ab235627
Bilirubin (Total and Direct) Colorimetric Assay Kit

For the measurement of Bilirubin concentration in serum.

This product is for research use only and is not intended for diagnostic use.
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1. Overview

Bilirubin (Total and Direct) Colorimetric Assay Kit (ab235627) utilizes the Jendrassik-Grof principle to detect bilirubin. Total bilirubin (unconjugated + conjugated) concentration is determined in the presence of a catalyst, where bilirubin reacts with a diazo-salt to form azobilirubin, which absorbs at 600 nm. Direct bilirubin (conjugated) is determined in the absence of catalyst (550 nm).

Prepare samples.

↓

Prepare Bilirubin Standard Curve.

↓

Prepare Total Bilirubin Reagent Mix or Direct Bilirubin Reagent Mix and add to the standards, samples and background controls.

↓

Measure end point absorbance at 600 nm for Total Bilirubin or end point absorbance at 550 nm for Direct Bilirubin.
2. Materials Supplied and Storage

Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage temperature (before prep)</th>
<th>Storage temperature (after prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin Reagent 1</td>
<td>2.5 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Bilirubin Reagent 2</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Catalyst</td>
<td>15 mL</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>Total Bilirubin Probe</td>
<td>10 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Direct Bilirubin Probe</td>
<td>20 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Bilirubin Standard (0.2 μg/μL)</td>
<td>2 x 200 μL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>DMSO (Anhydrous)</td>
<td>3.5 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- 96-well black plate with flat bottom.
- Multi-well spectrophotometer (ELISA reader).
4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide: www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.
5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

5.1 Bilirubin Reagent 1
1. Ready to use as supplied.
2. Warm to room temperature before use.

5.2 Bilirubin Reagent 2
1. Ready to use as supplied.
2. Light sensitive.
3. Warm to room temperature before use.

5.3 Catalyst
1. Ready to use as supplied.
2. Warm to room temperature before use.

5.4 Total Bilirubin Probe
1. Ready to use as supplied.
2. Warm to room temperature before use.

5.5 Direct Bilirubin Probe
1. Ready to use as supplied.
2. Warm to room temperature before use.

5.6 Bilirubin Standard
1. Ready to use as supplied.
2. Warm to room temperature before use.
   \( \Delta \text{Note:} \) Light and Oxygen sensitive. Aliquot into amber vials and store at -20°C.

5.7 DMSO
1. Ready to use as supplied.
2. Warm to room temperature to liquefy completely before use.
6. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

1. Dilute 150 µL of 0.2 µg/µL Bilirubin standard with 150 µL 100% DMSO (provided).
2. Using 0.1 µg/µL Bilirubin standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Bilirubin Standard (µL)</th>
<th>50% DMSO (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End amount of Bilirubin standard in well (µg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7.5</td>
<td>142.5</td>
<td>150</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>135</td>
<td>150</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>120</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>90</td>
<td>150</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>30</td>
<td>150</td>
<td>4</td>
</tr>
</tbody>
</table>

Each dilution has enough standard to set up duplicate readings (2 x 50 µL).
7. Sample Preparation

General sample information:
We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
We recommend that you use fresh samples for the most reproducible assay.

7.1 Serum:
1. Add 2-50 µL of undiluted serum to desired well(s) in a 96-well plate.
2. Adjust the volume to 50 µL/well with 50 % DMSO (mix 500 µL 100% DMSO (provided) and 500 µL ddH₂O for about 20 wells).

Note: Bilirubin concentration varies over a wide range depending on the patient’s age, gender, and pathological conditions. In healthy patients, bilirubin concentrations (in mg/dL) are: Total: (0.1-1.2); Indirect: (0.1-0.7); Direct: (0.1-0.4). For unknown samples, we recommend doing a pilot experiment and testing several doses to ensure the readings are within the Standard Curve range.
8. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

8.1 Total Bilirubin Reagent Mix:
1. For Total Bilirubin determination, mix enough reagents for the total number of well(s) to be assayed including Standards, Samples, and Background Controls.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin Reagent 1</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>Bilirubin Reagent 2</td>
<td>5 µL</td>
<td>-</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-L</td>
<td>5 µL</td>
</tr>
<tr>
<td>Catalyst</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

2. Add 125 µL of Reagent Mix to each well. Mix well.
3. Incubate plate for 30 minutes at room temperature (~25°C).
4. Add 75 µL of Total Bilirubin Probe to all Standards, Samples and Background Control.
5. Incubate plate at room temperature for 20 minutes, protected from light.
6. Measure end point absorbance at 600 nm in a plate reader.
8.2 Direct Bilirubin Reagent Mix:

1. For Direct Bilirubin determination, mix enough reagents for the total number of well(s) to be assayed including Standard, Samples and Background Controls.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin Reagent 1</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>Bilirubin Reagent 2</td>
<td>5 µL</td>
<td>-</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-L</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

2. Add 25 µL of Reagent Mix to each well. Mix well.
3. Incubate plate for 30 minutes at room temperature (~25°C).
4. Add 175 µL of Direct Bilirubin Probe to all Standards, Samples and Background Control.
5. Incubate plate at room temperature for 20 minutes, protected from light.
6. Measure end point absorbance at 550 nm in a plate reader.
9. Data Analysis

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (Standard #1) from all standards, controls and sample readings. This is the corrected absorbance.
3. If significant, subtract the sample background control from sample readings.
4. Plot the Linear Total Bilirubin Standard Curve.
5. Apply the corrected OD to the Total Bilirubin Standard Curve to get B µg of Total Bilirubin in the sample well.
6. Fit the Direct Bilirubin Standard values to a polynomial trend-line and solve the quadratic equation of the polynomial Standard curve (Figure 2) to obtain B µg of Direct Bilirubin in the sample well.

Total or Direct Bilirubin Concentration = \( \frac{B}{V} \times D = \mu g/mL \)

Where:
B = amount of Total/Direct Bilirubin in the sample well (µg).
V = sample volume added in the sample wells (µL).
D = sample dilution factor.

Total Bilirubin = Unconjugated Bilirubin + Conjugated Bilirubin

Bilirubin Molecular Weight: 584.7 kDa

10 mg/mL ≡ 10 µg/µL ≡ 10000 µg/mL ≡ 1 g/dL
10. FAQs / Troubleshooting

General troubleshooting points are found at www.abcam.com/assaykitguidelines.
11. Typical Data

Data provided for demonstration purposes only.

Figure 1. Total Bilirubin Standard Curve (0 - 4 µg).
**Figure 2.** Direct Bilirubin Standard Curve (0 - 4 µg).
**Figure 3.** Total and Direct Bilirubin concentrations in Normal Human Serum. Different volumes of human serum (10 - 50 µL) were assayed following kit protocols. Reported concentrations (in mg/dL): Total Bilirubin: 4; Direct Bilirubin: 1.5. Experimental concentrations (calculated as the average of estimated bilirubin in five different human serum volumes ranging from 10 to 50 µL): Total: 3.8 mg/dL; Direct: 1.75 mg/dL.
12. Notes
Technical Support

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