Total and Direct Bilirubin Colorimetric Assay Kit

Item No. 701720

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400494	Reagent 1 (R1)	1 vial/8 ml	-20°C
400495	Reagent 2 (R2)	1 vial/2 ml	-20°C
400506	Bilirubin Assay Catalyst	1 vial/15 ml	-20°C
400505	Total Bilirubin Probe	1 vial/12 ml	RT
400498	Direct Bilirubin Buffer	1 vial/8 ml	RT
400778	Bilirubin Standard	2 ea	-20°C
400499	Bilirubin Assay DMSO	1 vial/5 ml	RT
400014	96-Well Solid Plate (Colorimetric Assay)	2 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Total and Direct Bilirubin Colorimetric Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 525 and 600 nm. If the plate reader does not have a built-in function to program "wait before read", a timer or a stopwatch is recommended for timing the direct bilirubin assay.
2. Adjustable pipettes and multichannel pipettes
3. Dilution plates and reagent reservoirs suitable for the multichannel pipettes are highly recommended.
4. A source of pure water; glass-distilled water or deionized water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. An incubator
6. Materials used for **Sample Preparation** (see page 8)
7. Aluminum foil

INTRODUCTION

Background

Bilirubin is a yellow product of heme catabolism formed when heme is cleaved by heme oxygenase in cells of the reticuloendothelial system.[1,2] Heme is hydrolyzed to biliverdin, which is then reduced by biliverdin reductase to form unconjugated or indirect bilirubin.[2] Unconjugated bilirubin is hydrophobic and must bind to albumin to be transported to the liver, where it is conjugated with glucuronic acid to form conjugated, or direct, bilirubin. Conjugated bilirubin is water soluble and can be incorporated into bile or further catabolized and excreted in the urine. Unconjugated hyperbilirubinemias are associated with disordered hemolysis and dyserythropoiesis, as well as disorders of microsomal conjugation of bilirubin, including Gilbert syndrome and Crigler-Najjar disease.[3] Conjugated hyperbilirubinemias are associated with neonatal immaturity of UDP-glucuronosyltransferase (UGT) isoform 1A (UGT1A), hepatitis, cirrhosis, Dubin-Johnson syndrome, and cholestasis of pregnancy. Hyperbilirubinemias, both conjugated and unconjugated, induce yellow discoloration of the skin and eyes, known as jaundice. Decreased serum levels of bilirubin positively correlate with increased risk of cardiovascular and metabolic disease.[4]

About This Assay

Cayman's Total and Direct Bilirubin Colorimetric Assay provides a rapid, reliable, and reproducible colorimetric method for measuring total and direct bilirubin levels in serum and cell lysates. This kit uses a modified Jendrassik-Gróf method, in which total bilirubin concentration is determined by diazo salt in the presence of a catalyst and direct bilirubin is determined in the absence of the catalyst.[5,6] The Jendrassik-Gróf method is a widely used clinical method.[7] However, the results may not be comparable to the results of analytical methods such as mass spectrometry or NMR, and this kit is designed for research use only (not to be used for clinical purposes).

The assay has a dynamic range of 0.045-8 mg/dl (0.022-4 µg/well) for both total and direct bilirubin.

Principle Of This Assay

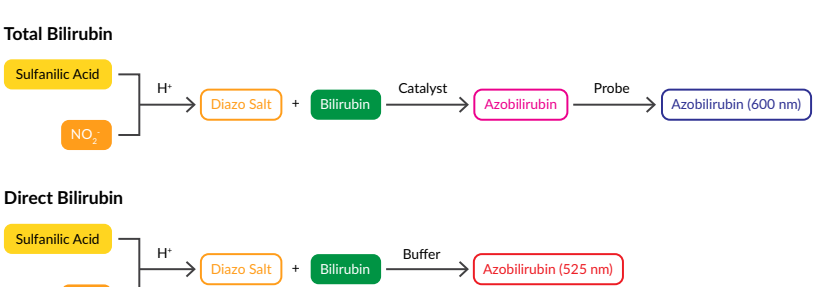


Figure 1. Principle of the assay

PRE-ASSAY PREPARATION

Sample Preparation

Serum

Bilirubin concentrations may vary depending on age, gender, pathological conditions, *etc*. For healthy human samples, the total bilirubin levels are usually below 1.2 mg/dl and the direct bilirubin levels are below 0.3 mg/dl. Samples from newborns (3-5 days) could contain up to 13 mg/dl total bilirubin.[8-10] Avoid using hemolytic or lipidemic serum as these interfere with the assay.

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Transfer the top yellow serum layer without disturbing the white buffy layer. Store serum on ice and protect from light. If not assaying the same day, store at -80°C. Avoid repeated freeze/thaw cycles.
4. Serum samples can be diluted in pure water, if necessary.

Cell Lysates

Cells can be lysed in a lysis buffer such as RIPA or M-PER™ following the manufacturer's protocol. Protease inhibitors shown in Table 1 on page 21 can be added. Pellet cellular debris by centrifugation and transfer the supernatant to clean tubes. These lysates may then be diluted with pure water to fall within the range of the standard curve.

NOTE: RIPA Buffer Concentrate (Item No. 10010263) is available for purchase from Cayman.

Reagent Preparation

Equilibrate all reagents to room temperature prior to preparation.

1. **Reagent 1 (R1) - (Item No. 400494)**

   This vial contains 8 ml of R1. Thaw and let equilibrate to room temperature prior to use. It is normal for R1 to contain crystalline salts after thawing. These will completely dissolve after thorough mixing. If not using all at once, prepare aliquots and store at -20°C, limiting freeze-thaw cycles to three.

2. **Reagent 2 (R2) - (Item No. 400495)**

   This vial contains 2 ml of R1. Thaw and let equilibrate to room temperature prior to use. If not using all at once, prepare aliquots and store at -20°C, limiting freeze-thaw cycles to three.

3. **Bilirubin Assay Catalyst - (Item No. 400506)**

   This vial contains 15 ml of Bilirubin Assay Catalyst. Thaw and let equilibrate to room temperature prior to use. If not using all at once, prepare aliquots and store at -20°C, limiting freeze-thaw cycles to three.

4. **Total Bilirubin Probe - (Item No. 400505)**

   This vial contains 12 ml of Total Bilirubin Probe. It is ready to use as supplied

5. **Direct Bilirubin Buffer - (Item No. 400498)**

   This vial contains 8 ml of Direct Bilirubin Buffer. It is ready to use as supplied.

6. **50% DMSO**

   Each vial of Bilirubin Assay DMSO (Item No. 400499) contains 5 ml of DMSO. Mix 600 µl of Bilirubin Assay DMSO with 600 µl of pure water for one standard curve (two signal wells and one background well for each standard).

   NOTE: Heat is generated during mixing; equilibriate to room temperature prior to use in the assay.

7. **Reagent Mix**

   Immediately before use, combine 2.4 ml of R1 and 600 µl of R2 (4:1 ratio). This is a sufficient volume to assay one plate for either total or direct bilirubin. Scale as needed. The Reagent Mix will be stable for 15 minutes at room temperature.

8. **Background Reagent**

   Immediately before use, combine 1.2 ml of R1 and 300 µl of pure water (4:1 ratio). This is a sufficient volume to assay one plate for either total or direct bilirubin. Scale as needed. The Background Reagent will be stable for 15 minutes at room temperature.

9. **Standard Preparation**

   Each vial of Bilirubin Standard (Item No. 400778) contains a lyophilized powder of bilirubin. Reconstitute the contents of the vial with 300 µl DMSO to obtain a 16 mg/dl bulk standard. The reconstituted standard is stable for at least one month when stored at -20°C. Avoid more than three freeze-thaw cycles. *NOTE: The bulk standard is dissolved in 100% DMSO. However, subsequent serial dilutions of the bilirubin standard can be prepared in 50% DMSO. This is required for optimal solubility. The difference in DMSO concentrations does not impact assay performance.*

   To prepare standards for the assay, obtain six clean tubes and label them S1 through S6. Add 180 µl of 50% DMSO to each tube. Transfer 180 µl of 16 mg/dl bilirubin standard to S1 and mix well. Transfer 180 µl from S1 to S2 and mix well. Then, transfer 180 µl from S2 to S3 and mix well. Repeat this process for tubes S4-5. Do not add any bilirubin to S6.

   The Bilirubin Standard is light sensitive. Protect diluted standards from light. They will be stable for four hours at room temperature.

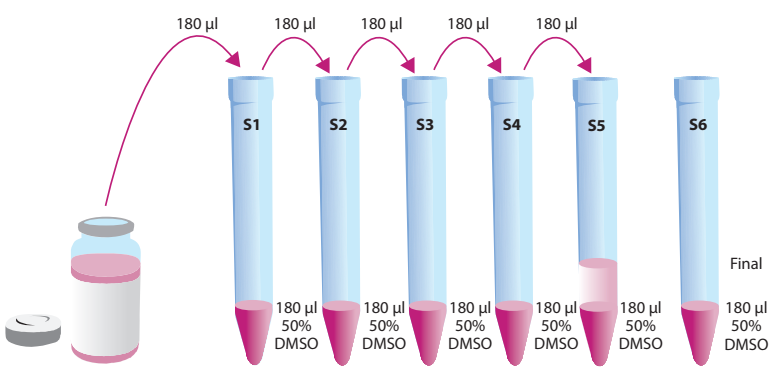


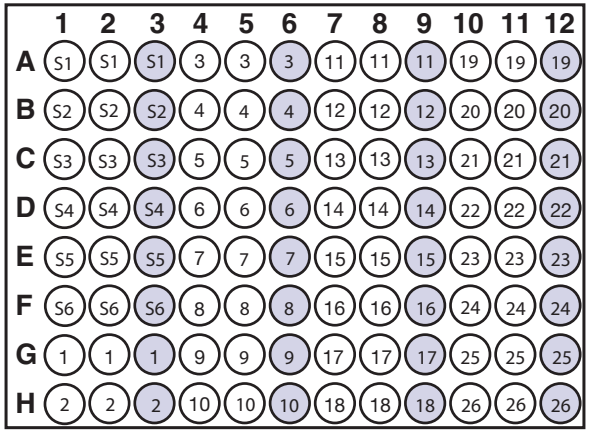
Figure 2. Preparation of the bilirubin standards

ASSAY PROTOCOL

Plate Set Up

Two plates are provided in this kit. There is no specific pattern for using the wells on the plate. It is recommended that the total and direct bilirubin assays be set up on separate plates if both assays will be performed simultaneously. Each standard or sample must contain at least two signal wells and one background well.

A typical layout is shown on Figure 3. It is suggested that the contents of each well are recorded on the template sheet provided (see pages 25 and 27).



S1-S6 = Standard Wells
1-26 = Sample Wells
◯ = Signal Wells
◯ = Background Wells

Figure 3. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- If adding a new reagent to reagent(s) existing in a well for mixing by pipetting, discard tips after mixing and use new tips for the next step to avoid contamination of reagents.

General Information

- The final volume of the assay is 250 µl in all of the wells.
- The assay is performed at room temperature (22-24°C). For better reproducibility, it is recommended to use an incubator or a plate reader with temperature control features.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the standards and samples be assayed at least in duplicate.
- Total or direct bilirubin levels of 26 samples can be assayed in duplicate on one plate.

Performing the Assay

Total Bilirubin

NOTE: Background reactions are required for each sample and standard. Use a multi-channel pipette to dispense reagents. Aluminum foil should be used to cover the plate to protect from light during the incubation steps.

1. Add 50 µl of each standard or sample to three wells.
2. Add 100 µl of Bilirubin Assay Catalyst (Item No. 400506) to all wells. Mix thoroughly.
3. Incubate for 10 minutes at room temperature, protected from light.
4. Add 25 µl of freshly prepared Reagent Mix or Background Reagent to designated signal or background wells for both standards and samples. Mix thoroughly. A white precipitate may form upon addition of the reagents but will disappear with continued mixing.
5. Incubate the plate for 15 minutes at room temperature, protected from light.
6. Add 75 µl of Total Bilirubin Probe to all wells. Mix thoroughly.
7. Incubate the plate for 5 minutes at room temperature, protected from light.
8. Read the absorbance at 600 nm.

Direct Bilirubin

NOTE: The direct bilirubin assay is time and temperature sensitive. If possible, set the instrument to read absorbance by columns from left to right, and initiate reactions by columns with a multichannel pipette from left to right. For best results, set up and read no more than half of the plate at one time.

1. Program the plate reader: "shake 5 seconds, wait for 5 minutes, and measure absorbance at 525 nm at room temperature". *NOTE: If a programmable plate reader is not available, a timer or a stopwatch is recommended for accurate timing.*
2. Add 50 µl of each standard or sample to three wells.
3. Add 125 µl pure water to all wells. Mix thoroughly.
4. Add 50 µl of Direct Bilirubin Buffer to all wells. Mix by pipetting up and down.
5. Add 25 µl of freshly prepared Reagent Mix or Background Reagent to designated signal or background wells for both standards and samples. Mix thoroughly.
6. Immediately read absorbance as described in step 1.

ANALYSIS

Calculations

1. Average the absorbance of each standard and sample. Subtract the absorbance of the corresponding background well from each averaged absorbance value to obtain corrected absorbance.
2. Plot the corrected absorbance of the standards *versus* bilirubin concentration. Because of the background correction (Step 1), there is no need to subtract the absorbance of S6 (no bilirubin containing standard) from the standards again.
3. Calculate the bilirubin concentration of the samples using the linear regression equation of the standard curve.

$$\text{Total/Direct bilirubin (mg/dl)} = \frac{\text{Corrected sample absorbance - (y-intercept)}}{\text{Slope}} \text{ X Sample dilution}$$

1 mg/dl = 0.01 mg/ml = 0.01 µg/µl = 17.1 µmole/l (µM)

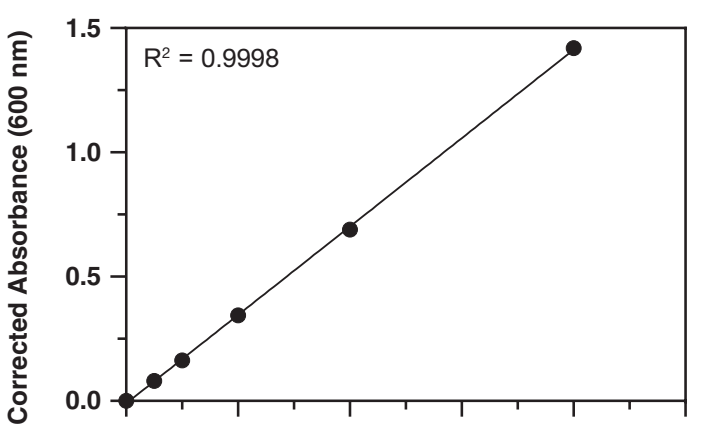


Figure 4. Typical standard curve - total bilirubin

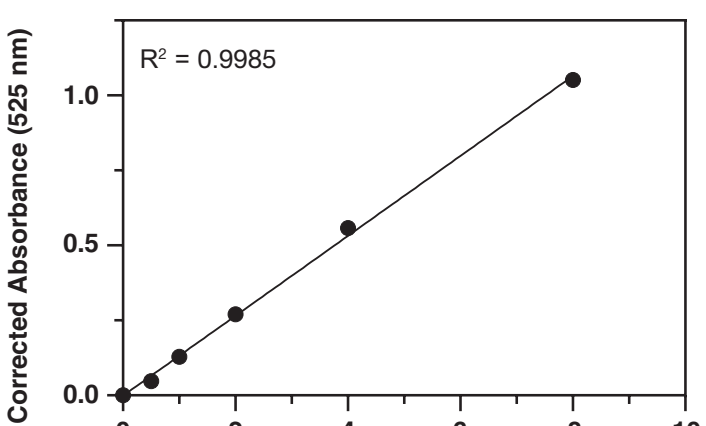


Figure 5. Typical standard curve - direct bilirubin

Performance Characteristics

Sensitivity:

The Lower Limit of Detection (LLOD) of the Total Bilirubin assay is 0.036 µg/well or 0.071 mg/dl.

The Lower Limit of Quantification (LLOQ) of the Total Bilirubin assay is 0.125 µg/well or 0.25 mg/dl.

Parallelism:

Human serum samples were serially diluted with pure water and evaluated using the total bilirubin assay. Measured concentrations were plotted as a function of sample dilution. The results are shown in Figure 6 below.

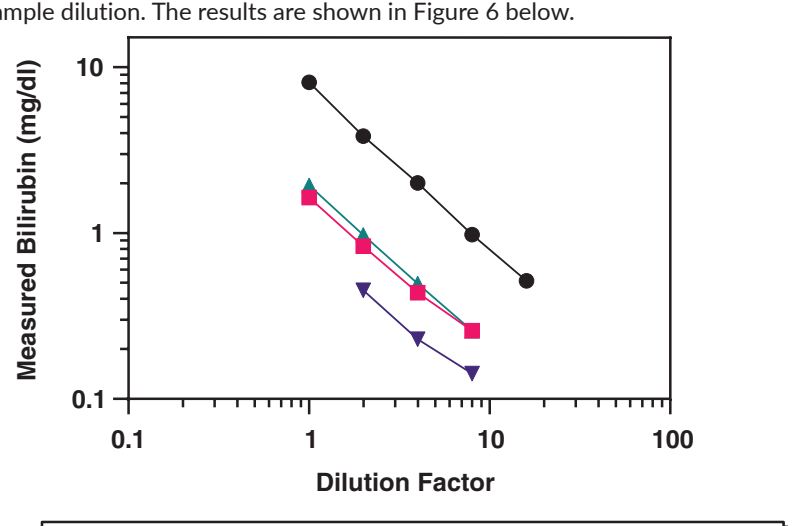


Figure 6. Parellelism of various sera in the Total Bilirubin Assay

Spike and Recovery:

MCF-7 cell lysates were prepared in RIPA lysis buffer containing protease inhibitors and spiked with different amounts of bilirubin. The total bilirubin levels were assessed using this kit. The results are shown below. The error bars represent standard deviations obtained from different dilutions of each spike.

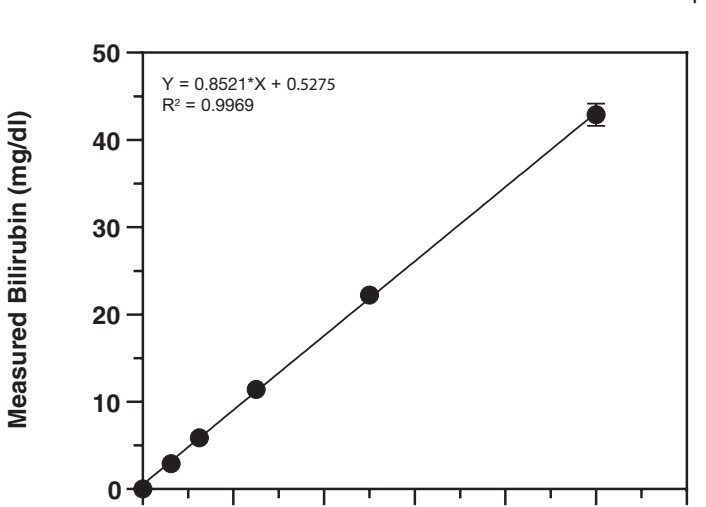


Figure 7. Spike and recovery of bilirubin in MCF-7 lysates

Interferences

The following reagents were tested for the interference in this assay

Reagent		Will Interfere
Buffers	RIPA Buffer	No
	M-PER™	No
	PBS, pH 7.5	No
Protease Inhibitors/Enzymes	Antipain (50 µg/ml)	No
	Bestatin (40 µg/ml	No
	Chymostatin (60 µg/ml)	No
	E-64 (10 µg/ml)	No
	Leupeptin (0.5 µg/ml)	No
	Pepstatin (0.7 µg/ml)	No
	Phosphoramidon (330 µg/ml)	No
	Pefabloc SC (1 mg/ml)	No
	Aprotinin (2 µg/ml)	No

Table 1. Interference

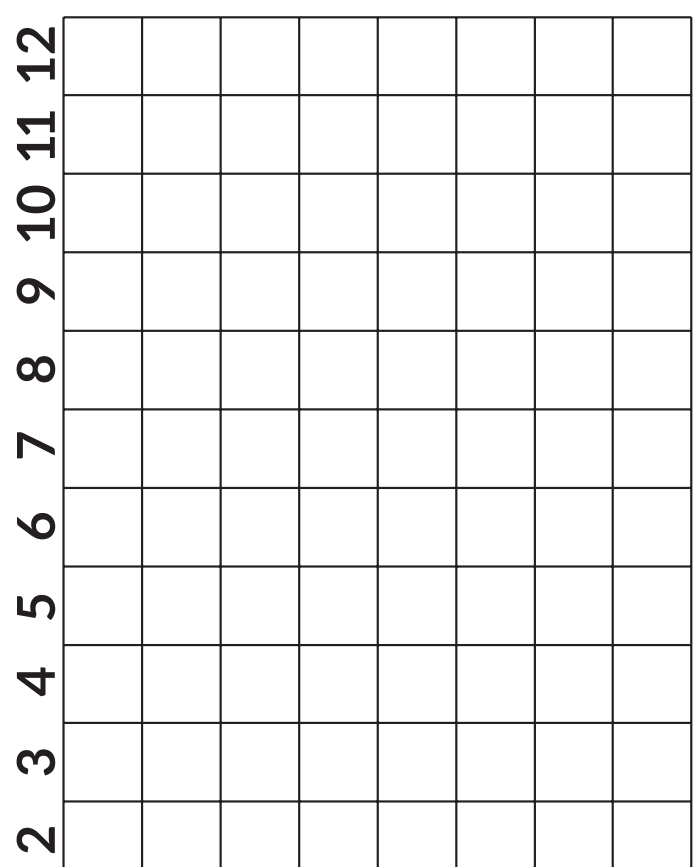
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
High background	A. Hemolytic or lipidemic sample B. Contamination of the background reagent by R2 during preparation, especially if significantly increased background signal was observed during the time of the assay. C. Bubbles in the well(s)	A. Prepare fresh reagent mix and background reagent, avoid contamination by changing tips B. Make sure that the reagent mix and background reagent are correctly added to the respective wells C. See recommended solutions for bubbly wells
Bilirubin not detected	A. High background B. Sample was too dilute C. Improper preparation of reagent mix or background reagent	A. See recommended solutions for high background signal B. Use more concentrated sample C. Ensure the R2 was added to the reagent mix and was not added to the background reagent D. Make sure that the reagent mix and background reagent are correctly added to the respective wells

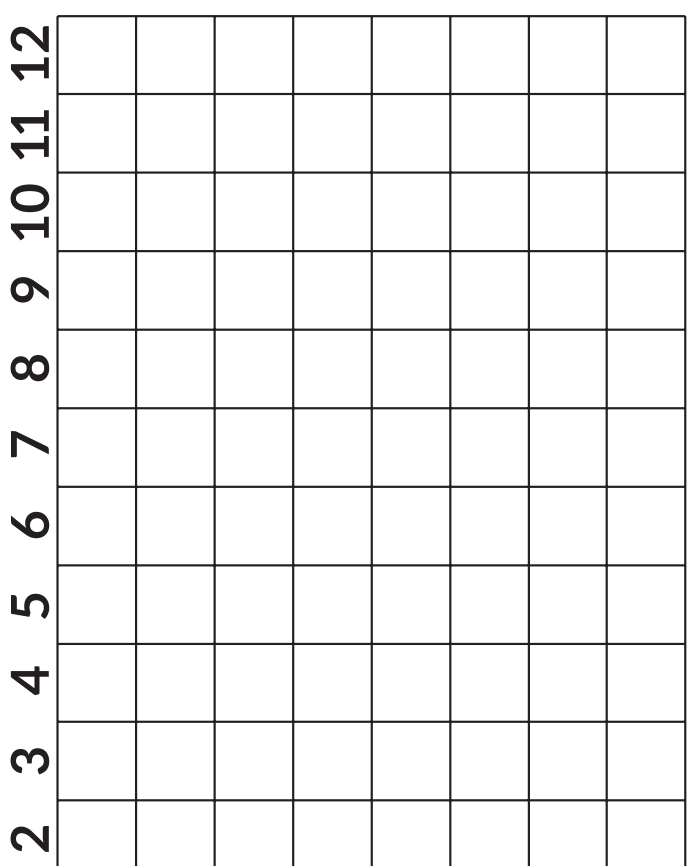
Reagent/ Procedure	Standard	Standard Background	Sample	Sample Background	Comments
Standard/ Sample	50 µl	50 µl	50 µl	50 µl	
Assay Catalyst	100 µl	100 µl	100 µl	100 µl	Pipette up and down to mix well
Incubate for 10 minutes at room temperature, protected from light					
Reagent Mix	25 µl	--	25 µl	--	Pipette up and down until the precipitate goes away
Background Reagent	--	25 µl	--	25 µl	Pipette up and down until the precipitate goes away
Incubate for 15 minutes at room temperature, protected from light					
Total Bilirubin Probe	75 µl	75 µl	75 µl	75 µl	Pipette up and down to mix well
Incubate for 5 minutes at room temperature, protected from light					
Absorbance	600 nm at room temperature				

Table 2. Total bilirubin summary



Reagent/ Procedure	Standard	Standard Background	Sample	Sample Background	Comments
Standard/ Sample	50 µl	50 µl	50 µl	50 µl	Pipette up and down to mix well
Pure Water	125 µl	125 µl	125 µl	125 µl	
Direct Bilirubin Buffer (5X)	50 µl	50 µl	50 µl	50 µl	Pipette up and down to mix well
Background Reagent	--	25 µl	--	25 µl	Pipette up and down to mix well
Bring reagent mix and plate to the plate reader					
Reagent Mix	25 µl	--	25 µl	--	Pipette up and down to mix well
Shake for 5 seconds, incubate for 5 minutes at room temperature protected from light					
Absorbance	525 nm at room temperature				

Table 3. Direct bilirubin summary



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NOTES

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