RayBio®
Human IFN-gamma
ELISA Kit

User Manual (for Cell Lysate and Tissue Lysate)
(Revised Feb 1, 2009)

RayBio® Human IFN-gamma ELISA Kit Protocol

(Cat#: ELH-IFNgamma-001C)

RayBiotech, Inc.

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TABLE OF CONTENTS

I. Introduction……..…….2

II. Reagents……..……..2

III. Storage.…….…….3

IV. Additional Materials Required……..3

V. Reagent Preparation…….……..4

VI. Assay Procedure……... 5

VII. Assay Procedure Summary……...7

VIII. Calculation of Results

A. Typical Data…..…….…….…..8

B. Sensitivity…….…….……8

C. Recovery……..……..8

D. Linearity…….…..... .9

E. Reproducibility……..9

IX. Specificity……. 9

X. References…….....…10

XI. Troubleshooting Guide.……...…...…11

I. INTRODUCTION

IFN-gamma is produced mainly by T-cells and natural killer cells activated by antigens, mitogens, or alloantigens. It is produced by lymphocytes expressing the surface antigens CD4 and CD8. IFN-gamma is a dimeric protein with subunits of 146 amino acids. The protein is glycosylated at two sites. The pI is 8.3-8.5. IFN-gamma inhibits the growth of B-cells induced by IL-4. IFN-gamma inhibits the proliferation of smooth muscle cells of the arterial intima in vitro and in vivo and therefore probably functions as an endogenous inhibitor for vascular overreactions such as stenosis following injuries of arteries.

The RayBio® Human IFN-gamma ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human IFN-gamma cell lysate and tissue lysate. This assay employs an antibody specific for human IFN-gamma coated on a 96-well plate. Standards and samples are pipetted into the wells and IFN-gamma present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IFN-gamma antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IFN-gamma bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. REAGENTS

1. IFN-gamma Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human IFN-gamma.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution
3. Standards (Item C): 2 vials, recombinant human IFN-gamma.

4. Sample Diluent Buffer (Item D): 10 ml of 5x concentrated buffer. For Standard/Sample (cell lysate/tissue lysate) diluent.
5. Assay Diluent (Item E): 15 ml of 5x concentrated buffer. For Detection Antibody (Item F) and HRP-Streptavidin concentrate (Item G) diluent.
6. Detection Antibody IFN-gamma (Item F): 2 vial of biotinylated anti-human IFN-gamma (each vial is enough to assay half microplate).
7. HRP-Streptavidin concentrate (Item G): 8 µl 20,000x concentrated HRP-conjugated streptavidin.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 2 M sulfuric acid.
10. Cell lysate buffer (Item J): 5 ml 2x cell lysate buffer.

III. STORAGE

May be stored for up to 6 months at 2° to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20 ^{o}C or -80 ^{o}C (recommended at –80 ^{o}C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.
Note: the kit can be used within one year if the whole kit is stored at -20 ^{o}C . Avoid repeated freeze-thaw cycles.

IV. ADDITIONAL MATERIALS REQUIRED

1 Microplate reader capable of measuring absorbance at 450 nm.
2 Precision pipettes to deliver 2 µl to 1 ml volumes.
3 Adjustable 1-25 ml pipettes for reagent preparation.
4 100 ml and 1 liter graduated cylinders.
5 Absorbent paper.
6 Distilled or deionized water.

7 Log-log graph paper or computer and software for ELISA data analysis.
8 Tubes to prepare standard or sample dilutions.

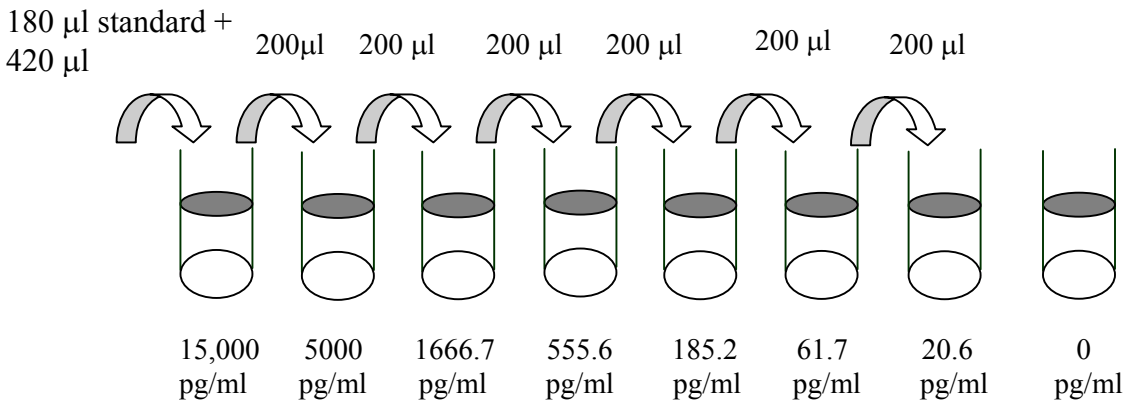
V. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.

2. Sample dilution: Tissue lysate and cell lysate sample should be diluted at least 5-fold with 1x Sample Diluent Buffer.

3. Sample Diluent Buffer (Item D) and Assay Diluent (Item E) should be diluted 5-fold with deionized or distilled water before use.

4. Preparation of standard: **Briefly spin the vial of Item C**. Add 400 µl 1x Sample Diluent Buffer (Item D) into Item C vial to prepare a 50 ng/ml standard. **Dissolve the powder thoroughly by a gentle mix.** Add 180 µl IFN-gamma standard from the vial of Item C, into a tube with 420 µl Sample Diluent Buffer to prepare a 15,000 pg/ml stock standard solution. Pipette 400 µl 1x Sample Diluent Buffer into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1x Sample Diluent Buffer serves as the zero standard (0 pg/ml).



5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.

6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1x Assay Diuent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diuent and used in step 4 of Part VI Assay Procedure.

7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) before use. HRP-Streptavidin concentrate should be diluted 20,000-fold with 1x Assay Diuent.

   For example: Briefly spin the vial (Item G) and pipette up and down to mix gently . Add *2 µl of HRP-Streptavidin concentrate into a tube with 198.0 µl 1x Assay Diluent B to prepare a 100-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix through and then pipette 70 µl of prepared 100-fold diluted solution into a tube with 14 ml 1x Assay Diluent B to prepare a final 20,000 fold diluted HRP-Streptavidin solution.*

8. Cell lysate buffer should be 2-fold with deionized or distilled water (for cell lysate and tissue lysate).

VI. ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.

2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.

   We recommend using 50-500 µg/ml of total protein for lysate sample. The amount of sample used depends on the abundance of target protein. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.

7. Discard the solution. Repeat the wash as in step 3.

8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

2. Add 100 µl standard or sample to each well.
   Incubate 2.5 hours at room temperature or over night at 4°C.

3. Add 100 µl prepared biotin antibody to each well.
   Incubate 1 hour at room temperature.

4. Add 100 µl prepared Streptavidin solution.
   Incubate 45 minutes at room temperature.

5. Add 100 µl TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

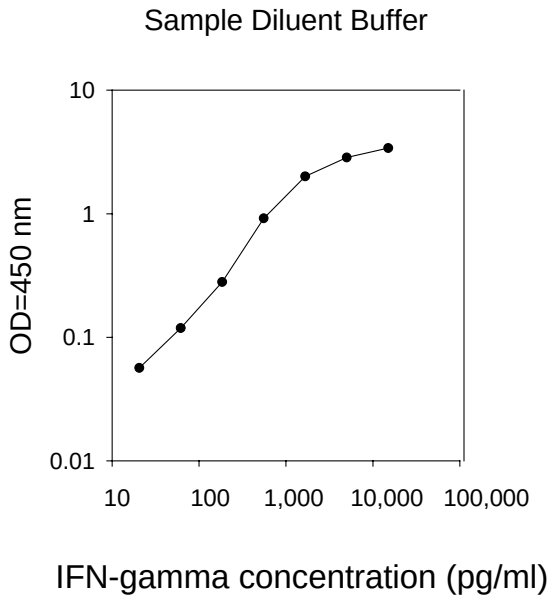
6. Add 50 µl Stop Solution to each well.
   Read at 450 nm immediately.

VIII. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable dose of IFN-gamma is typically less than 15 pg/ml.

C. RECOVERY

Recovery was determined by spiking various levels of human IFN-gamma into tissue lysate and cell lysate. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Tissue lysate	91.73	80-101
Cell lysate	92.25	82-103

D. LINEARITY

Sample Type		Tissue Lysate	Cell lysate
1:2	Average % of	91	92
	Expected Range (%)	80-101	81-102
1:4	Average % of	96	95
	Expected Range (%)	84-103	83-104

E. REPRODUCIBILITY

Intra-Assay: CV<10%
Inter-Assay: CV<12%

IX. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested *(e.g.*, human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, MCP-1, MCP-2, MCP-3, MDC, MIP-1α, MIP-1 β, MIP-1δ, PARC, PDGF, RANTES, SCF, TARC, TGF-β, TIMP-1, TIMP-2, TNF-α, TNF-β, TPO, VEGF).

X. REFERENCES:

1. De Maeyer E and De Maeyer-Guignard J Interferon-gamma. Current Opinion in Immunology 4: 321-6 (1992).

2. Dayton MA et al Human B cell lines express the interferon gamma gene Cytokine 4: 454-60 (1992).

3. Gu D and Sarvetnick N Epithelial cell proliferation and islet neogenesis in IFN-gamma transgenic mice. Development 118: 33-46 (1993).

XI. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting	1. Check pipettes
	2. Improper standard dilution	2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; assay procedure step 2 change to over night
	2. Inadequate reagent volumes or improper dilution	2. Check pipettes and ensure correct preparation
3. Large CV	1. Inaccurate pipetting	1. Check pipettes
4. High background	1. Plate is insufficiently washed	1. Review the manual for proper wash. If using an a plate washer, check that all ports are unobstructed.
	2. Contaminated wash buffer	2. Make fresh wash buffer
5. Low sensitivity	1. Improper storage of the ELISA kit	1. Store your standard at<-20°C after reconstitution, others at 4 ^{o}C. Keep substrate solution protected from light
	2. Stop solution	2. Stop solution should be added to each well before measure

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