GFR Method: Plasma Clearance 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-Gadolinium DOTA | FIT-0201 | _____ |
| HRP-Gadolinium DOTA | FIT 0202 | _____ |
| Gadolinium DOTA Concentrate | FIT-0203 | _____ |
| HRP Substrate Reagent | FIT-0002 | _____ |
| HRP Stop Reagent | FIT-0003 | _____ |
| Plate Sealer | FIT-0004 | _____ |
| Goat anti-rabbit Fc 96-well coated plate | FIT-0006 | _____ |
| Gd-DOTA Controls (High and Low)* | FIT-0204 | _____ |
| Standard and Sample Diluent* | FIT-0001 | _____ |
| Wash Buffer Concentrate* | FIT-0005 | _____ |

*Sold separately

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

**Note: The dilution factor will vary depending on the dose of Gd-DOTA administered. For example, at a dose of 10 µl of Gd-DOTA/kg, serum samples require a dilution of 1/300 and the more concentrated urine samples require a dilution of 1/3,000. In contrast, using a standard dose of 100 µl of Gd-DTPA per patient independent of the patient's mass, serum samples require a dilution of 1/75 and urine samples require a dilution of 1/750. Research should validate the required dilutions for their system.**

5. Prepare a standard curve using 1.00mg/ml Gadolinium-DOTA Concentrate (Cat. No. FIT-0203). Perform an initial dilution of 1 to 1000 by pipetting 10 ul of Gadolinium-DOTA Concentrate into 10 ml of Diluent to yield a concentration of 1 ug/ml. Then dilute this initial dilution into Standard and Sample Diluent to the following concentrations in ng/ml:1, 0.3, 0.1, 0.03, 0.01. Detailed instructions for making the standards are given in Appendix 1 (see page 4).

6. Remove Goat anti-rabbit Fc 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 Gd-DOTA Standards into each well as designated in your plate layout.

8. *Optional*: Vortex Gd-DOTA Controls to mix. Pipette 50µl Controls as designated by your plate layout.

9. Pipette 50µl diluted Subject serum samples as designated by your plate layout.

10. Pipette 50µl HRP-Gadolinium DOTA **(yellow)** into **all wells (except Blanks)**. Tap plate gently to mix contents of each well and observe wells for consistency.

11. Pipette 50µl Rabbit anti-Gadolinium DOTA **(blue)** into **all wells (including Blanks)**. Tap plate gently to mix contents of each well and observe wells for consistency. Peel paper backing from Plate Sealer and carefully place Plate Sealer (sticky side down) over plate. Use paper backing to cover plate and protect wells from light.

12. Place plate on Plate Shaker set to 500 rpm and time for 90 minutes.

13. When plate incubation from Step 12 is complete, wash plate three times with 350µl Wash Buffer per well.

14. 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.

15. 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.

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

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

SCHEME 1: REPRESENTATIVE PLATE LAYOUT

Gd-DTPA Standard Curve & Controls |----------Subject serum samples: one color per Subject----------|

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | B | B | | | | | | | | | | |
| **B** | E 0.01 | E 0.01 | | | | | | | | | | |
| **C** | 0.03 | 0.03 | | | | | | | | | | |
| **D** | 0.1 | 0.1 | | | | | | | | | | |
| **E** | 0.3 | 0.3 | | | | | | | | | | |
| **F** | 1 | 1 | | | | | | | | | | |
| **G** | | | | | | | | | | | | |
| **H** | | | | | | | | | | | | |

**Kit Lot Number: _____Kit Expiration Date: _____**
**Subjects Tested (GFR):_____**

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

1. Refer to Figure 1 for schematic. Set up five glass culture tubes in a row, leave a space, then two more glass culture tubes in a row.
2. Label the first five tubes with the following concentrations of Gadolinium-DOTA (in ng/ml): 100, 10, 1, 0.1, 0.01. Then label the last twothree tubes: 0.3, 0.03.
3. Mark 0.01 with **B**, 0.03 with **C**, 0.1 with **D**, 0.3 with **E**, and 1 with **F**. These letters correspond to the row each of the five 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 three columns of the 96-well plate.
4. **IMORPANT: Vortex microtube containing Gadolinium-DOTA Concentrate (1 mg/ml), Catalog # FIT-0203. Perform an initial dilution of Gadolinium-DOTA Concentrate (1 mg/ml) of 1 to 1000 by pipetting 10 ul of Gadolinium-DOTA Concentrate into a 15 ml centrifuge tube containing 10 ml of Diluent to yield a concentration of 1 ug/ml. Vortex well. Label as Gadolinium-DOTA stock (1 ug/ml)**
5. Add 900µl Diluent to every tube except tube labeled "0.3 / E." Add 970µl Diluent to tube labeled "0.3 / E."
6. Withdraw 100µl of Gadolinium-DOTA stock and add to tube labeled "100" and vortex to mix.
7. Withdraw 100µl from tube labeled "100" and add to tube labeled "10" and vortex to mix.
8. Withdraw 100µl from tube labeled "10" and add to tube labeled "1" and vortex to mix.
9. Withdraw 100µl from tube labeled "1" and add to tube labeled "0.1" and vortex to mix.
10. Withdraw 100µl from tube labeled "0.1" and add to tube labeled "0.01" and vortex to mix.
11. Withdraw 30µl from tube labeled "10" and add to tube labeled "0.3" and vortex to mix.
12. Withdraw 100µl from tube labeled "0.3" and add to tube labeled "0.03" and vortex to mix.
13. Transfer 850µl of all dilutions marked with **B-F** into the first 5 positions of a string of 8 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"
14. Standard curve is now prepared and is ready to be used in assay.

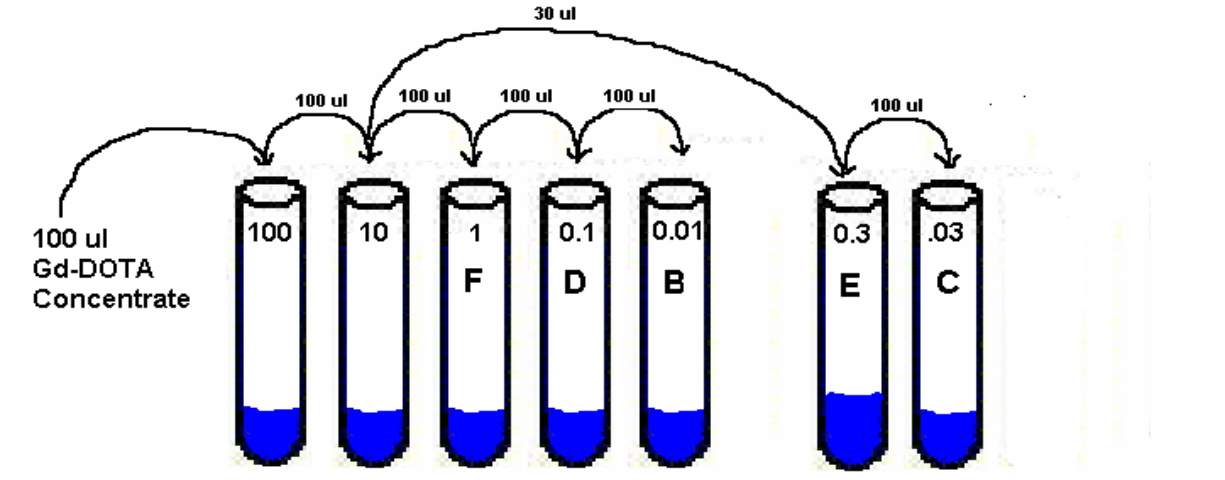


**Figure 1: Schematic representation of Standard Curve preparation**