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 (5X)	1 vial/8 ml	RT
400497	Bilirubin Standard	4 vials/200 µl	-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).*

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

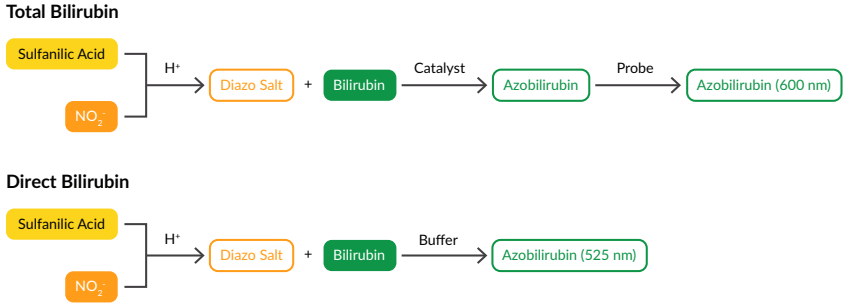

Figure 1. Principle of the assay

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 d) 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. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice, 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 2 on page 20 can be added. Pellet cellular debris by centrifugation and transfer 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 at room temperature prior to preparation.

1. **50% DMSO**

   One 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.

2. **Reagent Mix**

   One vial of Reagent 1 (R1) (Item No. 400494) contains 8 ml of R1 and one vial of Reagent 2 (R2) (Item No. 400495) contains 2 ml of R2. Both R1 and R2 are ready to use as supplied.

   Combine R1 and R2 at a 4:1 ratio (*e.g.* 800 µl R1 and 200 µl R2; see Note 2 for calculation) prior to each experiment. Use Reagent Mix within 15 minutes of preparation.

3. **Background Reagent**

   Combine R1 and pure water at a 4:1 ratio (*e.g.* 400 µl R1 and 100 µl pure water; see Note 2 for calculation) prior to each experiment. Use Background Reagent within 15 minutes of preparation.

Notes

1. R1 may contain undissolved particles if not completely equilibrated at room temperature after thawing, which could affect results. Make sure that the R1 solution is clear before making the reagent mix.
2. Calculate Reagent Mix and Background Reagent needed based on number of wells (see Sample Plate, Figure 3, on page 11):

   (# signal wells + # standard wells) X 25 = **µl of Reagent Mix needed**

   (# background wells + # standard wells) X 25 = **µl of Background Reagent needed**

3. Preparation of extra Reagent Mix and Background Reagent is recommended when using multichannel pipettes.

4. **Standard Preparation**

   Each vial of Bilirubin Standard (Item No. 400497) contains 200 µl of 16 mg/dl bilirubin which is sufficient for one standard curve for either a total or direct bilirubin assay - two signal wells (sg) and one background well (bg) for each standard. Thaw standards as needed and avoid repeated freeze-thaw cycles.

   To prepare standards for the assay, obtain six clean tubes (or one dilution plate) and label tubes (wells) S1 through S6. Add 180 µl of 50% DMSO to each tube (well). 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 (wells) 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.

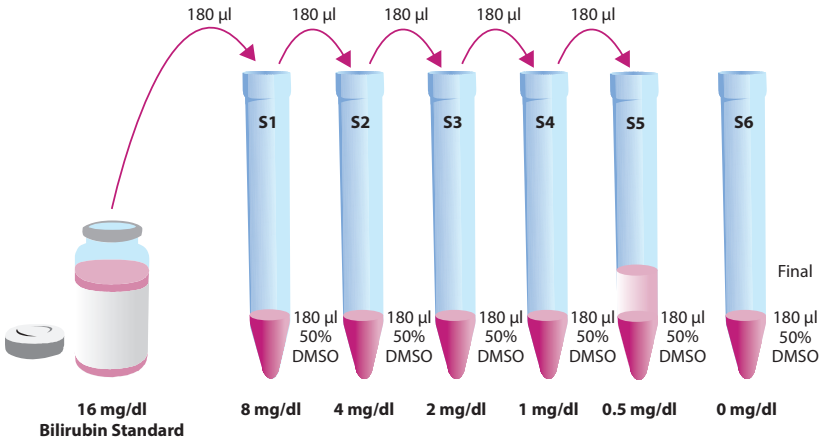

Figure 2. Preparation of the bilirubin standards

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 to set up the total bilirubin assay and direct bilirubin assay on separate plates if planning to perform simultaneously. A typical layout of bilirubin standards and samples to be measured in duplicate on a single plate is given below in Figure 3. It is suggested that the contents of each well are recorded on the template sheet provided (see pages 25 and 27).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1 sg	S1 sg	S1 bg	3 sg	3 sg	3 bg	11 sg	11 sg	11 bg	19 sg	19 sg	19 bg
B	S2 sg	S2 sg	S2 bg	4 sg	4 sg	4 bg	12 sg	12 sg	12 bg	20 sg	20 sg	20 bg
C	S3 sg	S3 sg	S3 bg	5 sg	5 sg	5 bg	13 sg	13 sg	13 bg	21 sg	21 sg	21 bg
D	S4 sg	S4 sg	S4 bg	6 sg	6 sg	6 bg	14 sg	14 sg	14 bg	22 sg	22 sg	22 bg
E	S5 sg	S5 sg	S5 bg	7 sg	7 sg	7 bg	15 sg	15 sg	15 bg	23 sg	23 sg	23 bg
F	S6 sg	S6 sg	S6 bg	8 sg	8 sg	8 bg	16 sg	16 sg	16 bg	24 sg	24 sg	24 bg
G	1 sg	1 sg	1 bg	9 sg	9 sg	9 bg	17 sg	17 sg	17 bg	25 sg	25 sg	25 bg
H	2 sg	2 sg	2 bg	10 sg	10 sg	10 bg	18 sg	18 sg	18 bg	26 sg	26 sg	26 bg

S1-S6 - Standard Wells
1-26 - Total or Direct Bilirubin Sample Wells
sg - Signal Wells (add Reagent Mix)
bg - Background Wells (add Background Reagent)

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. If the room temperature is significantly higher or lower than 24°C, an incubator at 24°C is recommended for reproducible results.
- 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

1. To a 96-well plate, add 100 µl of Bilirubin Assay Catalyst (Item No. 400506) to each well.
2. **Standard Wells:** Add 50 µl of each standard to the designated wells on the plate (see **Sample plate format**, Figure 3, on page 11). Mix by pipetting up and down at least 10 times.

   Sample Wells: Add 50 µl of each sample to the designated wells on the plate (see **Sample plate format**, Figure 3, on page 11). Mix by pipetting up and down at least 10 times.
3. Incubate the plate for 10 minutes at room temperature, protected from light.
4. **Signal Wells:** Add 25 µl of freshly prepared Reagent Mix to the signal wells (sg) for both standards and samples (see **Sample plate format**, Figure 3, on page 11). Pipette up and down rapidly 25 times without introducing bubbles. A white precipitate will form upon adding the reagents and will disappear with continued pipetting.

   Background Wells: Add 25 µl of freshly prepared Background Reagent to background wells (bg) (see **Sample plate format**, Figure 3, on page 11). Pipette up and down rapidly 25 times without introducing bubbles until the white precipitate disappears.
5. Incubate the plate for 15 minutes at room temperature, protected from light.
6. Add 75 µl of Total Bilirubin Probe (Item No. 400505) to all wells. Pipette up and down rapidly at least 10 times without introducing bubbles.
7. Incubate the plate for 5 minutes at room temperature, protected from light.
8. Read the absorbance at 600 nm (A_{600}).

Direct Bilirubin

1. Program the plate reader: "shake 5 seconds, wait for 5 minutes, and measure absorbance at 525 nm (A_{525}) at room temperature".*
2. In the 96-well plate, add 125 µl pure water to each well.
3. Add 50 µl of Direct Bilirubin Buffer (5X) (Item No. 400498). Mix well by pipetting up and down at least 10 times.
4. **Standard Wells:** Add 50 µl of each standard to the designated wells on the plate (see **Sample plate format**, Figure 3, on page 11). Mix by pipetting up and down at least 10 times.

   Sample Wells: Add 50 µl of each sample to the designated wells on the plate (see **Sample plate format**, Figure 3, on page 11). Mix by pipetting up and down at least 10 times.
5. **Background Wells:** Add 25 µl of Background Reagent. Mix well by pipetting without introducing bubbles.
6. **Signal Wells:** Add 25 µl Reagent Mix to initiate the reaction. Mix well by pipetting rapidly without introducing bubbles.
7. Immediately run the preset program on the plate reader.

Important Notes

It is important to ensure that the reagents are mixed well at each step to obtain reproducible results. Pipette up and down rapidly without introducing bubbles to ensure the completion of the reaction.

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. When setting up a plate with more than six columns (four signal columns), consider initiating half of the signal wells and obtaining A_{525} before initiating the other half.

*If a programmable plate reader is not available, a timer or a stopwatch is recommended for accurate timing (set up a timer for 5 minutes 5 seconds, shake for 5 seconds by quickly tapping the edge of the plate, and incubate in the dark before reading).

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 against 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.

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

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

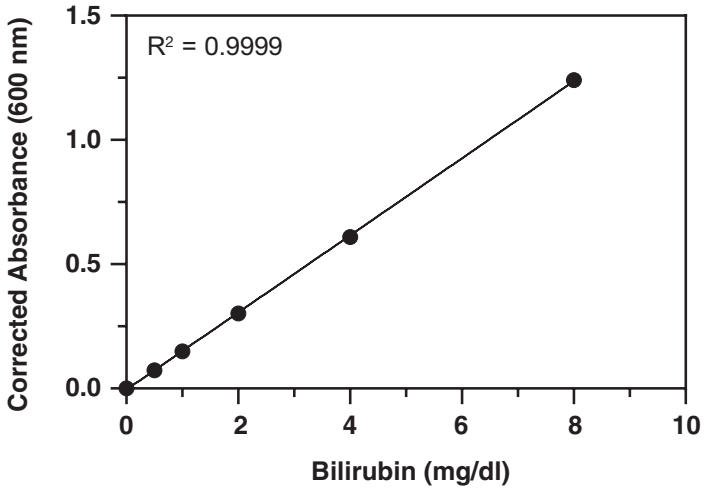

Figure 4. Typical standard curve - total bilirubin

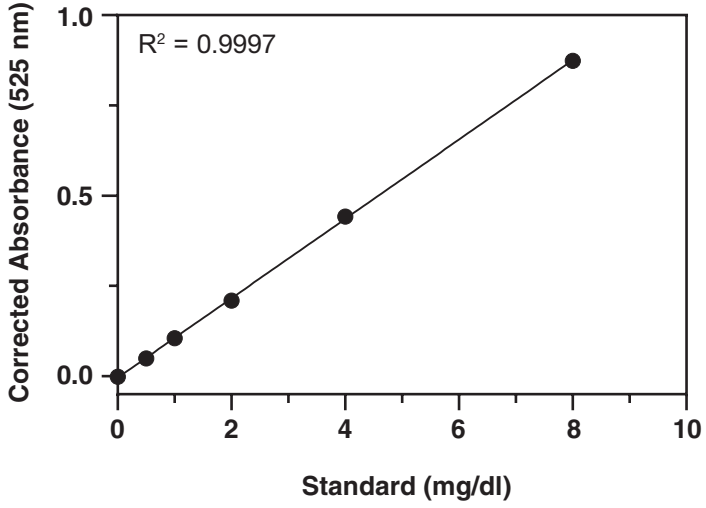

Figure 5. Typical standard curve - direct bilirubin

Performance Characteristics

Sensitivity:

The Lower Limit of Detection (LLOD) for this assay is 0.022 µg/well or 0.045 mg/dl.

The Lower Limit of Quantification (LLOQ) for this assay is 0.125 µg/well or 0.25 mg/dl.

Linearity:

Jaundice serum samples were serially diluted with pure water and evaluated for linearity using the total bilirubin assay. The results are shown in Table 1 below.

Sample	Dilution Factor	Bilirubin (mg/dl)	Dilution Linearity (%)
Jaundice serum sample 1	1	1.637	100%
	2	1.662	101%
	4	1.748	107%
	8	2.063	126%
Jaundice serum sample 2	1	1.937	100%
	2	1.943	100%
	4	1.980	102%
	8	2.060	106%

Table 1. Total bilirubin linearity

NOTE: Linearity has been calculated using the following formula:

%Linearity = (Observed concentration value, dilution adjusted / First observed concentration value in the dilution series, dilution adjusted) X 100

Spike and Recovery

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

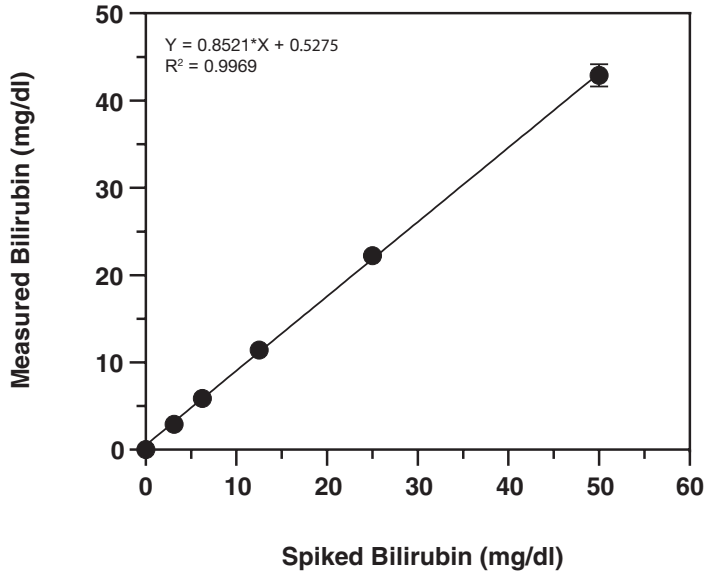

Figure 6. Spike and recovery of bilirubin in MCF-7 lysates

Interferences

The following reagents were tested for the interference in this assay

Reagent		Will Interfere
Buffers	RIPA Buffer	No
	M-PER™	No
	10 mM 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
Medications	Naproxen	Yes
	L-DOPA	Yes
	α-methyl DOPA	Yes
Other	Hemoglobin	Yes
	Indican	Yes
	Metal Ions	Yes
	Sample Turbidity	Yes

Table 2. Interference

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	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 C. If 10 µl gel-loading pipette tips are available, dip the tip in tiny amount of ethanol, and gently break large bubbles without inserting the tip into the reaction matrix. Only applicable to the total bilirubin assay D. Mix the components as described in the protocol. Consider additional mixing if needed E. Repeat the direct bilirubin assay if nessary

Problem	Possible Causes	Recommended Solutions
High background signal	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. If samples are hemolytic, draw blood carefully and avoid fine needles, rapid drawing, shaking or frothing of blood. Exclude lipidemic samples B. Prepare fresh reagent mix and background reagent, avoid contamination by changing new tips after transferring of every reagent C. Make sure that the reagent mix and background reagent are correctly added to the respective wells D. See recommended solutions for bubbly wells
Bilirubin not detected	A. High background signal B. Sample was too dilute C. Improper preparation of reagent mix or background reagent	A. See recommended solutions for high background signal B. Use less diluted sample C. Ensure the R2 was added to the reagent mix and was not added to the background mix D. Make sure that the reagent mix and background reagent are correctly added to the respective wells

References

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9. Pincus, M.R., Tierno, P., and Dafour, R. Evaluation of liver function. In Henry's Clinical Diagnosis and Management by Laboratory Methods, 24th Edition. 269 (2007).
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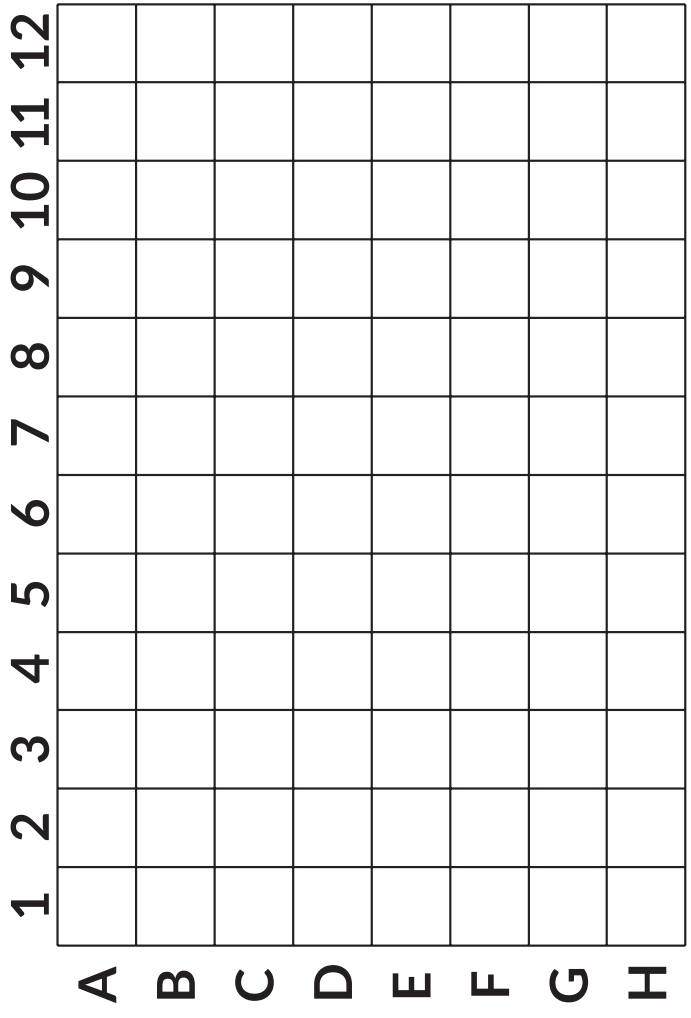
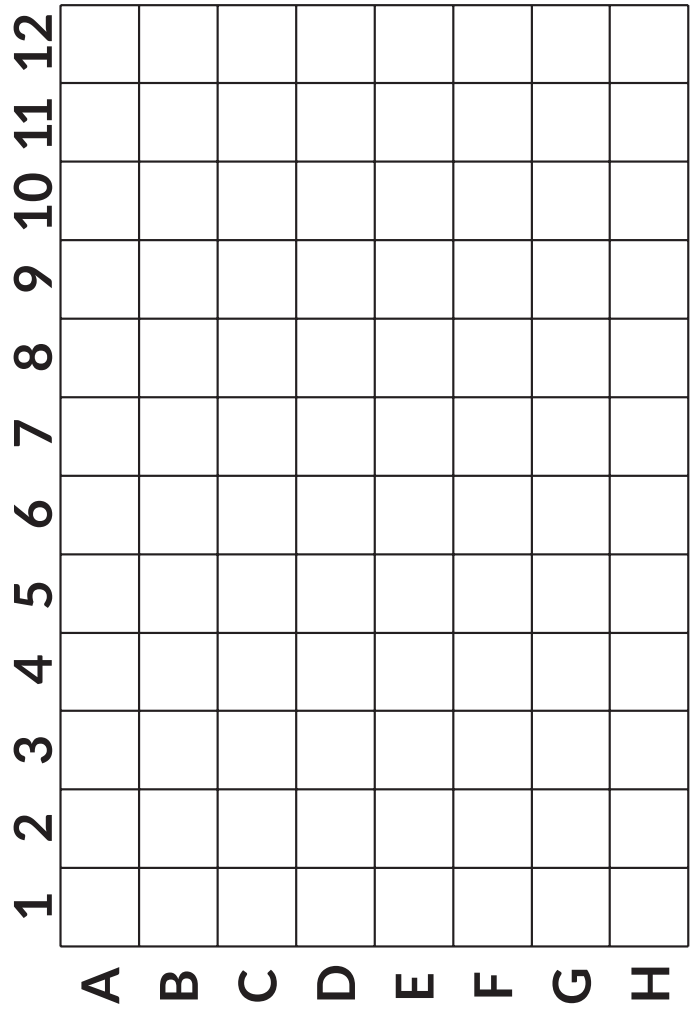
Procedure	Standard	Standard Background	Sample	Sample Background	Comments
Assay Catalyst	100 µl	100 µl	100 µl	100 µl	
Standard/ Sample	50 µl	50 µl	50 µl	50 µl	Pipette up and down at least 10 times
Incubate for 10 minutes at room temperature, protected from light					
Reagent Mix	25 µl	--	25 µl	--	Pipette up and down at least 25 times until the precipitate goes away
Background Reagent	--	25 µl	--	25 µl	Pipette up and down at least 25 times 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 at least 10 times
Incubate for 5 minutes at room temperature, protected from light					
Absorbance	600 nm at room temperature				

Table 3. Total bilirubin summary



Procedure	Standard	Standard Background	Sample	Sample Background	Comments
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 at least 10 times
Standard/ Sample	50 µl	50 µl	50 µl	50 µl	Pipette up and down at least 10 times
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 4. Direct bilirubin summary



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.