Bile Acids Assay

For the quantitative determination of total bile acids in human stool samples.

For Research Use Only

Catalog Number: 80-BILHU-E01, E10
Size: 96 wells, 10 x 96 wells
Version: 1.1

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INTENDED USE

The Bile Acids Assay is an enzymatic assay intended for the quantitative determination of total bile acids in human stool samples. The assay can be run manually or using an automated procedure for the Dynex DSX instrument. For Research Use Only. Not for use in diagnostic or therapeutic procedures.

PRINCIPLE OF THE PROCEDURE

This assay measures the amount of Thio-NADH produced in the presence of bile acids, Thio-NAD+, and an enzyme. Thio-NADH formation is measured by the absorbance (OD) at 405nm, and the amount produced is proportional to total bile acids in the stool extract. A standard curve plotting endpoint OD (or alternatively, OD rate) versus concentration is generated using the values obtained from known standards. Total bile acids concentration for each sample is extrapolated directly from this curve.

MATERIALS SUPPLIED

80-BILHU-E01			
Component	**Quantity**	**Preparation**	**Storage**
Microtiter Plate	One (12 strips x 8 well) Plate	Ready to use	18-28˚C
Standards A-F	1 vial, 500 µL	Ready to use*	2-8˚C
Control Levels 1-2	1 vial, 500 µL	Ready to use*	2-8˚C
Enzyme Mix	**1 vial, 9 mL**	**Ready to use**	**-20˚C**
Substrate Mix	**1 vial, 19 mL**	**Ready to use**	**-20˚C**

80-BILHU-E10			
Component	**Quantity**	**Preparation**	**Storage**
Microtiter Plate	Ten (12 strips x 8 well) Plates	Ready to use	18-28˚C
Standards A-F	10 vials, 500 µL each	Ready to use*	2-8˚C
Control Levels 1-2	10 vials, 500 µL each	Ready to use*	2-8˚C
Enzyme Mix	**10 vials, 9 mL each**	**Ready to use**	**-20˚C**
Substrate Mix	**10 vials, 19 mL each**	**Ready to use**	**-20˚C**

* Please refer to the Certificate of Analysis enclosed with each kit for lot-specific standard concentrations and control ranges.

MATERIALS REQUIRED BUT NOT PROVIDED

Feces Sample Collection:

1. Sample collection tube
2. Transport container

Manual Feces Preparation:

1. Disposable, breakable sterile inoculation loops or wooden stick
2. Disposable polypropylene screw cap tubes, 14 ml
3. Eppendorf tubes (1 - 1.5 ml)
4. Sensitive digital scale (40-150 mg)
5. Extraction buffer (10-EXBUF-225)
6. Vortex mixer or shaker
7. Centrifuge (1000 – 3000 x *g*)
8. Freezer (-20ºC, -80ºC)

Easy Stool Extraction Method:

1. ALPCO Easy Stool Extraction Device (30-EZEX-100)
2. Eppendorf tubes (1 - 1.5 ml)
3. Vortex mixer or shaker
4. Centrifuge (1000 – 3000 x *g*)
5. Freezer (-20ºC, -80ºC)

Equipment for Assay Measurements

1. Precision pipettes for dispensing 20-1000 µL (with disposable tips)
2. Dynex DSX machine (automated procedure) or Colorimetric plate reader (manual procedure)

PRECAUTIONS

1. Follow universal precautions. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency virus), HBV (Hepatitis B virus), and HCV (Hepatitis C virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are BSE negative. However, all materials should be treated as potentially infectious.
3. Avoid ingestion and direct contact with skin.
4. Reagents from this kit are lot-specific and must not be substituted.
5. Do not use reagents beyond the expiration date.
6. Variations to the test procedure are not recommended and may influence the test results.
7. Stool samples are generally heterogeneous. Mechanical homogenization using an applicator, inoculation loop, or similar device prior to sampling is recommended.
8. The ALPCO Easy Stool Extraction Devices should not be used for the extraction of liquid/watery stool samples. Samples of this consistency should be extracted using the manual weighing extraction method.

9. **The Substrate and Enzyme steps must be pipetted without stopping. Any interruption in the procedure will invalidate the result.**

STORAGE CONDITIONS

The Enzyme Mix and Substrate Mix must be stored at -20˚C and can withstand a total of two freeze-thaw cycles without any appreciable loss in performance. Once thawed, both mixes can be stored at 2-8°C for up to 7 days. Remaining liquid kit components should be stored at 2-8°C. Unopened reagents are stable until the expiration date on the component label when stored at the recommended temperature.

SAMPLE HANDLING

Extraction of the stool sample is required. Two acceptable extraction methods are outlined under ASSAY PROCEDURE – EXTRACTION. If a diluted sample has a greater concentration of analyte than the highest standard, the sample should be further diluted in 1X working ALPCO extraction buffer and the analysis should be repeated. Final results should be adjusted for any additional dilution.

Raw stool samples should be collected and analyzed within 3 days at room temperature (15–30°C), 7 days at 2–8°C, or within 14 days at -20°C. Avoid more than three freeze-thaw cycles.

QUALITY CONTROL

It is recommended that the Controls provided with the Bile Acids Assay be included in every assay. The concentration ranges of the controls are provided on the lot-specific Certificate of Analysis provided with each kit.

ASSAY PROCEDURE – EXTRACTION

Stool samples can be extracted by using:

A. The Manual Weighing / Standard Extraction Procedure or
B. The ALPCO Easy Extract Procedure (Catalog Number: 30-EZEX-100)

A) The Manual Weighing / Standard Extraction Procedure:

A1. Label and weigh (tare) the empty polypropylene tube together with the inoculation loop.

A2. Take out 50 to 100 mg of the thoroughly homogenized raw stool sample by means of the inoculation loop and place it into the pre-weighed tube.

A3. Estimate the net amount of sample, break off the inoculation loop and leave the lower part of the loop in the tube. Add Extraction Buffer (99 times the weight volume) to the tube and close the tube.

A4. Homogenize the sample on a multi-tube vortex by vigorous shaking (at highest speed) for 30 minutes.

A5. Centrifuge the extract in the tube for 5 minutes at 3000 x g.

A6. Decant the supernatant into fresh labeled tubes and continue with the assay procedure within 3 days, or store aliquots of the extract at 2-8°C for ≤ 7 days or at ≤ -20°C for 14 days.

This procedure results in a 1:100 dilution. This sample can be tested directly in the Bile Acids Assay. No additional dilution is required.

B) The ALPCO Extraction Device Procedure:

Please refer to the detailed instructions provided with the ALPCO extraction device, then follow steps A5 and A6 above.

These procedures result in a 1:100 dilution. This sample can be tested directly in the Bile Acids Assay. No additional dilution is required.

AUTOMATED ASSAY PROCEDURE

All reagents and samples should be equilibrated to room temperature (18-28°C) prior to use. Samples extracted using the ALPCO Easy Stool Extraction Device may be loaded directly onto the DSX instrument, however, be sure to select the appropriate sample tube type (see below for instructions). Kit standards and controls must be run alongside samples, and all standards, controls, and samples should be run in duplicate. Thoroughly vortex each standard and control before loading onto the machine.

1. This IFU outlines the steps required for use on the automated Dynex DSX instrument. An automated assay file (.asy) for the DSX instrument is available upon request from ALPCO technical support (603-893-8914 or ts@alpco.com). **The Substrate and Enzyme steps must be pipetted without stopping. Any interruption in the automated procedure will invalidate the run.**

2. Allow frozen Substrate and Enzyme Mix to thaw for at least 1.5 hours at room temperature. This process may be accelerated by placing the Substrate and Enzyme Mix bottles in a bath of room temperature water for no more than 60 minutes. Both Mixes must be fully thawed before use. Allow remaining materials stored at 4°C to equilibrate to room temperature for at least 30 minutes. **All reagents and samples should be equilibrated to room temperature (18-28°C) prior to use.**

3. Prepare a plate with sufficient strips to test the required number of standards, controls, and sample extracts in duplicate.

4. Switch ‘ON’ the DSX instrument.

5. Open Revelation DSX 6.25 Software and select ‘Connect to DSX.’

6. After device startup, select ‘File → New.’

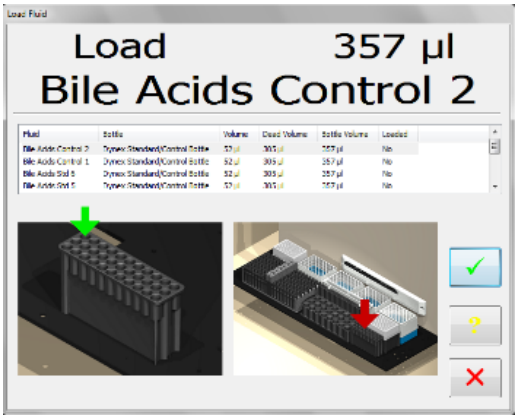
7. When prompted, select ‘Worklist.’

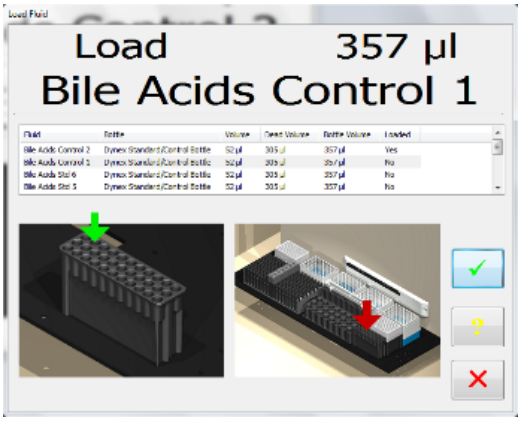
8. Select ‘Add assays using a new batch of samples’ and appropriate sample caddy definition. **ALPCO extraction device tubes may be directly loaded into the sample caddy, however, be certain to select the appropriate tube definition at this step**. The minimum sample volume required in each extraction tube is 250µl. Select ‘OK.’

9. Enter unique sample identifiers into each box that correspond to their sample positions in the caddy. A caddy consists of seven rows labeled A-G and fourteen columns numbered 1-14. At the top of the prompt, select the ‘New Plate’ tab and open the “ALPCO TBA” assay (.asy) file. Next, assign each sample for testing by clicking the corresponding boxes in column labeled ‘Test.’ A checkmark will appear in each ‘Test’ box. Select ‘OK.’

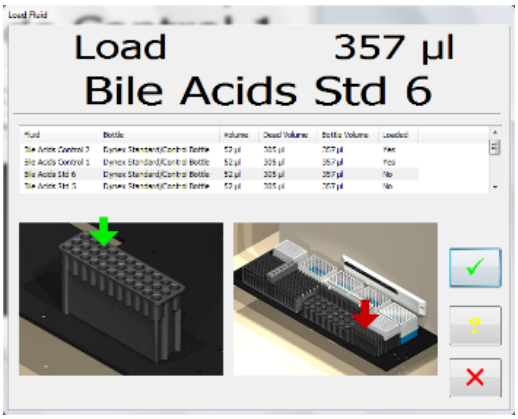
10. Once the prompt clears, click the green rightward triangle thumbnail to build the assay timeline. After a few seconds, click the green forward thumbnail to bring up another prompt to assign a lot name to the plate. Once the plate has been assigned, click the green checkmark and follow the prompts to load standards, controls, samples, and Substrate and Enzyme Mix into their assigned positions (see illustrations below).

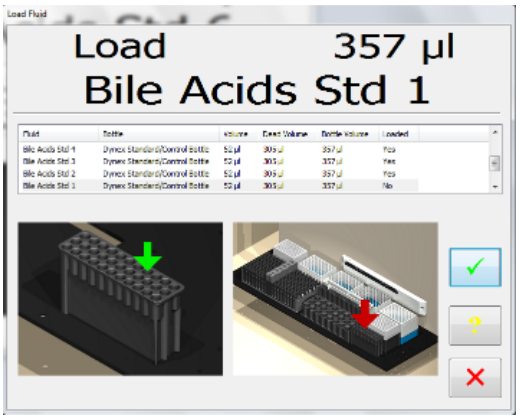
a. First, the program will direct you to load Kit Controls 2 & 1.



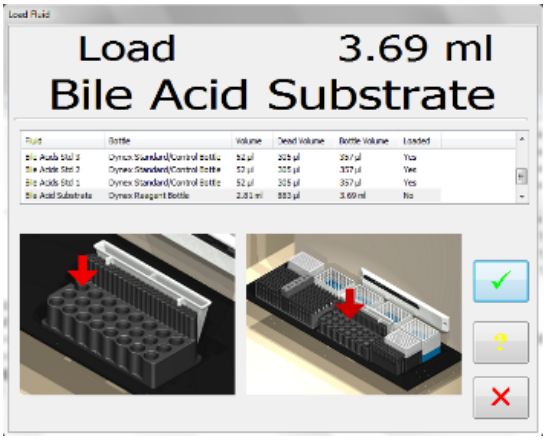


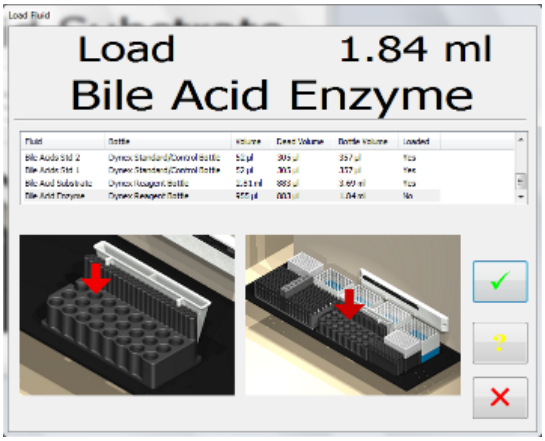
b. Next, load Standards; Standard vials are labelled A-F, but the DSX will ask for Standards 1-6. Standard F=6, Standard E=5, and so on.



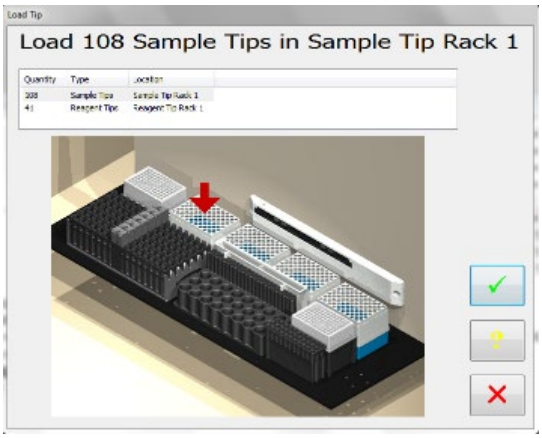


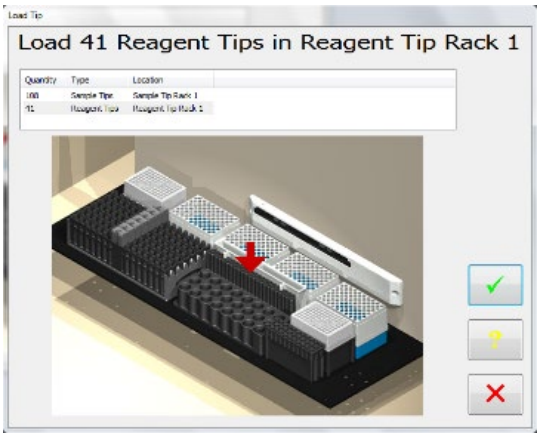
c. Then, carefully pour both Substrate and Enzyme Mixes into two, separate, labelled 20mL Dynex tubes. Load each into its corresponding position.





d. Lastly, ensure that Sample and Reagent pipette tips have been replenished.





11. Prior to beginning the assay, close the cover of the instrument and empty the waste tip container. Click ‘OK’ to start the assay protocol.

12. When the assay is completed, the data are automatically saved as a .dat file. OD Results are provided for each standard, control, and sample replicate, while control and sample values are automatically calculated off the standard curve and reported under the ‘Curve Fit’ column.

MANUAL ASSAY PROCEDURE

Kit standards and controls must be run alongside samples, and all standards, controls, and samples should be run in duplicate. Thoroughly vortex each standard and control before loading onto the machine. **The Substrate and Enzyme steps must be pipetted without stopping. Any interruption in the procedure will invalidate the results.**

Allow frozen Substrate and Enzyme Mix to thaw for at least 1.5 hours at room temperature. This process may be accelerated by placing the Substrate and Enzyme Mix bottles in a bath of room temperature water for no more than 60 minutes. Both Mixes must be fully thawed before use. Allow remaining materials stored at 4°C to equilibrate to room temperature for at least 30 minutes. **All reagents and samples should be equilibrated to room temperature (18-28°C) prior to use.**

1. Prepare a plate with sufficient strips to test the required number of standards, controls and sample extracts.
2. Add 20 µL of standards, controls, and samples to appropriate wells.
3. Add 150 µL of Substrate Mix to each well.
4. Cover the plate with a plate sealer and incubate for 5 minutes on a 3mm orbital plate shaker set at 550 rpm at room temperature (18-28˚C). Plate shaker settings may need to be optimized based on plate shaker model.
5. Remove and discard the plate sealer. Add 50 µL of Enzyme Mix to each well.
6. Cover the plate with a plate sealer.

7. If a **kinetic read** is available for analysis, incubate for **1 minute** at room temperature (18-28˚C) on a 3mm orbital plate shaker set at 550 rpm. Remove and discard the plate sealer. Perform a kinetic read at 405nm every 30 seconds for 10 minutes.

   If an **endpoint read** is required, incubate for **10 minutes** at room temperature (18-28˚C) on a 3mm orbital plate shaker set at 550 rpm. Remove and discard the plate sealer. Read the plate at 405nm.

CALCULATION OF RESULTS

For assays performed on the DSX, average the values for each replicate measure of controls and samples. Report the value for each sample as the average of the two back-calculated values. Confirm that both control levels fall within the ranges indicated in the Automated Analysis section of the Certificate of Analysis.

For a manual **kinetic read**, plot standard concentration vs. mOD/min. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the

samples. A 4-Parameter Logistic (4PL) curve fit with a fixed weighting of $1/y^2$ should be applied to generate the curve and to determine the concentration of bile acids in the samples. Sample concentrations can be read directly off the curve, as standard concentrations assume a 1:100 sample dilution. Confirm that both control levels fall within the ranges indicated in the Manual Kinetic section of the Certificate of Analysis.

For a manual **endpoint read**, plot standard concentration vs. OD. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples. A Sigmoid fit should be applied. Sample concentrations can be read directly off the curve, as standard concentrations assume a 1:100 sample dilution. Confirm that both control levels fall within the ranges indicated in the Manual Endpoint section of the Certificate of Analysis.

PERFORMANCE CHARACTERISTICS

Performance studies were conducted using the manual protocol with kinetic read. To compare analysis methods, 27 samples spanning the analytical measuring range of the assay were analyzed using the manual kinetic procedure, the manual endpoint procedure, and the automated DSX procedure. Slope and r^2 based on comparison to the manual kinetic procedure are outlined below.

	Manual Kinetic	Manual Endpoint	Automated DSX
Slope	1.00	0.97	0.99
r	1.00	0.99	0.99

Sensitivity

The LOD is 1.4 µM. The LLoQ of the assay is 5 µM.

Precision: Within run (intra-assay) variation

Intra-assay precision was determined by testing 16 replicates of three samples with either low (10µM), medium (40µM), or high (96µM) bile acids.

	Sample 1	Sample 2	Sample 3
Mean	9.9 µM	38.9 µM	86.6 µM
CV (%)	6.5%	3.5%	5.3%

Precision: Between run (inter-assay) variation

Intra-assay precision was determined by testing 16 replicates of three samples with either low (10µM), medium (40µM), or high (96µM) bile acids across two days.

	Sample 1	Sample 2	Sample 3
Mean	10.0 µM	40.2 µM	95.8 µM
CV (%)	7.5%	4.6%	10.8%

Linearity

Four stool extracts containing varying levels of bile acids (42 – 108 µM) were serially diluted from 1:2 to 1:16. Mean recoveries are listed below.

Dilution	Mean Recovery
1:2	108.0%
1:4	104.8%
1:8	101.0%
1:16	85.8%

Spike and Recovery

Five stool extracts were spiked with three levels of bile acids along with analysis of an extraction buffer control. Mean recoveries are listed below.

Spike (µM)	Expected Values (µM)	Mean Recovery
20	28-36	101.6%
73	80-88	105.2%
128	136-144	101.8%

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
B	Std B	Std B	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
C	Std C	Std C	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
D	Std D	Std D	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
E	Std E	Std E	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
F	Std F	Std F	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
G	Ctrl 1	Ctrl 1	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM
H	Ctrl 2	Ctrl 2	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM	SAM

Std= Standard
Ctrl = Control
SAM = Samples