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Introduction

Human Interleukin-12 (p70) (hIL-12), also known as natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMRF), is a potent regulator of cell-mediated immune responses. Biologically active IL-12 is secreted by activated B lymphocytes and macrophages as a 70 kD heterodimeric glycoprotein composed of disulfide-bonded 35 kD (p35) and 40 kD (p40) subunits. The BD OptEIA™ kit specifically measures only this biologically active p70.

IL-12 stimulates the growth of activated CD4\(^+\) and CD8\(^+\) T cells and NK cells. IL-12 also promotes the development of proinflammatory-Th1-like CD4\(^+\) cells and cytotoxic CD8\(^+\) T cells. Other effects include increasing the lytic activity of NK cells and antibody-dependent cell-mediated cytotoxic cells, synergizing with IL-2 in the generation of lymphokine-activated killer (LAK) cells, and inducing NK and T cells to secrete large amounts of the proinflammatory cytokine, IFN-\(\gamma\).

The BD OptEIA™ human IL-12 (p70) ELISA Kit is for the \textit{in vitro} quantitative determination of human IL-12 (p70) in serum, plasma, and cell culture supernatant.

Principle of the Test

The BD OptEIA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes a monoclonal antibody specific for IL-12 (p70) coated on a 96-well plate. Standards and samples are added to the wells, and any IL-12 (p70) present binds to the immobilized antibody. The wells are washed and a mixture of biotinylated anti-human IL-12 (p70) antibody and Streptavidin-horseradish peroxidase is added, producing an antibody-antigen-antibody “sandwich”. The wells are again washed and a substrate solution is added, which produces a blue color in direct proportion to the amount of IL-12 (p70) present in the initial sample. The Stop Solution changes the color from blue to yellow, and the wells are read at 450 nm.
Reagents Provided

Antibody Coated Wells: 2 plates of 96 breakable wells (12 strips × 8 wells) coated with anti-human IL-12 (p70) monoclonal antibody

Detection Antibody: 30 mL of biotinylated anti-human IL-12 (p70) monoclonal antibody with ProClin™-150 as preservative

Enzyme Concentrate (250×): 150 μL of 250× concentrated Streptavidin-horseradish peroxidase conjugate with BSA* and ProClin™-150 as preservative

Standards: 4 vials lyophilized recombinant human IL-12 (p70)

Standard Diluent: 30 mL of a buffered protein base with 0.15% ProClin™-150 as preservative

Wash Concentrate (20×): 100 mL of 20× concentrated detergent solution with ProClin™-150 as preservative

Substrate Reagent A: 13 mL of hydrogen peroxide in buffered solution

Substrate Reagent B: 13 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution

Stop Solution: 13 mL of 1 M phosphoric acid

Plate Sealers: 4 sheets with adhesive backing

*Source of all serum proteins is from USDA inspected abattoirs located in the United States

Materials Required but not Provided

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 50 μL and 100 μL volumes
- Adjustable 1 mL, 5 mL, 10 mL, 25 mL pipettes for reagent preparation
- Deionized or distilled water
- Wash bottle or automated microplate washer
- Log-log graph paper or automated data reduction
- Tubes to prepare standard dilutions
- Laboratory timer
- Absorbent paper

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Storage Information

1. Store unopened kit at 2 - 8°C. Do not use kit after expiration date.

2. Before use, bring all reagents to room temperature (18 - 25°C). Immediately after use, return to proper storage conditions.

3. Lyophilized standards are stable until kit expiration date. After reconstitution, use freshly reconstituted standard within 12 hours (stored at 2 - 8°C).

Warnings and Precautions

1. Reagents that contain preservatives may be toxic if ingested, inhaled, or come in contact with skin.

2. Avoid contact of skin, eyes, or clothing with Stop Solution or Substrate Reagents.

3. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.

4. Warning

Wash Concentrate (20X) (component 51-9003738) contains 0.002% (w/w), Human IL-12 (p70) Lyophilized Standard (component 51-26816E) contains 0.03% (w/w), Standard Diluent (component 51-2604KC) contains 0.003% (w/w), and Detection Antibody Biotin Anti-Human IL-12 (p70) (component 51-26812E) contains 0.003% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

Hazard statements

May cause an allergic skin reaction.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

Avoid breathing mist/vapours/spray.

If skin irritation or rash occurs: Get medical advice/attention.

IF ON SKIN: Wash with plenty of water.

Dispose of contents/container in accordance with local/regional/national/international regulations.

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5. **Danger**

Stop Solution (component 51-2608KC) contains 15.23% phosphoric acid (w/w).

**Hazard statements**

Causes severe skin burns and eye damage.

**Precautionary statements**

Wear protective gloves / eye protection.

Wear protective clothing.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

Dispose of contents/container in accordance with local/regional/national/international regulations.

6. **Danger**

Substrate Reagent B (component 51-2607KD) contains 33.05% methanol (w/w).

**Hazard statements**

Flammable liquid and vapor.

Toxic if swallowed, in contact with skin or if inhaled.

Causes damage to the central nervous system. Route of exposure: Oral.

**Precautionary statements**

Keep away from heat/sparks/open flames/hot surfaces. - No smoking.

Wear protective gloves / eye protection.

Wear protective clothing.

Do not breathe mist/vapours/spray.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic. Samples with expected values higher than the top standard, 300 pg/mL, should be diluted with Standard/Sample Diluent prior to running the assay.

Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum tube (eg, BD Vacutainer® Cat. No. 366430) and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 × g. Remove serum and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin as anticoagulant. Centrifuge for 10 minutes at 1000 × g within 30 minutes of collection. Assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Reagent Preparation

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

2. Standards

   a. Reconstitute 1 vial lyophilized Standard with required volume (noted on vial label) of Standard Diluent to prepare a 500 pg/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex to mix.

   b. Add 300 µL Standard Diluent to 6 tubes. Label as 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 pg/mL.

   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. The undiluted standard serves as the high standard (500 pg/mL). The Standard Diluent serves as the zero standard (0 pg/mL).

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3. Serial dilutions of the standard within the plate may also be performed. Add 100 µL Standard Diluent to each well designated for standards, except for the high standard. Add 100 µL of the high standard to these wells and also to the designated 250 pg/mL standard wells. Mix by rinsing pipette tip 3×, then transfer 100 µL to 125 pg/mL wells. Continue in this fashion to the 7.8 pg/mL wells. Discard the extra 100 µL from these wells.

4. Working Detector
   
   Note: One-step incubation of Biotin/Streptavidin reagents. See Assay Procedure, step 4.

5. Wash Buffer
   
   Note: If the Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute required quantity of 20× Wash Concentrate with deionized or distilled water, mix. (To prepare 2,000 mL, add 100 mL Wash Concentrate to 1,900 mL water. At least 500 mL solution should be prepared for a full 96-well plate).

6. Substrate Solution
   
   Within 15 minutes prior to use, mix equal volumes of Substrate Reagent A and Substrate Reagent B in a clean glass tube or flask. Make only the amount required for each run (for a full 96-well plate, prepare 6 mL + 6 mL = 12 mL solution). Discard any remaining working solution after use. Avoid prolonged exposure to light or contact with metal, air, or extreme temperature as color may develop.

Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25°C) prior to use. It is recommended that all standards and samples be run in duplicate. A standard curve is required in each assay run.

2. Remove required quantity of test strips/wells, place in well holder.
   
   Note: Wells are provided in breakable 8-well strips. Strips may be “broken” into individual wells, replaced in well holder, and assayed. Return any unused wells to sealed pouch for 2 - 8°C storage.

3. Pipette 100 µL of each standard (see Reagent Preparation, step 2), sample, and control into appropriate wells. Cover wells with Plate Sealer and incubate for 2 hours at room temperature.

4. Prepare Working Detector:
   
   Within 15 minutes prior to use, pipette required volume of Detection Antibody into a clean tube or flask. Add in required quantity of Enzyme Concentrate (250×), vortex or mix well. For a full 96-well plate, add 48 µL of Enzyme Concentrate into 12 mL of Detection Antibody.

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5. Decant or aspirate contents of wells. Wash wells by filling with at least 300 μL/well prepared Wash Buffer (see Reagent Preparation, step 5) and then decanting/aspirating. Repeat wash 4 times for a total of 5 washes. After the last wash, blot plate on absorbent paper to remove any residual buffer. Complete removal of liquid is required for proper performance.

6. Add 100 μL of prepared Working Detector (see step 4 above) to each well. Cover wells with Plate Sealer and incubate for 1 hour at room temperature.

7. Wash wells as in Step 5, but wash a total of 7 times, soaking the wells for 30 seconds to 1 minute for each wash. Thorough washing at this step is very important.

8. Add 100 μL of prepared Substrate Solution (see Reagent Preparation, step 6) to each well. Incubate plate (without Plate Sealer) for 30 minutes at room temperature in the dark.

9. Add 50 μL of Stop Solution to each well.

10. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract A (570) nm from A (450 nm).

**Assay Procedure Summary**

1. Add 100 μL standard or sample to each well. Incubate 2 hours at room temperature.

2. Aspirate and wash 5 times.

3. Add 100 μL prepared Working Detector to each well. Incubate 1 hour at room temperature.

4. Aspirate and wash 7 times.

5. Add 100 μL Substrate Solution to each well. Incubate 30 minutes at room temperature.

6. Add 50 μL Stop Solution to each well. Read at 450 nm within 30 minutes. λ correction 570 nm.
Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance (ie, plate background) from each.

Plot the standard curve on log-log graph paper, with IL-12 (p70) concentration on the x-axis and absorbance on the y-axis. Draw the best fit straight line through the standard points.

To determine the IL-12 (p70) concentration of the unknowns, find the unknown mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the IL-12 (p70) concentration. If samples were diluted, multiply the IL-12 (p70) concentration by the dilution factor.

Computer data reduction may also be employed, utilizing linear regression analysis.

Typical Data

This standard curve is for demonstration only. A standard curve must be run with each assay.

<table>
<thead>
<tr>
<th>Concentration (pg/mL)</th>
<th>OD1</th>
<th>OD2</th>
<th>Mean</th>
<th>Zero Standard Subtracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.031</td>
<td>0.033</td>
<td>0.032</td>
<td>0.000</td>
</tr>
<tr>
<td>7.8</td>
<td>0.063</td>
<td>0.060</td>
<td>0.062</td>
<td>0.030</td>
</tr>
<tr>
<td>15.6</td>
<td>0.103</td>
<td>0.092</td>
<td>0.098</td>
<td>0.066</td>
</tr>
<tr>
<td>31.3</td>
<td>0.162</td>
<td>0.149</td>
<td>0.155</td>
<td>0.123</td>
</tr>
<tr>
<td>62.5</td>
<td>0.289</td>
<td>0.280</td>
<td>0.284</td>
<td>0.252</td>
</tr>
<tr>
<td>125</td>
<td>0.562</td>
<td>0.557</td>
<td>0.559</td>
<td>0.527</td>
</tr>
<tr>
<td>250</td>
<td>1.060</td>
<td>0.999</td>
<td>1.030</td>
<td>0.998</td>
</tr>
<tr>
<td>500</td>
<td>1.870</td>
<td>1.817</td>
<td>1.843</td>
<td>1.811</td>
</tr>
</tbody>
</table>

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Limitations of the Procedure

1. Samples that generate absorbance values higher than the standard curve should be diluted with Standard Diluent and re-assayed.

2. Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.

3. This kit is intended for use as an integral unit. Do not mix reagents from different kit lots. Reagents from other manufacturers/other available clones should not be used in this kit.

Performance

Limit of Detection

The minimum detectable dose of hIL-12 (p70) was determined to be 4 pg/mL. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

Recovery

Human IL-12 (p70) was added to various matrices at three different levels in the assay range. The serum and plasma were diluted 1:10 with Standard Diluent prior to IL-12 addition. Results are compared with same amounts of IL-12 added to Standard Diluent, as follows:

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Observed in Standard Diluent (pg/mL)</th>
<th>Observed in given matrix (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>500</td>
<td>424</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>87</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9</td>
<td>84</td>
</tr>
<tr>
<td>Plasma</td>
<td>500</td>
<td>401</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>81</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>84</td>
</tr>
<tr>
<td>Cell culture supernatant</td>
<td>425</td>
<td>355</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>91</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>12</td>
<td>72</td>
</tr>
</tbody>
</table>
Linearity

Human IL-12 (p70) was added to the following matrices, which were then serially diluted with Standard Diluent and run in the BD OptEIA Human IL-12 (p70) Kit. The serum and plasma were diluted 1:10 with Standard Diluent prior to IL-12 addition.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Expected (pg/mL)</th>
<th>Observed (pg/mL)</th>
<th>% of Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>undiluted</td>
<td>-</td>
<td>424</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>212</td>
<td>204</td>
<td>96</td>
</tr>
<tr>
<td>1:4</td>
<td>106</td>
<td>102</td>
<td>97</td>
</tr>
<tr>
<td>1:8</td>
<td>53</td>
<td>47</td>
<td>89</td>
</tr>
<tr>
<td>1:16</td>
<td>27</td>
<td>23</td>
<td>89</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>undiluted</td>
<td>-</td>
<td>401</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>201</td>
<td>209</td>
<td>104</td>
</tr>
<tr>
<td>1:4</td>
<td>100</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>1:8</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>1:16</td>
<td>25</td>
<td>24</td>
<td>98</td>
</tr>
<tr>
<td>Cell culture media</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>undiluted</td>
<td>-</td>
<td>355</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>177</td>
<td>188</td>
<td>106</td>
</tr>
<tr>
<td>1:4</td>
<td>89</td>
<td>99</td>
<td>111</td>
</tr>
<tr>
<td>1:8</td>
<td>44</td>
<td>52</td>
<td>117</td>
</tr>
<tr>
<td>1:16</td>
<td>22</td>
<td>29</td>
<td>132</td>
</tr>
</tbody>
</table>

Specificity

Cross Reactivity

The factors listed below were spiked in Standard Diluent at 50 ng/mL to test for any cross reactivity with the BD OptEIA Human IL-12 (p70) assay. No cross reactivity was identified.

Recombinant Human

sCD23, Eotaxin, sFas, GM-CSF, Gro-α, Gro-β, Gro-γ, I-309, IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p40), IL-15, IL-16, IP-10, MCP-1, MCP-2, MCP-3, MIG, MIP-1α, MIP-1β, NAP-2, PF-4, SDF-1α, TNF-α, TNF-β

Recombinant Mouse

IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), GM-CSF, MCP-1, MIG, MIP-1α, MIP-1β, TCA-3, TNF-α

Recombinant Rat

IL-2, IL-10, GM-CSF, MCP-1, RANTES
Interfering Substances

The following substances at the following levels were added to Standard Diluent containing 125 pg/mL hIL-12 (p70). No effect on assay results was observed.

- Bilirubin 80 mg/mL
- Human hemoglobin 12.5 mg/mL
- Human transferrin 50 mg/mL
- Triglycerides 12 mg/mL
- Heparin 40 units/mL
- Sodium Citrate 10 mg/mL
- EDTA 12.5 mg/mL

Precision

Intra-assay

Twenty-four replicates each of three different levels of IL-12 (p70) were tested in one plate. The following results were observed:

<table>
<thead>
<tr>
<th>Number of Replicates (n)</th>
<th>24</th>
<th>24</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Concentration</td>
<td>249.5 pg/mL</td>
<td>99.1 pg/mL</td>
<td>24.3 pg/mL</td>
</tr>
<tr>
<td>SD</td>
<td>6.9</td>
<td>5.8</td>
<td>1.6</td>
</tr>
<tr>
<td>%CV</td>
<td>2.7</td>
<td>5.8</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Inter-assay

Three different levels of IL-12 (p70) were tested in four different assays. The following results were observed:

<table>
<thead>
<tr>
<th>Number of Replicates (n)</th>
<th>32</th>
<th>32</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Concentration</td>
<td>247.5 pg/mL</td>
<td>98.2 pg/mL</td>
<td>22.6 pg/mL</td>
</tr>
<tr>
<td>SD</td>
<td>9.1</td>
<td>4.3</td>
<td>1.8</td>
</tr>
<tr>
<td>%CV</td>
<td>3.7</td>
<td>4.4</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Standardization

This immunoassay is calibrated against recombinant human IL-12 (p70).

The NIBSC/WHO First International Standard 95/544 (recombinant human IL-12 (p70)) was evaluated in this kit. The conversion factor for NIBSC material is as follows:

NIBSC (95/544) equivalent value (IU/mL) = 0.01 × BD OptEIA™ IL-12 (p70) value (pg/mL)

∴ 1.0 µg NIBSC IL-12 (p70) = 1.0 µg BD OptEIA™ IL-12 (p70)

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Experimental Results

Serum

Eleven serum and plasma (both EDTA and heparin) samples were tested in this assay. One sample measured 4 pg/mL, all other samples measured less than 4 pg/mL.

Twenty high-titer rheumatoid factor serum samples and 12 high titer heterophilic antibody serum samples were tested in this assay. The mean value was 5 pg/mL, with a range from 0 to 64 pg/mL. Twenty-six samples measured less than 4 pg/mL (minimum sensitivity level).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Samples</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>11</td>
<td>10 samples &lt; 4.0 pg/mL; 1 sample = 4 pg/mL</td>
</tr>
<tr>
<td>High-titer heterophilic Ab serum</td>
<td>12</td>
<td>10 samples &lt; 4.0 pg/mL; 2 samples 11 to 34 pg/mL</td>
</tr>
<tr>
<td>High-titer rheumatoid factor serum</td>
<td>20</td>
<td>16 samples &lt; 4.0 pg/mL; 4 samples 5 to 64 pg/mL</td>
</tr>
</tbody>
</table>

Cell Culture Supernatants

Human peripheral blood mononuclear cells were cultured in RPMI 1640 complete medium with 10% fetal bovine serum at 1×10^6 cells/mL, and activated with TPA at 50 ng/mL, A23187 at 1 µg/mL. After 24 hours, culture supernatants were collected and stored at -70°C. BD OptEIA assay results are as follows:

<table>
<thead>
<tr>
<th>Donor#</th>
<th>IL-12 (p70) (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>239</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>268</td>
</tr>
</tbody>
</table>
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Source</th>
<th>Corrective Action</th>
</tr>
</thead>
</table>
| Poor Precision   | • Inadequate washing / aspiration of wells  
• Inadequate mixing of reagents  
• Imprecise / inaccurate pipetting  
• Imprecise sealing of plate   | • Check function of washing system  
• Ensure adequate mixing  
• Check / calibrate pipettes  
• Ensure complete sealing of plate |
| Poor Standard Curve | • Improper standard handling / dilution  
• Incomplete washing / aspiration of wells  
• Imprecise / inaccurate pipetting  | • Ensure correct preparation of standards  
• Check function of washing system  
• Check / calibrate pipettes |
| Low Signal       | • Inadequate reagent volumes added to wells  
• Incorrect incubation times / temperature  
• Overly high wash / aspiration pressure from automated plate-washer.  | • Check / calibrate pipettes  
• Ensure sufficient incubation times / reagents warmed to room temperature  
• Utilize manual washing |
References


Plate Templates

For Research Use Only. Not for use in diagnostic or therapeutic procedures.