GFR Method: Assay ID:_____ Ex. _____
Technician name *Date (YYMMDD01)*

Kit Lot Number: _____Kit Expiration Date: _____

Subjects Tested (GFR):_____

Note: These instructions are for a specific plate configuration with a predetermined number of samples. With slight modification, these instructions can be used to run any plate configuration.

1. Standard and Sample Diluent (0.1% BSA, 0.01% Thimerosal in PBS, Cat. No. FIT-0001) should be stored at 2-8°C and a new aliquot should be poured and brought to room temperature before use.

2. Wash Buffer (0.05% Tween 20 in PBS), if not already prepared, needs to be made by diluting Wash Buffer Concentrate (0.5% Tween 20 in 10x PBS, Cat. No. FIT-0005) tenfold into distilled water.

3. Prepare kit components by allowing them to reach room temperature. Record lot number for each component as follows:

	Cat. No.	Lot No.
Rabbit anti-Iohexol	FIT-0501	_____
Goat anti-Rabbit IgG-HRP	FIT 0417	_____
Iohexol Standard Concentrate	FIT-0503	_____
HRP Substrate Reagent	FIT-0002	_____
HRP Stop Reagent	FIT-0003	_____
Plate Sealer (2 units)	FIT-0004	_____
Iohexol 96-well coated plate	FIT-0516	_____
Standard and Sample Diluent*	FIT-0001	_____
Wash Buffer Concentrate*	FIT-0005	_____

*Sold separately

4. Prepare Subject serum/urine samples by diluting to appropriate concentrations that fall within the active region of the standard curve

Note: The suggested dose of iohexol (10 mg/ml) for the measurement of GFR is 0.1 ml/kg. Based on this dosage, the suggested dilution for blood samples is 1/100 and the suggested dilution for urine samples is 1/1,000. Sample dilutions should be prepared in advance of the assay. Research should validate the required dilutions for their system.

5. Prepare a standard curve using 0.1 mg/ml iohexol Concentrate (Cat. No. FIT-0503) diluted into Standard and Sample Diluent to the following concentrations in µg/ml: 10.0, 3.0, 1.0, 0.3, 0.1, 0.03, 0.01. Detailed instructions for making the standards are given on page 4.

6. Remove Iohexol 96-well coated plate from sealed bag. *Optional:* Use a black permanent marker to partition plate as designated by your own plate layout. Scheme 1 shows a representative plate layout.

7. Pipette 50µl Iohexol Standards into each well as designated in your plate layout.

8. Pipette 50µl diluted Subject serum/urine samples as designated by your plate layout.

9. Pipette 50µl Rabbit anti-Iohexol (Cat. No.:FIT-0501) into **all wells (except Blanks)**.

10. Cover the plate with a Plate Sealer and incubate on an orbital shaker for 1 hour.

11. Upon completion of the 1 hour incubation period, remove and discard the Plate Sealer. Aspirate solution from all wells. Wash/aspirate with 350 µl 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.

12. Pipette 100µl Goat anti-Rabbit IgG-HRP (Cat. No.: FIT 0417) into **all wells (except Blanks)**.

13. Cover the plate with a Plate Sealer and incubate on an orbital shaker for 30 minutes.

14. Upon completion of the 30 minute incubation period, remove and discard the Plate Sealer. Aspirate solution from all wells. Wash/aspirate with 350 µl 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.

15. Check HRP Substrate Reagent to be sure it is colorless. Do not use if blue (contaminated). Pipette 100µl into **all wells (including Blanks)**. Tap gently to mix contents of each well.

16. Incubate substrate for 30 minutes at room temperature (no shaking). Before end of 30-minute incubation, look over plate for any bad wells and circle on your plate layout for future reference.

17. Pipette 100µl of HRP Stop Reagent into **all wells (including Blanks)**. Tap gently to mix contents of each well.

18. Read plate at 450nm and use 4-parameter curve-fit for data reduction.

SCHEME 1: REPRESENTATIVE PLATE LAYOUT

Blanks & Inulin Standard Curve |----------Subject serum/urine samples----------|

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank										
B	0.01	0.01										
C	0.03	0.03										
D	0.1	0.1										
E	0.3	0.3										
F	1.0	1.0										
G	3.0	3.0										
H	10.0	10.0										

Plate Notes:

Appendix 1: Detailed instructions on how to make a Standard Curve

1. Refer to Figure 1 for schematic. Set up four glass culture tubes in a row, leave a space, then three more glass culture tubes in a row.
2. Label the first four tubes with the following concentrations of Iohexol (in µg/ml): 10, 1, 0.1, 0.01. Then label the last three tubes: 3, 0.3, and 0.03.
3. Mark 0.01 with **B**, 0.03 with **C**, 0.1 with **D**, 0.3 with **E**, 1 with **F**, 3 with **G**, and 10 with **H** These letters correspond to the row each of the seven Standards falls on in the Representative Plate Layout (Scheme 1). Although each individual researcher's plate configuration may change, it is strongly recommended the positioning of the standard curve remain in the first two columns of the 96-well plate.
4. Add 1,800µl diluent to test "10 / **H**" and add 700µl diluent to tube "3 / **G**." Add 900µl diluent to each of the remaining tubes.
5. Vortex vial containing Iohexol Concentrate (10µg/ml), Catalog # FIT-0503. Withdraw 200µl of Iohexol Concentrate from the vial and add to tube labeled "10 / **H**" and vortex to mix.
6. Withdraw 100µl from tube labeled "10 / **H**" and add to tube labeled "1 / **F**" and vortex to mix.
7. Withdraw 100µl from tube labeled "1 / **F**" and add to tube labeled "0.1 / **D**" and vortex to mix.
8. Withdraw 100µl from tube labeled "0.1 / **D**" and add to tube labeled "0.01 / **B**" and vortex to mix.
9. Withdraw 300µl from tube labeled "10 / **H**" and add to tube labeled "3 / **G**" and vortex to mix.
10. Withdraw 100µl from tube labeled "3 / **G**" and add to tube labeled "0.3 / **E**" and vortex to mix.
11. Withdraw 100µl from tube labeled "0.3 / **E**" and add to tube labeled "0.03 / **C**" and vortex to mix.
12. For long term storage transfer 850µl of all dilutions marked with **B**-**H** into a string of 7 microtubes, placing concentrations in ascending order: 1st microtube: 850µl "B/ 0.01"; 2nd microtube: 850µl "C/ 0.03"; 3rd microtube: 850µl "D/0.1"; 4th microtube: 850µl "E/0.3"; 5th microtube: 850µl "F/1"; 6th microtube: 850µl "G/3"; 7th microtube: 850µl "H/10".
13. Standard curve is now prepared and is ready to be used in assay.

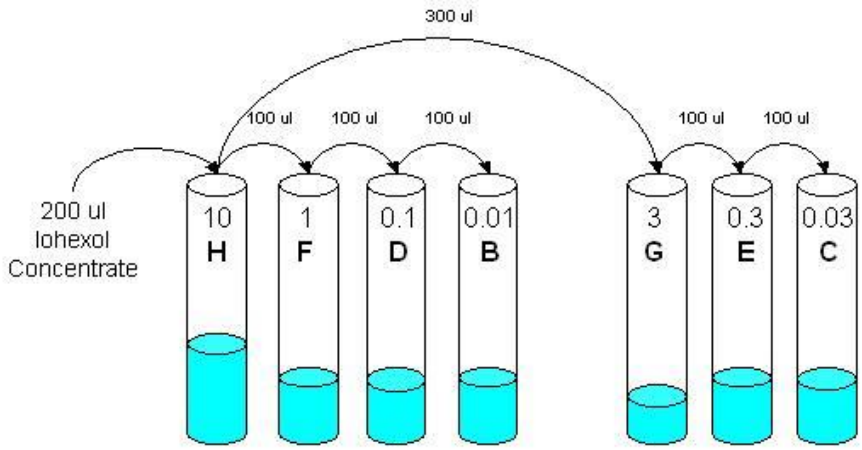

Figure 1: Schematic representation of Standard Curve preparation Add 1,800µl diluent to test "10 / **H**" and add 700µl diluent to tube "3 / **G**" and then add 900µl diluent to each of the remaining tubes.