



EAGLE
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Vitamin B₂ (Riboflavin) HPLC Assay

Catalog Number: VB231-H100

100 Tests

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

v. 1.0

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1. Intended purpose

The Eagle Biosciences Vitamin B₂ (Riboflavin) HPLC Assay is intended for the quantitative determination of Vitamin B₂ (Riboflavin) in EDTA-blood. The Vitamin B₂ (Riboflavin) HPLC Assay kit is for research use only and not to be used in diagnostic procedures.

2. Introduction

Vitamin B₂ is a collective term for the molecules Riboflavin, Flavin Adenine Dinucleotide (FAD) and Flavin Adenine Mononucleotide (FMN). Riboflavin belongs to the group of Flavonoids (plant pigments). It only can be synthesized by plants and microorganisms. Humans and animals ingest it via the gut and convert it to the coenzymes FAD and FMN. They are distributed within the whole body with maximum concentrations in liver, kidney and heart.

FAD and FMN are important coenzymes in protein and energy metabolism. In the central nervous system vitamin B₂ is involved in the control of neurohormones and biogenic amines. A lack of vitamin B₂ leads to unspecific symptoms as fatigue and listlessness. Also, erythema, itching and gingival inflammation are recognized. The metabolic pathways of carbohydrates, fatty acids, proteins, and iron and vitamin B₆ metabolism are affected. A serious lack of the B₂ vitamins leads to inflammation of the mucous membrane in mouth and nose, skin disease (dermatitis), ophthalmic disorder (reduction of the acuteness of vision, cataract), neurological disorders and disturbance of the iron metabolism.

Vitamin B₂ (Riboflavin) HPLC Assay kit makes it possible to determine the vitamin in an easy, fast and precise way. Vitamin B₂ (Riboflavin) HPLC Assay kit includes all reagents in ready to use form for preparation and separation of the samples with exception of the columns (IC2300rp) and the controls (IC2300ko). Both can be supplied by Eagle Biosciences. Beside the complete test kits it is possible to order all components separately. Please request our single component price list.

3. Warnings and precautions

- All reagents of the Vitamin B₂ (Riboflavin) HPLC Assay kit are strictly intended for research use only and are not to be used for diagnostic procedures.
- Test kit and column are concerted . Using alternative columns might cause in insufficient separation, resulting in false high results. The given test characteristics might not be fulfilled.
- Do not interchange Vitamin B₂ (Riboflavin) HPLC Assay kit components from different lots.
- Calibrator and controls contain human blood. It was tested and found negative for HBsAg, anti-HIV-1/2, and anti-HCV. No test can guarantee the absence of HBsAg or HIV, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.



- The precipitating reagent contains acid and has to be handled carefully. It is corrosive and causes burns. It should be handled with gloves, eye protection, and appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician.
- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- Reagents should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Materials Provided

Catalog #	Component	Description	Size
IC2300lm	ELU	Mobile phase	1000 ml
IC2300nk	CAL	Calibrator (lyoph. 2 ml)	5 vials
IC2300fr	PREC	Precipitation reagent	30 ml
IC2300sl	STAB	Stabilization solution	50 ml
IC2300re	RECON	Reconstitution solution	10 ml



5. Additional special equipment

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with Fluorescence-detector
- HPLC column Vitamin B2 (IC2300rp)
- Vortex mixer

6. Reagent preparation

- Reconstitute the **calibrator (CAL)** in **2 ml** reconstitution solution (RECON), divide the calibrator in several portions and store them at -20 °C. Avoid repeated freeze-thaw cycles. The concentration of vitamin B2 might have minor changes from lot to lot. The concentration is given in the attached product specification.
- All other test reagents are stable at 2-8 °C, up to the date of expiry stated on the label. The reconstituted calibrator is stable for 10 days at -20 °C.

7. Specimen

- EDTA blood could be used in this Vitamin B₂ (Riboflavin) HPLC Assay kit.
- Riboflavins are light sensitive; therefore samples have to be protected from light and cooled and centrifuged immediately.
- The samples are stable in the dark at 2-8°C for 1 day. For longer storage samples should be frozen at -20 °C. The sample may not be frozen several times.

8. Procedure

Principle of the method

For the determination of vitamin B₂ a precipitation step to remove high molecular substances is performed first. After centrifugation the supernatant is mixed with a stabilization solution and injected into the HPLC system. The isocratic separation via HPLC at 30°C lasts 10 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed with the delivered whole blood calibrator; the concentration is calculated via integration of the peak heights respectively areas.



Sample preparation

1. Pipette into 1.5 ml reaction tubes:
300 µl PREC. The precipitation reagent should be refrigerated (2-8 °C).
+
200 µl sample, CAL or CTRL
2. Mix well (30 s on a Vortex-mixer)
3. Add **500 µl** STAB, vortex briefly and centrifuge for 5 min at 10.000 g.
4. Inject **50 µl** of the supernatant for chromatography into the HPLC-system.

The supernatant is stable in the dark for 24 hours at 20-25 °C, for 48 hours at 2-8°C and for minimum 30 days at -20 °C. Once frozen samples may only be thawed one time.

Chromatographic settings

Column material:	Vitamin B2 column (IC2300rp)	
Column dimension:	125 mm x 4 mm	
Flow rate:	1.0 ml/min	
Fluorescence detection:	Excitation	450 nm
	Emission	530 nm
Injection volume:	50 µl	
Running time:	10 min	
Temperature:	30 °C	

Treatment of the column

After the analysis the column should be flushed with 15 ml deionized water (1 ml/min) and stored in 85% acetonitrile in deionized water (approx. 15 ml, flow 0.7 ml/min). Before use, the system should be equilibrated with approx. 30 ml ELU.

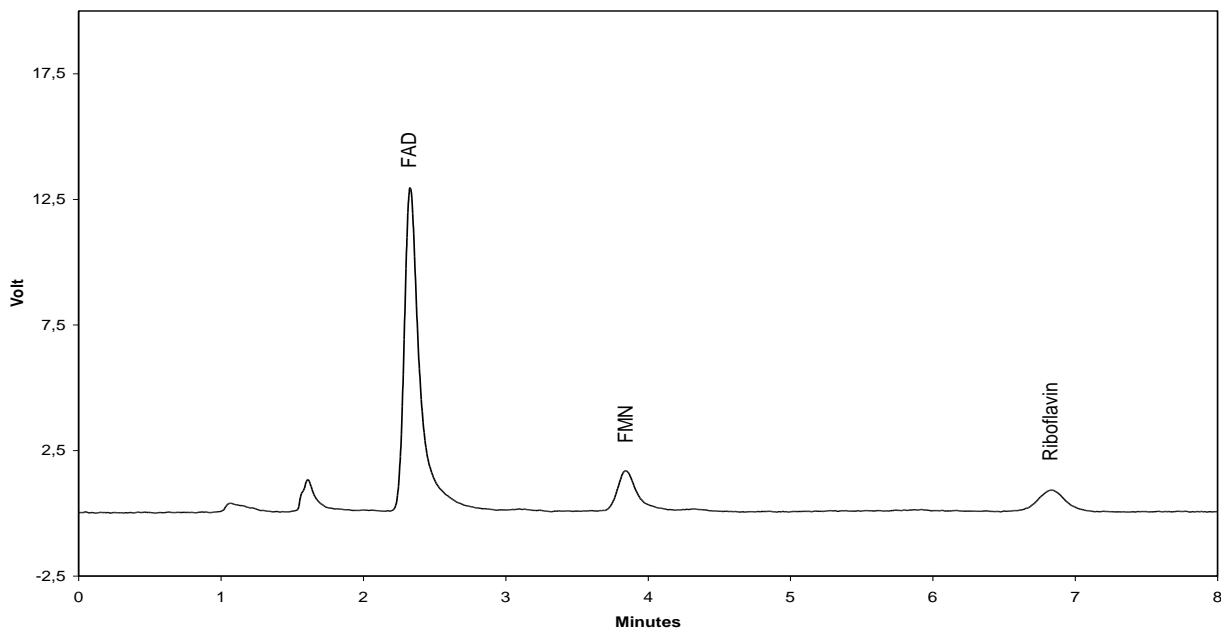


9. Calculation of analytical results

Calculation

$$\text{Conc. sample (ng/ml)} = \frac{\text{peak area patient} * \text{conc. calibrator (ng/ml)}}{\text{peak area calibrator}}$$

Typical chromatogram



Flavin Adenine Dinucleotide (FAD)	2.4 min
Flavin Adenine Mononucleotide (FMN)	3.9 min
Riboflavin	6.9 min

10. Internal quality Control

Reference intervals

FAD: 180 - 295 ng/ml

We recommend that each laboratory should develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data.



11. Validation data

Precision and reproducibility

Intra-Assay VK:	4.1 % (211 ng/ml)	[n = 6]
	4.3 % (293 ng/ml)	[n = 6]
Inter-Assay VK:	4.7 % (214 ng/ml)	[n = 6]
	4.6 % (287 ng/ml)	[n = 6]

Linearity

Up to 500 ng/ml

Detection limit

8 ng/ml

12. Limitations of the method

Lipemic samples should not be measured.

13. Disposal

The mobile phase (ELU) must be disposed as non-halogenated solvent. The precipitation solution (PREC) could be neutralized with NaOH and if the pH value is neutral it can be disposed as salt solution. (**Important:** Reaction will produce heat, be careful)
Please refer to the appropriate national guidelines.

14. Troubleshooting

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase



	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump delivers imprecise	Check pump, degas the system
	System is not in steady state yet	Rinse system mobile phase for 15 min
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
Continue baseline is drifting	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flowcell is dirty	Clean flow cell

15. Literature references

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- S. Fidanza, M. S. Simonetti, A. Floridi, M. Codini, R. Fidanza: Comparison of methods for thiamin and riboflavin nutriture in man, Int. J. Vitam. Nutr. Res. 59(1) (1989) 40-47.

For further information about this kit, its application or the procedures in this Vitamin B2 (Riboflavin) HPLC Assay insert, please contact the Technical Service Team at Eagle Biosciences at info@eaglebio.com or at 866-411-8023.