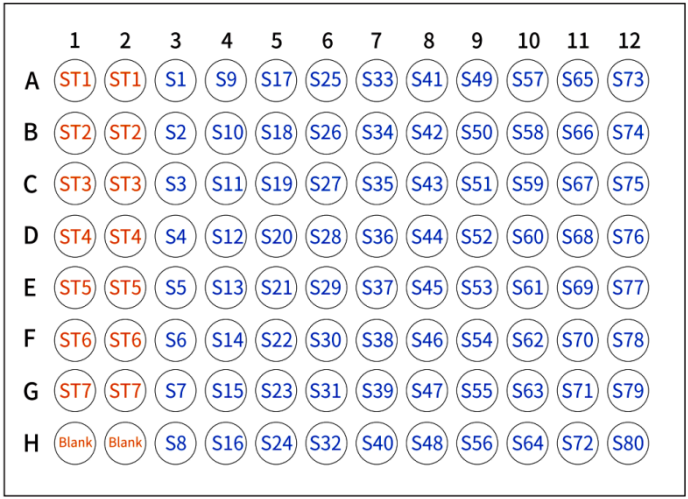
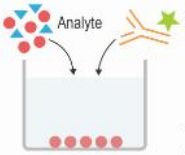


Figure: Example Plate Layout

ST: Standard curve well; Blank: Blank well (0 pg/mL).

S: Sample well.

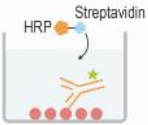
Assay Procedure Summary



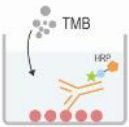
1. Add 50μL standard or sample to the wells, immediately add 50μL Biotinylated Detection Ab working solution to each well. Incubate for 45 min at 37°C



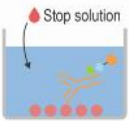
2. Aspirate and wash the plate for 3 times



3. Add 100μL HRP conjugate working solution. Incubate for 30 min at 37°C. Aspirate and wash the plate for 5 times



4. Add 90μL Substrate Reagent. Incubate for 15 min at 37°C



5. Add 50μL Stop Solution



6. Read the plate at 450nm immediately. Calculation of the results

Calculation of results

1. Data Processing

- Calculate the mean optical density (OD) of duplicate readings for each standard and sample.

2. Standard Curve

- Plot a four-parameter logistic (4PL) curve with standard concentrations on the x-axis (log scale optional) and OD values on the y-axis.

3. Sample Concentration

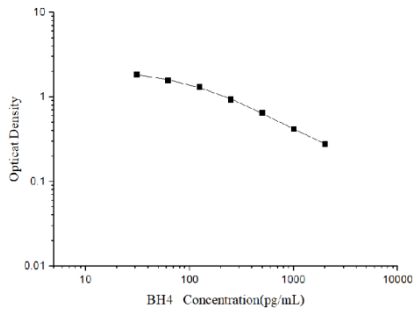
- Determine the concentration of each sample from the standard curve.
- If the sample OD falls below the lower limit of the standard curve, repeat the assay using an appropriate dilution.
- The final concentration is calculated using the following equation:

Final concentration = Measured concentration × Dilution factor

Typical data

The following data was generated by the Quality Control Department, under controlled laboratory conditions (ambient temperature: 18-25 ° C, relative humidity: 35-75%) using standardized procedures (TMB reaction at 37 °C in the dark for 15 minutes, followed by termination and OD measurement). These values are provided for reference only. Actual results may vary due to differences in laboratory conditions, operator technique, and equipment. Users are required to generate a standard curve using their own experimental data.

pg/mL	OD1	OD2	Mean OD
2000	0.281	0.273	0.277
1000	0.429	0.425	0.427
500	0.637	0.623	0.630
250	0.929	0.939	0.934
125	1.292	1.246	1.269
62.5	1.613	1.553	1.583
31.25	1.807	1.801	1.804
0	2.166	2.116	2.141



Performance

■ Precision

Intra-assay Precision (Within-run Precision): Three samples representing low, mid, and high concentrations of BH4 were tested 20 times on a single plate.

Inter-assay Precision (Between-run Precision): Three samples representing low, mid, and high concentrations of BH4 were tested on three separate plates, with 20 replicates per plate, to assess variability among assays.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
Numbers	20	20	20	20	20	20
Mean (pg/mL)	100.55	276.36	963.04	93.45	291.80	1025.24
Standard deviation	5.03	19.26	60.00	8.30	24.22	71.87
CV (%)	5.00	6.97	6.23	8.88	8.30	7.01

■ **Recovery**

The recovery of BH4 was evaluated by spiking samples at low, mid, and high concentrations across the assay range in various sample matrices. The assay performance was assessed by comparing the measured concentrations to the expected spiked amounts to determine the percent recovery.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=8)	89-104	96
EDTA plasma (n=8)	88-103	96
Cell culture media (n=8)	93-108	100

■ Linearity

Linearity of the assay was evaluated by spiking samples with high concentrations of BH4 and performing serial dilutions using Standard & Sample Diluent to produce concentrations spanning the assay's dynamic range. The measured values were then compared to the expected concentrations to assess the linearity of response.

		Serum (n=5)	EDTA plasma (n=5)	Cell culture media(n=5)
1:2	Range (%)	93-107	93-108	91-106
	Average (%)	100	99	97
1:4	Range (%)	89-103	94-109	88-101
	Average (%)	96	100	93
1:8	Range (%)	94-107	88-100	90-103
	Average (%)	101	94	98
1:16	Range (%)	89-101	94-106	87-97
	Average (%)	96	100	92

■ Stability

Each kit batch is subjected to accelerated stability testing and real-time stability monitoring. Sample performance is evaluated after storage at 37 °C for 10 days to assess the impact of elevated temperature on assay reliability and reagent integrity.

	Variation range of 37°C mean concentration / 2-8°C mean
Sample 1 (n=16)	95.56-106.77
Sample 2 (n=16)	86.24-96.81

Declaration

1. Due to current technological and methodological limitations, comprehensive identification and analysis of all raw materials cannot be guaranteed. Users should be aware of potential qualitative and technical risks associated with kit use.
2. This assay is designed to minimize interference by factors present in biological samples; however, until all potential interfering substances are fully evaluated, their influence cannot be entirely excluded.
3. Experimental results are dependent on reagent quality, operator technique, and laboratory conditions. The manufacturer is responsible only for the performance of the kit itself, not for the samples utilized during the assay. Users should estimate and reserve sufficient sample volume for their experiments.
4. Optimal results are achieved only when all reagents supplied with the kit are used and the instructions are strictly followed.
5. Inaccurate results may occur due to improper reagent preparation, incorrect sample or reagent loading, or misconfigured microplate reader parameters. Users should carefully review instructions and calibrate instruments prior to assay.
6. Variability may occur even when the same operator performs separate assays. Consistent execution of all procedural steps is essential for reproducible results.
7. All kits undergo rigorous quality control; however, variations in results may arise from differences in transportation, laboratory equipment, or environmental conditions. Intra-assay variability may also occur between

different kit batches.

8. Results may differ when using kits from other manufacturers or alternative assay methods for the same analyte, as comparative evaluations have not been performed.
9. This kit is intended for research use only. The manufacturer is not responsible for any outcomes resulting from use in clinical diagnostics or other non-research applications.