ELISA Kit
Catalog # KAC1231 (96 tests)

Human IFN-γ

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Contents and Storage

Storage
Store at 2 to 8°C.

Contents

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<thead>
<tr>
<th>Reagents Provided</th>
<th>96 Test Kit</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard 0</strong> in human serum, with benzamidin and thymol. Lyophilized. Refer to vial label for quantity and reconstitution volume.</td>
<td>3 vials</td>
<td>Black</td>
</tr>
<tr>
<td><strong>Standards 1 - 5</strong> in human serum, with benzamidin and thymol. Lyophilized. Refer to vial label for concentration and reconstitution volume. 1 U of the standard preparation is equivalent to 1 IU NIBSC 87/586.</td>
<td>5 vials</td>
<td>Yellow</td>
</tr>
<tr>
<td><strong>Controls 1 and 2</strong> in human serum, with benzamidin and thymol. Lyophilized. Refer to vial label for reconstitution volume and range.</td>
<td>2 vials</td>
<td>Silver</td>
</tr>
<tr>
<td><strong>IFN-γ Antibody-Coated Wells</strong>, 96 wells per plate.</td>
<td>1 plate</td>
<td>Blue</td>
</tr>
<tr>
<td><strong>Anti-IFN-γ-HRP Conjugate in Tris-Maleate Buffer with BSA and thymol; 6 mL per bottle.</strong></td>
<td>1 bottle</td>
<td>Red</td>
</tr>
<tr>
<td><strong>Wash Buffer Concentrate (200x); 10 mL per bottle.</strong></td>
<td>1 bottle</td>
<td>Brown</td>
</tr>
<tr>
<td><strong>Concentrated Chromogen</strong>, Tetramethylbenzidine (TMB) in DMF, 1 mL per vial.</td>
<td>1 vial</td>
<td>Green</td>
</tr>
<tr>
<td><strong>Substrate Buffer</strong>: H₂O₂ in acetate/citrate buffer; 21 mL per bottle</td>
<td>3 bottles</td>
<td>White</td>
</tr>
<tr>
<td><strong>Stop Solution</strong>, 1.8 N H₂SO₄; 6 mL per bottle</td>
<td>1 bottle</td>
<td>Black</td>
</tr>
</tbody>
</table>

**Note:** Standard 0 is recommended for sample dilutions

**Note**
Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

**Safety**
All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Avoid any skin contact with Stop Solution (H₂SO₄) and Concentrated Chromogen (TMB), Substrate Buffer, and Chromogenic Solution. In case of contact wash thoroughly with water.
Introduction

Purpose
An immunoenzymometric assay for the quantitative measurement of human interferon gamma (IFN-γ) in serum, plasma, cell culture medium or other biological fluids.

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Principle of the Method
The Invitrogen IFN-γ kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs 1) directed against distinct epitopes of IFN-γ are used. The use of a number of distinct MAbs avoids hyperspecificity and allows high sensitive assays with extended standard range and short incubation time. The oligoclonal antibody has been coated onto the wells of the microtiter strips provided.

During the first incubation, standards of known IFN-γ content, controls, and unknown samples are pipetted into the wells together with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). An incubation period allows the formation of a sandwich: coated MAbs 1 - IFN-γ - MAb 2 – HRP. After washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of IFN-γ present in the original specimen.

Background Information
IFN-γ (type 2, immune IFN) is structurally and functionally distinct from type 1 (alpha/beta) interferons and acts on a separate receptor. Only one IFN-γ gene has been identified, coding for a 146 AA protein that is post-translationally processed into two glycosylated species of 20 and 25 Kd. Native IFN-γ is pH2-labile, highly basic, and can aggregate to form dimers that are biologically active. IFN-γ is a real lymphokine produced by activated T (and NK) cells. Despite its clear antiviral and cellular growth regulating activities, its immunomodulatory properties are believed to be the most important. IFN-γ is the principal activator of macrophage function (Macrophage Activating Factor, MAF), and it also regulates the pathway of differentiation of myeloid cells. It plays an important role in the growth and differentiation of cytotoxic (and possibly suppressor) T cells, activates NK cells and acts as a B cell maturation factor. It regulates Ig isotype production and inhibits IgE responses. One of the modes of action of IFN-γ is to induce the expression of membrane proteins, such as class 1 and class 2 MHC antigens and adhesion molecules on various cell types, high affinity Fc receptors for IgG on myelomonocytic cells, etc. Integrated in the cytokine network, IFN-γ interacts with other cytokines, in either a synergistic (e.g. TNF) or antagonistic (e.g. IL-4) way (1-5).
Methods

Materials Needed But Not Provided
• Microtiter plate reader (at or near 450 nm) with software
• Horizontal microtiter plate shaker capable of 700 rpm ± 100 rpm
• Calibrated adjustable precision pipettes
• Distilled or deionized water
• Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
• Glass or plastic tubes for diluting solutions
• Absorbent paper towels
• Calibrated beakers and graduated cylinders

Procedural Notes
1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. Microtiter plates should be allowed to come to room temperature before opening the foil bags. Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. Do not mix or interchange different reagent lots from various kit lots.
9. Do not use reagents after the kit expiration date.
10. Absorbances should be read immediately, but can be read up to 2 hours after assay completion. For best results, keep plate covered in the dark.
11. In-house controls or kit controls, if provided, should be run with every assay. If control values fall outside pre-established ranges, the assay accuracy suspect.
12. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
13. Because Chromogen solution is light sensitive, avoid prolonged exposure to light. Avoid contact between chromogen and metal, or color may develop.

Directions for Washing
- Incomplete washing will adversely affect the test outcome. All washing must be performed with the Wash Buffer provided.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of each well. Take care not to scratch the inside of the well. After aspiration, fill the wells with at least 0.4 mL of diluted Wash Buffer. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under Assay Procedure. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- Alternatively, the diluted Wash Buffer may be put into a squirt bottle. If a squirt bottle is used, flood the plate with the diluted Wash Buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- If using an automated washer, follow the washing instructions carefully.
**Sample Preparation**

The IFN-γ kit may be used to measure IFN-γ in serum, plasma, cell culture supernatant as well as other biological fluids. Isolation and culture of peripheral blood mononuclear cells may be realized by usual methods. However, one should avoid an unintentional stimulation of the cells by the procedure. The use of pyrogen-free reagents and adequate controls are mandatory.

Sampling conditions can affect values measured in serum or plasma, therefore, strict precautions have to be taken during sampling to avoid impurities contained in sampling materials that would stimulate IFN-γ production by blood cells and thus falsely increase plasma IFN-γ values.

Serum must be removed as soon as possible from the clot of red cells after clotting and centrifugation, and kept at 4°C.

Collection tubes must be pyrogen-free. Plasma can be collected on sterile EDTA or heparin tubes (at 4°C) and rapidly separated after centrifugation. However, as batches of heparin are often contaminated with pyrogen, it is recommended to test each batch of heparin to avoid unintentional stimulation of blood cells. Other substances in the tube must be also pyrogen-free.

These recommendations are also valuable for other biological fluids (urine, etc.).

**Sample Dilution:** If samples generate values higher than the highest standard, dilute the sample with the Standard 0 (Diluent) and repeat the assay.

**Storage:** Serum/plasma samples must be kept at -20°C for maximum 2 months, and for longer storage (maximum one year) at -70°C. Samples with low protein levels (e.g. cell culture medium, urine, etc.) should be stored at -70°C (maximum one year).

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**Standards and Controls**

Reconstitute lyophilized Standard 0 according to the instructions on the vial.

Reconstitute the lyophilized Standards and Controls to the volume specified on the vial label with distilled water (0.5 mL). Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion. See vial label for exact concentration.

The reconstituted Standards and Controls are stable for 4 days at 2°C to 8°C. Aliquots held for longer periods of time should be frozen at -20°C (maximum 2 months) or at -70°C for longer storage (until expiration date). Avoid freeze-thaw cycles.

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**Wash Buffer**

Dilute 2 mL of Washing Solution Concentrate in 400 mL distilled water or all the contents of the Wash Solution Concentrate vial in 2000 mL distilled water (use a magnetic stirrer).

The Wash Solution Concentrate is stable at room temperature until expiration date. In order to avoid washerhead obstructions, it is recommended to prepare a fresh diluted Wash Solution each day.
Chromogen Solution

Pipette 0.2 mL of the Concentrated Chromogen (TMB) into one of the vials of Substrate Buffer (H₂O₂ in acetate/citrate buffer). Extemporaneous preparation is necessary. Use only at room temperature. Avoid direct exposure to sunlight.

The freshly prepared Chromogen Solution is stable for a maximum of 15 min. at room temperature and must be discarded afterwards.

Assay Procedure

Be sure to read the Procedural Notes section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame for current use. (Re-bag extra strips. Store these in the refrigerator for future use.)
2. Pipette 50 µL of each Standard, Control, or Sample into the appropriate wells.
3. Pipette 50 µL of anti-IFN-γ HRP Conjugate into all the wells.
4. Incubate for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See Direction for Washing.
6. Pipette 200 µL of freshly prepared Chromogen Solution into each well within 15 min.
7. Incubate the plate for 15 min. at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight. Note: Do not cover the plate with aluminum foil or metalized mylar. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument.
8. Pipette 50 µL of Stop Solution into each well. The solution in the wells should change from blue to yellow.
9. Read absorbance at 450 nm and 490 nm (reference filter: 630 or 650 nm) within 3 hours. Read the plate within 30 minutes after adding the Stop Solution.
10. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.
11. Read the concentrations for unknown samples and controls from the standard curve. (Samples producing signals greater than that of the highest standard (500 pg/mL) should be further diluted with Standard 0 and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)
12. If any Control or Sample has an absorbance greater than the absorbance of the last standard read at 450 nm, a second reading at 490 nm (reference filter: 630 or 650 nm) is needed. Construct a second standard curve at 490 nm using all the standard points. The segment of the curve drawn between the last standard read at 450 nm and the most concentrate standard will be considered at 490 nm. The concentration of Samples and Controls for which absorbance is included in this segment, is read at 490 nm. So, the first reading gives the high sensitivity of the assay and the second reading allows an extended standard range. Note: The readings at 490 nm are only for off-scale values at 450 nm (above the limit of reader linearity) and should not replace the reading at 450 nm for values below the limit of reader linearity.
The following data were obtained for the various standards over the range of 0 to 30 IU/mL IFN-γ.

<table>
<thead>
<tr>
<th>Standard IFN-γ (IU/mL)</th>
<th>Optical Density (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3.107</td>
</tr>
<tr>
<td>10</td>
<td>1.353</td>
</tr>
<tr>
<td>5</td>
<td>0.700</td>
</tr>
<tr>
<td>2</td>
<td>0.339</td>
</tr>
<tr>
<td>1</td>
<td>0.173</td>
</tr>
<tr>
<td>0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Typical Data (Example)**
Performance Characteristics

Analytical Sensitivity
The minimum detectable dose of IFN-γ is 0.03 IU/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

Precision
1. Intra-Assay Precision
Samples of IFN-γ were assayed in replicates of 20 to determine precision within an assay.

<table>
<thead>
<tr>
<th>Mean (pg/mL)</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.26 IU/mL</td>
<td>12.28 IU/mL</td>
</tr>
<tr>
<td>SD</td>
<td>0.03</td>
<td>0.35</td>
</tr>
<tr>
<td>%CV</td>
<td>3.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

SD = Standard Deviation  
CV = Coefficient of Variation

2. Inter-Assay Precision
Samples were assayed 20 times in multiple assays to determine precision between assays.

<table>
<thead>
<tr>
<th>Mean (pg/mL)</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.61 IU/mL</td>
<td>5.72 IU/mL</td>
</tr>
<tr>
<td>SD</td>
<td>0.03</td>
<td>0.51</td>
</tr>
<tr>
<td>%CV</td>
<td>5.8</td>
<td>8.8</td>
</tr>
</tbody>
</table>

SD = Standard Deviation  
CV = Coefficient of Variation
### Linearity of Dilution

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Theor. conc. (IU/mL)</th>
<th>Meas. conc. (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1/1</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>9</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>4.5</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>2.25</td>
<td>2.39</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>1.13</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>0.56</td>
<td>0.6</td>
</tr>
<tr>
<td>Plasma</td>
<td>1/1</td>
<td>-</td>
<td>11.72</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>5.86</td>
<td>5.89</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>2.93</td>
<td>3.17</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>1.47</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>0.73</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>0.37</td>
<td>0.41</td>
</tr>
</tbody>
</table>

### Recovery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added IFN-γ (IU/mL)</th>
<th>Recovery IFN-γ (IU/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>20.46</td>
<td>20.3</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>9.85</td>
<td>10.12</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>4.73</td>
<td>4.76</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>2.41</td>
<td>2.25</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>1.03</td>
<td>99</td>
</tr>
<tr>
<td>Plasma</td>
<td>20.46</td>
<td>19.6</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>9.85</td>
<td>9.53</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>4.73</td>
<td>4.81</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>2.41</td>
<td>2.43</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>1</td>
<td>96</td>
</tr>
</tbody>
</table>

### Antigenic Specificity

No significant cross-reaction was observed in presence of 50 ng of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, TNF-α, TNF-β, IFN-α, IFN-β, TGF-β, GM-CSF, OSM, MIP-1α, MIP-1β, LIF, MCP-1, G-CSF and RANTES. This IFN-γ assay is specific for human natural and recombinant IFN-γ.

### High Hook Dose Effect

A sample spiked with IFN-γ up to 500,000 IU/mL gives a response higher than that obtained for the last standard point.

### Expected Range

At the present stage of our studies, only preliminary results can be provided and we thus recommend that each laboratory establishes its own normal values. For guidance, the mean of 60 normal plasma was 0.08 IU/mL (SD = 0.12), ranging between 0 IU/mL and 0.89 IU/ml. This study was performed with samples collected in strict sampling condition.
Limitations of the Procedure

Do not extrapolate the standard curve beyond the top standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute all samples above the top standard point; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native IFN-γ in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.
# Appendix

## Troubleshooting Guide

<table>
<thead>
<tr>
<th>Issue</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
</table>
| Elevated background          | *Cause:* Insufficient washing and/or draining of wells after washing. Solution containing HRP conjugate can elevate the background if residual is left in the well.  
*Solution:* Wash according to the protocol. Verify the function of automated plate washer. At the end of each washing step, invert plate on absorbent tissue on countertop and allow to completely drain and tap forcefully if necessary to remove residual fluid.  
*Cause:* Contamination of substrate solution with metal ions or oxidizing reagents.  
*Solution:* Use distilled/deionized water for dilution of wash buffer and use plastic equipment. DO NOT COVER plate with foil.  
*Cause:* Contamination of pipette, dispensing reservoir or substrate solution with HRP conjugate.  
*Solution:* Do not use chromogen that appears blue prior to dispensing onto the plate. Obtain new vial of chromogen.  
*Cause:* Incubation time is too long or incubation temperature is too high.  
*Solution:* Reduce incubation time and/or temperature. |                                                             |
| Elevated sample/standard ODs | *Cause:* Incorrect dilution of standard stock solution; intermediary dilutions not followed correctly.  
*Solution:* Follow the protocol instructions regarding the dilution of the standard.  
*Cause:* Incorrect dilution of the HRP conjugate.  
*Solution:* Warm solution of HRP concentrate to room temperature, draw up slowly and wipe tip with laboratory wipe to remove excess. Dilute ONLY in HRP diluent provided.  
*Cause:* Incubation times extended.  
*Solution:* Follow incubation times outlined in protocol.  
*Cause:* Incubations carried out at 37°C when RT is dictated.  
*Solution:* Perform incubations at RT (= 25 ± 2°C) when instructed in the protocol. |                                                             |
| Poor standard curve          | *Cause:* Improper preparation of standard stock solution.  
*Solution:* Dilute lyophilized standard as directed by the vial label only with the standard diluent buffer or in a diluent that most closely matches the matrix of your sample.  
*Cause:* Reagents (lyophilized standard, standard diluent buffer, etc.) from different kits, either different analyte or different lot number, were substituted.  
*Solution:* NEVER substitute any components from another kit.  
*Cause:* Errors in pipetting the standard or subsequent steps.  
*Solution:* Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device. |                                                             |
Weak/no color develops

Cause: Reagents not at RT (25 ± 2°C) at start of assay.
Solution: Allow ALL reagents to warm to RT prior to commencing assay.

Cause: Incorrect storage of components, e.g., not stored at 2 to 8°C.
Solution: Store all components exactly as directed in protocol and on labels.

Cause: Working HRP conjugate solution made up longer than 15 minutes before use in assay.
Solution: Use the diluted HRP conjugate within 15 minutes of dilution.

Cause: TMB solution lost activity.
Solution 1: The TMB solution should be clear before it is dispensed into the wells of the microtiter plate. An intense aqua blue color indicates that the product is contaminated. Please contact Technical Support if this problem is noted. To avoid contamination, we recommend that the quantity required for an assay be dispensed into a disposable trough for pipetting. Any TMB solution left in the trough should be discarded.
Solution 2: Avoid contact of the TMB solution with items containing metal ions.

Cause: Attempt to measure analyte in a matrix for which the ELISA assay has not been optimized.
Solution: Please contact Technical Support for advice when using alternative sample types.

Cause: Wells have been scratched with pipette tip or washing tips.
Solution: Use caution when dispensing and aspirating into and out of microwells.

Poor Precision

Cause: Errors in pipetting the standards, samples or subsequent steps.
Solution: Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device. Check for any leaks in the pipette tip.

Cause: Repetitive use of tips for several samples or different reagents.
Solution: Use fresh tips for each sample or reagent transfer.

Cause: Wells have been scratched with pipette tip or washing tips.
Solution: Use caution when dispensing and aspirating into and out of microwells.

Technical Support

Contact Us
For more troubleshooting tips, information, or assistance, please call, email, or go online to www.invitrogen.com/ELISA.

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References


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Human IFNγ Assay Summary

Add 50 µL of standard, control or sample

Add 50 µL of Anti-IFN-γ-HRP conjugate
Incubate 2 hr at RT shaking

⇒ aspirate and wash 4x

Add 200 µL of Chromogen
Incubate 15 minutes at RT shaking

⇒

Add 50 µL Stop Solution
Read at 450 nm

Total time: 2 hr 15 minutes