

## Phenytoin - Free Phenytoin Application

## Order information

REF	CONTENT	Analyzer(s) on which <b>cobas c</b> pack(s) can be used
20737879 322	Phenytoin (200 tests)	System-ID 07 3787 9 COBAS INTEGRA 400 plus COBAS INTEGRA 800
20759015 322	COBAS FP Free Phenytoin Calibrators Calibrator A (1 × 3.5 mL) Calibrators B-F (5 × 1 × 1.5 mL)	System-ID 07 5901 5
20759023 322	COBAS FP Free Phenytoin Controls Level I (2 × 5 mL) Level II (2 × 5 mL) Level III (2 × 5 mL)	System-ID 07 5902 3
21986643 122	Serum Filters (50/Pack)	
20720720 322	COBAS FP Sample Dilution Reagent II (1 × 200 mL)	System-ID 07 2072 0

## English

## System Information

Test FPHNY, test ID 0–187.

## Intended use

In vitro test for the quantitative determination of free phenytoin in serum or heparinized plasma on COBAS INTEGRA systems.

## Summary

Phenytoin (diphenylhydantoin) has been used extensively for seizure control in patients having both grand mal epilepsy (major motor) and cortical focal seizures and temporal lobe epilepsy.<sup>1</sup> Serum level monitoring of the drug is essential in order to achieve maximal seizure control while maintaining minimal blood levels through a routine daily dosage.

Although total phenytoin is traditionally monitored, phenytoin is approximately 90 % protein bound in most patients. It is the unbound or free fraction of the drug that possesses the ability to cross membranes or bind receptors. Therefore, the pharmacological effects of phenytoin are related to the concentration of the unbound or free drug.<sup>2</sup>

## Test principle

Fluorescence polarization

COBAS INTEGRA therapeutic drug monitoring measurements are made on the COBAS INTEGRA systems using the principle of fluorescence polarization. When a fluorescent molecule, or fluorophore, is irradiated with light of the proper wavelength (the excitation wavelength) some of the light is absorbed. Within a few nanoseconds the absorbed light is emitted, although at a longer wavelength (the emission wavelength). Whether or not the emitted light is polarized depends on the freedom of the fluorophore to rotate in solution. A small molecule, such as fluorescein, can rotate rapidly before light emission occurs, resulting in depolarization of the emitted light. In contrast, a fluorescent macromolecule, such as a fluorescein-labeled protein, will rotate much more slowly. Thus, in the time frame between excitation and emission, the macromolecule will have rotated only very slightly and the emitted light will be polarized.<sup>3</sup> Fluorescence polarization is a reproducible function of the drug concentration, and is suitable for the quantitative determination of drug concentrations in serum for the purpose of therapeutic drug monitoring.

Surface active agents are used to ensure dissociation of the drug from serum proteins and to prevent nonspecific binding of the tracer.

## Reagents - working solutions

## R1 Antibody reagent

Anti-phenytoin monoclonal antibody (mouse) in buffer, pH 6.5, with stabilizer and preservative.

## SR Tracer reagent

Fluorescein-labeled phenytoin derivative in buffer, pH 7.5, with stabilizer, surfactant, and preservative.

R1 is in position B and SR is in position C.

## Precautions and warnings

Pay attention to all precautions and warnings listed in Section 1 / Introduction of this Method Manual.

For USA: Caution: Federal law restricts this device to sale by or on the order of a physician.

## Reagent handling

Ready for use

## Storage and stability

Shelf life at 2-8 °C

See expiration date on  
**cobas c** pack label

COBAS INTEGRA 400 plus system

On-board in use at 10-15 °C

12 weeks

COBAS INTEGRA 800 system

On-board in use at 8 °C

26 weeks

## Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable:

Unhemolyzed serum

Unhemolyzed heparinized plasma

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Sample preparation for the COBAS FP Free Phenytoin assay requires the separation of the free phenytoin fraction from the protein-bound fraction prior to analysis. The COBAS FP Free Phenytoin controls and calibrators do not require this separation step because all of the drug is in the free state. Methods to isolate the free fraction include equilibrium dialysis and ultrafiltration. Acceptable filters for ultrafiltration are listed in the "Order information" section.

Samples should be processed by ultrafiltration immediately upon receipt in the lab, if possible. Alternately, samples may be stored refrigerated at 2-8 °C for up to 7 days prior to ultrafiltration, if necessary. Frozen samples should be warmed to room temperature and mixed completely before ultrafiltration. Freezing samples for less than 16 weeks has been reported to have little effect on free phenytoin concentrations.<sup>4,5</sup> Samples should be securely capped and opening minimized prior to ultrafiltration to prevent pH changes.

## Procedure

1. Cut the cap off the COBAS INTEGRA sample cup and push the cup onto the bottom of the Serum Filter.
2. Add the patient sample (approximately 1 mL) to the sample reservoir of the assembled ultrafiltration device.
3. Place the filter in a fixed-angle rotor of a centrifuge, capable of maintaining the temperature at 25 °C ± 3 °C. The rotor and centrifuge must be equilibrated to 25 °C before adding the filters.
4. Centrifuge at 1000-2000 × g for 30 minutes at 25 °C ± 3 °C. At least 220 µL of ultrafiltrate should be obtained to ensure accurate recovery. Smaller volumes may not yield accurate recovery of the free drug.

**Note**

Ultrafiltrates containing free drug should be assayed immediately.

**Materials provided**

See "Reagents – working solutions" section for reagents.

**Materials required (but not provided)**

1. COBAS FP Sample Dilution Reagent (SDR II), Cat. No. 20720720322  
The SDR II is placed as special diluent in its predefined rack position and is stable for 7 days on-board COBAS INTEGRA 400 plus/800 analyzers.
2. Serum Filters, Cat. No. 21986643122, for ultrafiltration.  
The Serum Filters are complete, ready-to-use disposable filters which may be used with COBAS INTEGRA sample cups.

**Assay**

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

**Application for serum and plasma****COBAS INTEGRA 400 plus test definition**

Measuring mode	FP
Reaction mode	R1-SDR2/S-SR
Wavelength	485 nm (excitation) 515 nm (emission)
Reading cycle blank/test	29/45
Unit	µg/mL

**Pipetting parameters**

		Diluent (H <sub>2</sub> O)
R1	120 µL	10 µL
Sample	9 µL	5 µL
Special diluent (SDR II)	6 µL	
SR	15 µL	10 µL
Total volume	175 µL	

**COBAS INTEGRA 800 test definition**

Measuring mode	FP
Reaction mode	R1-SDR2/S-SR
Wavelength	485 nm (excitation) 515 nm (emission)
Reading cycle blank/test	40/60
Unit	µg/mL

**Pipetting parameters**

		Diluent (H <sub>2</sub> O)
R1	135 µL	10 µL
Sample	9 µL	5 µL
Special diluent (SDR II)	6 µL	
SR	15 µL	10 µL
Total volume	190 µL	

**Calibration**

Calibrators	COBAS FP Free Phenytoin Calibrators
Calibration mode	Exponential 5
Calibration replicate	Duplicate recommended
Deviation low/high	< 10 % at ≥ 0.5 µg/mL (≥ 2.0 µmol/L)

**Calibration interval**

Each lot, every 20 weeks, and as required following quality control procedures

Calibration interval may be extended based on acceptable verification of calibration by the laboratory.

A calibration curve must be prepared using the COBAS FP Free Phenytoin Calibrators. Calibrators must be placed from the highest concentration (F) first, to the lowest (A) last, on the CAL/QC rack. This curve is retained in memory by the COBAS INTEGRA systems and recalled for later use.

Traceability: The COBAS FP Free Phenytoin Calibrators are prepared to contain known quantities of phenytoin in normal human serum and are traceable to USP reference standards.

**Note**

Calibrators should be assayed within 2 hours after placing on-board the instrument.

**Quality control**

Quality control	COBAS FP Free Phenytoin Controls
Control interval	24 hours recommended
Control sequence	User defined
Control after calibration	Recommended

For quality control, use control materials as listed in the "Order information" section. In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

**Note**

Controls should be assayed within 2 hours after placing on-board the instrument.

**Calculation**

COBAS INTEGRA analyzers automatically calculate the analyte concentration of each sample. For more details, please refer to Data Analysis in the Online Help (COBAS INTEGRA 400 plus/800 analyzers).

Conversion factor: µg/mL × 3.96 = µmol/L

**Limitations - interference**

See the Specificity section of this method sheet for information on substances tested for cross-reactivity in this assay. There is the possibility that other substances and/or factors may interfere with the test and cause erroneous results (e.g., technical or procedural errors). Specimens with assay values greater than the highest calibrator will be flagged by the system and must be repeated after appropriate manual dilution of the ultrafiltrate of the sample with the zero calibrator. Specimens with high fluorescent backgrounds or those giving polarization values greater than the zero calibrator will also be flagged by the system.

**Serum/plasma**

Samples were filtered following the established protocol.

Criterion: Recovery within ± 10 % of initial value at a phenytoin concentration of 0.75 µg/mL (3.0 µmol/L) and 2.7 µg/mL (10.7 µmol/L).

Icterus:<sup>6</sup> No significant interference up to a bilirubin concentration of 441 µmol/L or 25.8 mg/dL.

Hemolysis:<sup>6</sup> No significant interference up to a hemoglobin concentration of 621 µmol/L or 1000 mg/dL.

Lipemia:<sup>6</sup> No significant interference up to a triglycerides concentration of 2264 mg/dL.

Total protein: No significant interference up to a total protein concentration of 12 g/dL.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

**ACTION REQUIRED**

**Special Wash Programming:** The use of special wash steps is mandatory when certain test combinations are run together on COBAS INTEGRA

analyzers. Refer to the CLEAN Method Sheet for further instructions and for the latest version of the Extra wash cycle list.

**Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.**

### Limits and ranges

#### Measuring range

COBAS INTEGRA 400 plus analyzer:

0.16-4 µg/mL (0.64-15.8 µmol/L)

COBAS INTEGRA 800 analyzer:

0.1-4 µg/mL (0.4-15.8 µmol/L)

#### Lower limits of measurement

Lower detection limit of the test:

COBAS INTEGRA 400 plus analyzer:

0.16 µg/mL (0.64 µmol/L)

COBAS INTEGRA 800 analyzer:

0.1 µg/mL (0.4 µmol/L)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from the zero calibrator at a 95 % confidence level.

### Expected values

A free phenytoin therapeutic range of 1.2-2.5 µg/mL (4.8-9.9 µmol/L) has been suggested to reflect the clinical status of the patient equal to or better than the total phenytoin concentration.<sup>7,8</sup> The normal protein binding equilibrium of a drug may be significantly affected by the concentration changes of serum proteins. As a result of varying protein concentration, the amount of free phenytoin in the circulation can increase between 5-10 fold.<sup>9</sup> Elevated free phenytoin levels have been found in patients with hypoalbuminemia and hepatic and renal impairments.<sup>10</sup> Co-administered drugs, particularly valproic acid, can displace protein bound phenytoin and alter the clinical response.<sup>10</sup>

In view of the potential for large individual differences in protein binding, the quantitation of free phenytoin levels is thought to be a more reliable indicator of clinical effectiveness.<sup>2,9,10</sup>

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

### Specific performance data

Representative performance data on the COBAS INTEGRA analyzers are given below. Results obtained in individual laboratories may differ.

#### Precision

Precision was determined using controls in accordance with the NCCLS EP5-T2<sup>11</sup> requirements with repeatability and intermediate precision (2 aliquots per run, 2 runs per day, 20 days). The following results were obtained on a COBAS INTEGRA 700 analyzer:

Repeatability	Mean µg/mL (µmol/L)	SD µg/mL (µmol/L)	CV %
Level 1	1.0 (4.0)	0.013 (0.051)	1.3
Level 2	2.0 (7.9)	0.024 (0.095)	1.2
Level 3	2.9 (11.5)	0.031 (0.12)	1.1

Intermediate precision	Mean µg/mL (µmol/L)	SD µg/mL (µmol/L)	CV %
Level 1	1.0 (4.0)	0.020 (0.079)	2.0
Level 2	2.0 (7.9)	0.040 (0.158)	2.0
Level 3	2.9 (11.5)	0.071 (0.281)	2.4

### Method comparison

Phenytoin values for human serum samples obtained on a COBAS INTEGRA 700 analyzer using the COBAS INTEGRA Phenytoin reagent (y) were compared with those determined using COBAS FP reagents on the COBAS FARA II analyzer (x).

Number of samples	209
Range of values	min. 0.07 µg/mL

max.	≥ 4 µg/mL
Slope	1.044
Intercept	-0.018 µg/mL
Correlation coefficient	0.996

### Analytical specificity

The following cross-reactive substance was evaluated on the COBAS INTEGRA systems in filtered normal human serum spiked with phenytoin at 1.9 µg/mL (7.5 µmol/L). The substance was tested at 10 times the highest concentration for its therapeutic or normal range, as per the protocol described by NCCLS.<sup>12</sup> The imprecision of the assay was taken into account when determining cross-reactivity.

$$\text{Cross-reactivity (\%)} = \frac{100 \times (\text{analytical result} - \text{analyte concentration})}{\text{concentration of interferent}}$$

Drug	Level tested µg/mL	Cross-reactivity %
5-p-(Hydroxyphenyl)- 5-phenylhydantoin (HPPH)	100	1.8

For additional cross-reactivity information, please refer to the method sheet for phenytoin.

### References

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- National Committee for Clinical Laboratory Standards. Interference Testing in Clinical Chemistry; Proposed Guideline. Villanova, PA.: NCCLS; 1986;6(13). NCCLS Publication EP7-P.

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.


### Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see <https://usdiagnostics.roche.com> for definition of symbols used):

# PHNY

## Phenytoin - Free Phenytoin Application

**cobas<sup>®</sup>**  
Therapeutic drug monitoring

CONTENT	Contents of kit
	Volume after reconstitution or mixing
GTIN	Global Trade Item Number

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