

**Functional Immunoassay Technology™ (FIT™)  
Effective Renal Blood Flow (RBF)  
FIT-RBF™ Kit (IODOHIPPURATE)**

Patents Pending

*An enzyme immunoassay test kit for the determination of **IODOHIPPURATE** in serum, EDTA, citrated or heparinized plasma and urine*

**FOR RESEARCH USE ONLY • NOT FOR USE IN DIAGNOSTIC PROCEDURES  
• THIS PACKAGE INSERT MUST BE READ IN ITS ENTIRETY BEFORE  
USING THIS PRODUCT**

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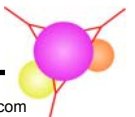
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## PROPRIETARY NAME

Functional Immunoassay Technology™ (FIT™)  
FIT-RBF™ Kit (**IODOHIPPURATE**)  
BioPhysics Assay Laboratory (BioPAL), Inc.  
Catalog Number: FIT-0715, 96-Well Test Kit

## INTENDED USE

The **IODOHIPPURATE** FIT-RBF assay is an enzyme immunoassay used for the determination of **IODOHIPPURATE** in many research settings including physiological studies (RBF measurements), toxicology studies, immunological studies, and pharmaceutical studies. The kit is especially useful to measure **IODOHIPPURATE** in serum, citrated or heparinized plasma and urine in determination of RBF. Following intravenous (IV) administration of **IODOHIPPURATE** for injection, sequential blood and/or urine samples are collected from the subject. The rate of change in the concentration of **IODOHIPPURATE** measured in collected samples over time is used to calculate the RBF.

**THE IODOHIPPURATE FIT-RBF™ ASSAY TEST KIT IS FOR RESEARCH USE ONLY.  
NOT FOR USE IN DIAGNOSTIC PROCEDURES**

## REAGENTS AND MATERIALS SUPPLIED

Test kit reagents are provided as a set sufficient to perform 96 determinations. Some reagents require preparation on the day of assay. Kits should be stored at 2-8°C in the dark. Each kit includes:

1. **IODOHIPPURATE 96-Well Coated Plate (one plate)**  
Catalog # FIT-0716
2. **IODOHIPPURATE Standard Concentrate (1 ml)**  
Concentrate (0.1 mg/ml) should be diluted using Standard and Sample Diluent to appropriate concentrations to run a complete Standard Curve.  
Catalog # FIT-0703
3. **Goat anti-Rabbit IgG-HRP (12 ml)**  
Store away from light.  
Catalog # FIT-0417
4. **Rabbit anti-IODOHIPPURATE (6 ml)**  
Catalog # FIT-0701
5. **HRP Substrate Reagent (12 ml)**  
Proprietary solution provided ready-to-use. Store away from light.  
Catalog # FIT-0002
6. **HRP Stop Reagent (12 ml)**  
Proprietary solution provided ready-to-use.  
Catalog # FIT-0003
7. **Plate Sealer (2 unit)**  
Catalog # FIT-0004
8. **FIT-RBF (IODOHIPPURATE) Kit Manual (1)**  
Catalog # FIT-0718



## ADDITIONAL KIT COMPONENTS AVAILABLE FOR PURCHASE

The following components are not included in the kit, but are necessary to run the assay. Researchers have the option of purchasing these reagents through BioPAL or preparing the reagents themselves. The reagent components are listed below.

1. **Standard and Sample Diluent (100 ml)**

Ready-to-use. Standard and Sample Diluent is composed of 0.1% bovine serum albumin, 0.01% thimerosal in PBS buffer (0.0098M dibasic sodium phosphate, 0.138M sodium chloride, 0.00268M potassium chloride).

Catalog # FIT-0001

2. **Wash Buffer Concentrate (100 ml)**

Dilute  $\frac{1}{10}$  with distilled water before using. Wash Buffer (unconcentrated) is composed of 0.05% Tween® 20 in PBS Buffer (0.0098M dibasic sodium phosphate, 0.138M sodium chloride, 0.00268M potassium chloride).

Catalog # FIT-0005

3. **IODOHIPPURATE for Injection (2 ml at 10 mg/ml and 5 ml at 100 mg/ml)**

Catalog # FIT-0704 and Catalog # FIT-0714

## MATERIALS REQUIRED TO RUN THE KIT, BUT NOT SUPPLIED

1. Precision pipettes with disposable tips to deliver 5 to 3000  $\mu$ l volumes
2. 50-200  $\mu$ l adjustable multi-channel pipette
3. Beaker, flask, cylinders necessary for preparation of reagents
4. 96-well plate washer/aspirator device
5. Mini-vortexer
6. Graph paper or computer software (for data reduction)
8. 96-well plate reader for measurement of absorbance at 450 nm
9. Labcor Non-Sterile Basins, 55 ml, Cat. No. 730-01 or equivalent
10. De-ionized or distilled water
11. Horizontal orbital microshaker

## PRECAUTIONS

1. SUBJECT SPECIMENS AND ALL MATERIALS COMING INTO CONTACT WITH THEM SHOULD BE HANDLED AS IF CAPABLE OF TRANSMITTING INFECTION AND DISPOSED OF USING PROPER PRECAUTIONS. Wear disposable gloves while handling specimens and wash hands afterwards.
2. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
3. Do not pipette by mouth.
4. Reagents containing thimerosal may be toxic if ingested.
5. Avoid contact of Specimens, **IODOHIPPURATE** Concentrate, Rabbit anti-**IODOHIPPURATE**, Goat anti-Rabbit IgG-HRP, Standard and Sample Diluent, HRP Substrate Reagent and HRP Stop Reagent with skin and mucous membranes. In case of contact, thoroughly wash the contaminated area.
6. Consult local regulations concerning disposal of kit reagents, assay materials and subject specimens.
7. Do not mix reagents from different kit lots.



8. Do not use kit components beyond expiration date.
9. Do not expose kit components and **IODOHIPPURATE** for injection to strong light during storage or incubation. Avoid contact of the HRP Substrate Reagent with oxidizing reagents. Do not allow HRP Substrate or Stop Reagents to contact any metal parts.
10. When running multiple plates, a standard curve should be run with each individual plate.
11. Do not pour unused HRP Substrate Reagent back into original container. Take care not to contaminate the HRP Substrate Reagent. If the solution is blue before use, DO NOT USE.
12. Take precautions to avoid microbial contamination when opening and removing aliquots from primary vials.

## STORAGE INSTRUCTIONS

1. Upon receiving, all kit reagents and components should be stored at 2-8°C. **IODOHIPPURATE** for Injection should be stored at room temperature.
2. DO NOT allow kit reagents to remain at room temperature for more than 1 hour before use.
3. All reagents must be brought to room temperature ( $24 \pm 2$  °C) before starting the assay. Unused material must be returned to appropriate storage conditions.
4. Protect Goat anti-Rabbit IgG-HRP (FIT-0417) from exposure to light.

## SPECIMEN COLLECTION AND HANDLING

There are a number of protocols a researcher can use to measure effective renal blood flow (RBF). These protocols include, but are not limited to, the UV/P method, the complete blood clearance method, and single blood sample method. BioPAL's FIT-RBF (**IODOHIPPURATE**) kit will support all standard protocols for measuring RBF. However, the choice of method is the researcher's prerogative.

A venous blood sample is collected aseptically. Serum, EDTA, heparin or citrated plasma are suitable for use in the assay. Remove the serum or plasma from the clot or red cells, respectively, as soon as possible after clotting and separation. Whichever experimental protocol is chosen, it is recommended that the method be used consistently, as subtle changes in recovery may be seen.

Urine samples can be collected as a clean catch or collected by catheterization.

Samples may be stored up to 30 days at 2-8°C. The recovery of **IODOHIPPURATE** from frozen samples may be variable. Each investigator should establish protocols for storage of samples that exceed 30 days.

Prior to assay, sera, plasma and/or urine should be brought to room temperature slowly and gently mixed by hand. Do not vortex or sharply agitate samples.



## PROCEDURAL NOTES

1. Assay each **IODOHIPPURATE** sample and standard in at least duplicate each time the assay is performed.
2. Since conditions can vary from assay-to-assay, a full standard curve must be established for every run and every plate.
3. Disposable pipette tips must be used to prevent cross contamination between reagents or specimens.
4. Reusable glassware must be washed and thoroughly rinsed free of all detergent before use.
5. Thorough washing and aspiration of wells following incubation is required.
6. Incubation times or temperatures other than those specified could cause erroneous results. Perform assay continuously, according to procedure and WITHOUT INTERRUPTION.

## INTERFERING CONDITIONS

1. SODIUM AZIDE INACTIVATES HORSERADISH PEROXIDASE.  
SPECIMENS CONTAINING SODIUM AZIDE SHOULD NOT BE USED IN THIS ASSAY.
2. Turbid specimens or those containing a visible precipitate must be centrifuged prior to use in this assay. DO NOT USE SERUM SPECIMENS WITH SUSPECTED MICROBIAL CONTAMINATION.

## PROCEDURE

### Reagent Preparation

#### Wash Buffer (1L)

Dilute Wash Buffer Concentrate (Cat. No.: FIT-0005)  $1/10$  by adding 100 ml Wash Buffer Concentrate to 900 ml distilled water. Mix thoroughly.

#### IODOHIPPURATE Standards

Prepare ~1 ml of the following dilutions of **IODOHIPPURATE** Concentrate (Cat. No.: FIT-0703, 0.1 mg/ml), using Standard and Sample Diluent:

- 10.0 µg/ml **IODOHIPPURATE**
- 3.0 µg/ml **IODOHIPPURATE**
- 1.0 µg/ml **IODOHIPPURATE**
- 0.3 µg/ml **IODOHIPPURATE**
- 0.1 µg/ml **IODOHIPPURATE**
- 0.03 µg/ml **IODOHIPPURATE**
- 0.01 µg/ml **IODOHIPPURATE**

**Note:** BioPAL's **IODOHIPPURATE** concentrate is based on **IODOHIPPURATE** for Injection. If other sources of **IODOHIPPURATE** are used to measure RBF, we suggest for good laboratory practice developing standards for that source. If using a multi-channel pipette for pipetting standards into wells of the plate, it is recommended that dilutions be transferred to micro-tubes (VWR Cat. # 89005-566).

## ASSAY PROTOCOL

BRING ALL REAGENTS AND SAMPLES TO ROOM TEMPERATURE BEFORE USE.

**Note:** All standards and samples are run in duplicate. Design plate layout to accommodate the desired number of subject samples. For example, consider a standard blood clearance protocol wherein four blood withdrawals and two urine



collections will be obtained per subject. As a result, each subject will utilize 12 wells. An additional 16 wells will be needed for running blanks and standards, and an additional four wells will be needed for running controls, if desired (see Scheme 1, Page 8). Using this protocol and plate design, six subjects may be run on one plate.

1. Mix all reagents thoroughly without foaming before use.
2. As prepared above, pipette 50  $\mu$ l **iodohippurate** Standards into each well in the first two columns as designated by Scheme 1 (Page 8). **DO NOT PIPETTE ANYTHING INTO THE WELLS DESIGNATED FOR BLANKS.**
3. Pipette 50  $\mu$ l of diluted subject sample (serum or urine) per well, as designated by Scheme 1 (Page 8).

**NOTE:** The suggested dose of **iodohippurate** for the measurement of RBF is 0.7 ml/kg bottled at 10 mg/ml or 0.07 ml/kg bottled at 100 mg/ml. Based on this dosage, the suggested dilution for blood samples is 1/50 and the suggested dilution for urine samples is 1/200. Researchers may need to adjust the dilution value to be within the active range of the standard curve. Sample dilutions should be prepared in advance of the assay.

4. Pipette 50  $\mu$ l Rabbit anti-**iodohippurate** (Cat. No.: FIT-0701) into all wells **except blanks**.
5. Cover the plate with a Plate Sealer (Cat. No.: FIT-0004) and incubate on an orbital shaker for 1 hour.
6. Upon completion of the 1 hour incubation period, remove and discard the Plate Sealer. Aspirate solution from all wells. Wash/aspirate with 350  $\mu$ l Wash Buffer per well for a total of three times. Whack plate upside-down on a clean paper towel to remove residual liquid in wells.
7. Pipette 100  $\mu$ l Goat anti-Rabbit IgG-HRP (Cat. No.: FIT-0417) into all wells **except blanks**. Store the HRP-conjugate in the dark until needed.
8. Cover the plate with a new, unused Plate Sealer (Cat. No.: FIT-0004) and incubate on an orbital shaker for 30 minutes.
9. Upon completion of the 30 minute incubation period, remove and discard the Plate Sealer. Aspirate the solution from all wells. Wash/aspirate with 350  $\mu$ l Wash Buffer per well for a total of three times. Whack plate upside-down on a clean paper towel to remove residual liquid in wells.
10. Pipette 100  $\mu$ l HRP Substrate Reagent (Cat. No.: FIT-0002) into all wells **including blanks**. Incubate substrate without shaking for 30 minutes.
11. Pipette 100  $\mu$ l HRP Stop Reagent (Cat. No.: FIT-0003) into all wells **including blanks**. Tap plate gently to mix contents of each well.
12. Read absorbance of wells at 450 nm. The absorbance should be read as soon as possible after the completion of the assay, but may be read up to 30 minutes after addition of HRP Stop Reagent when wells are kept protected from light. Subtract the averaged blank from the averaged standards and unknowns.



**NOTE:** It is important that HRP Stop Reagent be added to wells prior to reading at 450 nm. Addition of HRP Stop Reagent causes an increase in absorbance of the TMB component of the HRP Substrate Reagent and a shift in absorption spectrum.

13. Generate the standard curve by plotting the average absorbance obtained for each Standard concentration on the vertical (y) axis (linear normal scale) vs. the corresponding **IODOHIPPURATE** concentration ( $\mu\text{g/ml}$ ) on the horizontal (x) axis (log scale).

For best results, plot data using 4-parameter curve fitting statistical software. Manual plots on graph paper can also be utilized, but are not recommended. Determination of the **IODOHIPPURATE** amount in each sample can be done by either (1) automatically generating values through curve-fitting software, (2) by using equation generated by software and program, such as Excel®, to fit unknown values into equation, or (3) by manually interpolating from the absorbance value (y-axis) to **IODOHIPPURATE** concentration (x-axis) using the standard curve.

If the test sample was diluted, multiply the interpolated value obtained from the standard curve by the dilution factor to calculate  $\mu\text{g/ml}$  of **IODOHIPPURATE** in the sample.

**Scheme 1: Example of a standard UV/P protocol**

|          | 1          | 2          | 3              | 4              | 5              | 6              | 7              | 8              | 9              | 10             | 11             | 12             |
|----------|------------|------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| <b>A</b> | blank      | blank      | serum<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_3$ | serum<br>$t_3$ | urine<br>$t_1$ | urine<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_3$ | serum<br>$t_3$ |
| <b>B</b> | Standard 1 | Standard 1 | serum<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_4$ | serum<br>$t_4$ | urine<br>$t_2$ | urine<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_4$ | serum<br>$t_4$ |
| <b>C</b> | Standard 2 | Standard 2 | serum<br>$t_3$ | serum<br>$t_3$ | urine<br>$t_1$ | urine<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_3$ | serum<br>$t_3$ | urine<br>$t_1$ | urine<br>$t_1$ |
| <b>D</b> | Standard 3 | Standard 3 | serum<br>$t_4$ | serum<br>$t_4$ | urine<br>$t_2$ | urine<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_4$ | serum<br>$t_4$ | urine<br>$t_2$ | urine<br>$t_2$ |
| <b>E</b> | Standard 4 | Standard 4 | urine<br>$t_1$ | urine<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_3$ | serum<br>$t_3$ | urine<br>$t_1$ | urine<br>$t_1$ | Control 1      | Control 1      |
| <b>F</b> | Standard 5 | Standard 5 | urine<br>$t_2$ | urine<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_4$ | serum<br>$t_4$ | urine<br>$t_2$ | urine<br>$t_2$ | Control 2      | Control 2      |
| <b>G</b> | Standard 6 | Standard 6 | serum<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_3$ | serum<br>$t_3$ | urine<br>$t_1$ | urine<br>$t_1$ | serum<br>$t_1$ | serum<br>$t_1$ |                |                |
| <b>H</b> | Standard 7 | Standard 7 | serum<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_4$ | serum<br>$t_4$ | urine<br>$t_2$ | urine<br>$t_2$ | serum<br>$t_2$ | serum<br>$t_2$ |                |                |

**Key:**

|  |                           |
|--|---------------------------|
| <b>Columns 1&amp;2:</b>                                      | Blanks and Standard Curve |
| <b>Columns 3-12:</b>   | Subject Samples           |
| <b>Grey Wells:</b>   | Unused Wells or controls  |
| $t_x$ : timepoint ( 4 serum, 2 urine timepoints per Subject) |                           |
| Subject 1  | Subject 2                 |
| Subject 3  | Subject 4                 |
| Subject 5  | Subject 6                 |





## RESULTS

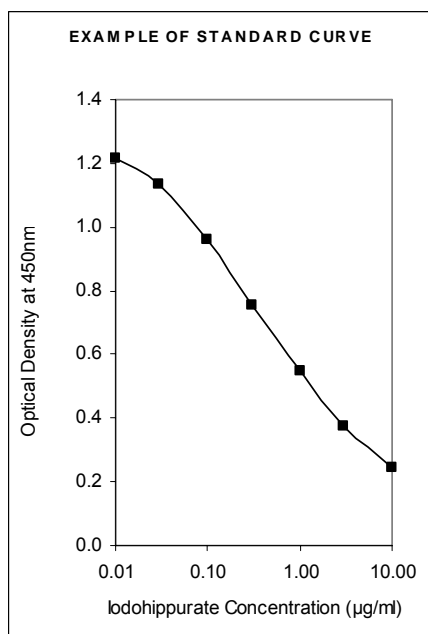
**IODOHIPPURATE** values are expressed in micrograms per milliliter ( $\mu\text{g/ml}$ ).

### A. STANDARD CURVE

1. Record the absorbance at 450 nm for each standard well.
2. Calculate and record the mean absorbance for each set of standard duplicates.
  - a. Correct all values by subtracting the mean value for the blank from each standard mean absorbance value.
  - b. Construct a standard curve by plotting the correct mean absorbance of each standard on the vertical (y) axis (linear) versus the corresponding **IODOHIPPURATE** concentration on the horizontal (x) axis (log). Use log-linear graph paper or plotting software.
  - c. Draw a point-to-point or smooth curve through the points on the graph or use a suitable curve-fitting program with 4-parameter fit to give best fit to the data.

EXAMPLE (Typical Standard Curve Data)

| Standards ( $\mu\text{g/ml}$ ) | 0.01  | 0.03  | 0.1   | 0.3   | 1.0   | 3.0   | 10.0  |
|--------------------------------|-------|-------|-------|-------|-------|-------|-------|
| OPTICAL DENSITY (450 nm)       |       |       |       |       |       |       |       |
|                                | 1.245 | 1.167 | 0.925 | 0.747 | 0.523 | 0.390 | 0.224 |
|                                | 1.279 | 1.201 | 0.962 | 0.760 | 0.527 | 0.387 | 0.225 |
|                                |       |       |       |       |       |       |       |
| <b>Average</b>                 | 1.262 | 1.184 | 0.943 | 0.754 | 0.525 | 0.386 | 0.225 |
|                                |       |       |       |       |       |       |       |
| <b>STANDARD DEVIATION</b>      | 0.024 | 0.024 | 0.026 | 0.009 | 0.003 | 0.002 | 0.001 |
| <b>% CV</b>                    | 1.89  | 2.03  | 2.75  | 1.22  | 0.54  | 0.55  | 0.31  |



**Note:** Do not use example values in place of standard curve determined at the time of the assay.



## B. SAMPLES

1. Record the absorbance at 450 nm for each specimen well.
2. Calculate and record the mean absorbance for each set of specimen duplicates. Correct all values by subtracting the mean value for the blank from each specimen value.
3. Locate the corrected mean absorbance value, which corresponds to each specimen on the vertical axis, and follow a horizontal line intersecting the Standard Curve. At the point of intersection, read the **IODOHIPPURATE** concentration from the horizontal axis. For best results, use curve-fitting software that automatically interpolates this data *via* a 4-parameter curve fit and equation. If sample was diluted, results need to be adjusted by multiplying by the dilution factor to determine actual **IODOHIPPURATE** concentration in the original sample.
4. The measured values of **IODOHIPPURATE** in each sample can then be used to calculate the effective renal blood flow using appropriate methods for the chosen protocol.

## LIMITATIONS

1. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.
2. Since assay conditions may vary from assay to assay, a standard curve must be established for every run.
3. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.
4. Reusable glassware must be washed and thoroughly rinsed of all detergent before use. Disposable flasks or glassware are preferred.
5. Thorough washing of the wells after the first 60 minute and second 30 minute incubations is required:
  - a. Completely aspirate well contents before dispensing fresh wash solution.
  - b. Fill with wash solution to the top of the well for each wash cycle (approximately 350  $\mu$ l).
  - c. Do not allow wells to sit uncovered or dry for extended periods between steps.
6. The 96-well plate is NOT re-useable.
7. The instructions regarding well volumes for reagents cannot be altered.
8. Do not run samples containing other iodinated contrast agents such as Iopamidol or Iothalamate.

## PERFORMANCE CHARACTERISTICS

### A. SENSITIVITY

The detection limit of **IODOHIPPURATE** is approximately 10 nanograms per ml.

### B. PRECISION

Intra-assay and inter-assay precision was determined by choosing two **IODOHIPPURATE** concentrations that fall on the active range of the standard curve and assaying 12 replicates of each. The chosen **IODOHIPPURATE** concentrations were 0.85  $\mu$ g/ml and 0.025  $\mu$ g/ml, and were prepared in Standard and Sample Diluent. The intra and inter-assay CVs were under 12%.



C. CROSS REACTIVITY OF VARIOUS IODINE CONTAINING COMPOUNDS WITH ANTI-**IODOHIPPURATE** ANTISERUM

| Inhibitor                  | Cross reactivity (%w/w) |
|----------------------------|-------------------------|
| <b>IODOHIPPURATE</b>       | 100                     |
| p-Aminohippurate           | <0.0001                 |
| Iopamidol                  | Not detected at 10µg/ml |
| Iohexol                    | Not detected at 10µg/ml |
| Iothalamate                | Not detected at 10µg/ml |
| Sodium iodide              | Not detected at 10µg/ml |
| L-Thyroxine                | Not detected at 10µg/ml |
| 3,3',5-Triiodo-L-thyronine | Not detected at 10µg/ml |
| 3,5-Diiodo-Ltyrosine       | Not detected at 10µg/ml |
| 3-Iodo-L-tyrosine          | Not detected at 10µg/ml |
| Inulin                     | <0.0001                 |
| Gd-DTPA                    | Not detected at 10µg/ml |
| Gd-DOTA                    | Not detected at 10µg/ml |

