Application Note:
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Quantitative Determination of Total Bile Acids with a Thermo Scientific Varioskan Flash Multimode Reader Using a Kinetic Enzyme Cycling Assay

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Introduction

Serum total bile acids (TBA) level is a long-established and accepted indicator of liver diseases. This application note presents a simple photometric measurement of serum TBA.

TBA concentration was determined with an enzyme cycling assay kit (Total Bile Acid Assay kit, DZ042A, Diazyme Laboratories, Poway, CA). This kit is designed for cuvette photometer use but for this study the kit was adapted to the 96-well microtiter plate format. This reduces the consumption of the reagents and therefore the assay costs at least 50% and at the same time increases the throughput remarkably since up to 43 samples can be analyzed simultaneously.

The assay is based on the oxidation of bile acids to 3-keto steroids by the enzyme 3-α-hydroxysteroid dehydrogenase (3-α-HSD) in the presence of Thio-NAD. The reaction is reversible and 3-α-HSD can also convert 3-keto steroids back to bile acids. However, in the presence of NADH, enzyme cycling occurs, as shown in Figure 1.

During enzyme cycling, Thio-NADH is formed when bile acids molecules are oxidized by 3-α-HSD to 3-keto steroids in the presence of Thio-NAD. When NADH is also present in the reaction, these 3-keto steroids are immediately reduced back to bile acids, which are again re-oxidized and re-reduced. This creates a kinetic oxidation-reduction cycle which leads to accumulation of high levels of Thio-NADH in the sample. In the presence of an excess of cofactors Thio-NAD and NADH, the kinetic of the reaction is directly proportional to the amount of bile acids present in the sample. This is calculated by measuring the rate of formation of Thio-NADH, which has an absorbance maximum at 405 nm.

Materials and Methods

- A bile acids standard (50 µmol/l) included in the kit was used for calibration and all bile acids concentrations were calculated against this 50 µmol/l standard.
- Three quality control samples at different concentrations were prepared from the commercial bile acids (Diazyme Labs) with following concentrations: CTR1 (10.6 ± 1.9 µmol/l), CTR2 (30.3 ± 5.1 µmol/l) and CTR3 (108.5 ± 18.4 µmol/l).
- 4 µl volumes of the standard, quality controls, serum samples, and water (as blank) were added in duplicate in a 96-well clear Thermo Scientific

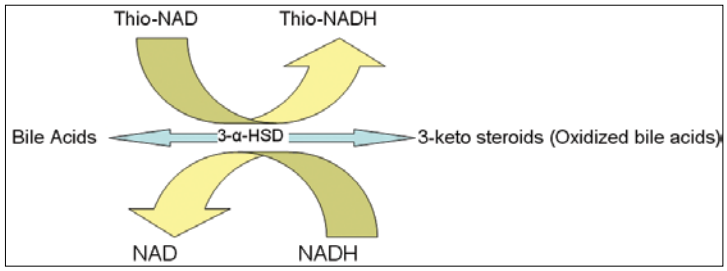

Figure 1. Principle of the enzyme cycling method for the quantitative determination of TBA in serum samples.

microtiter plate. The plate was then placed into the Thermo Scientific Varioskan Flash instrument that had been preset at 37 °C using the startup temperature option.

- The Varioskan® Flash instrument then dispensed 135 µl of Reagent 1 from the kit (containing Thio-NAD) in all wells.
- The plate was incubated at 37°C for five minutes to let the plate stabilize at the desired temperature.
- Then, 45 µl of Reagent 2 from the kit (containing 3-α-HSD and NADH) was added using a second automatic dispenser.
- Absorbance at 405 nm was recorded at exactly one and two minutes after the addition of the Reagent 2.

The Varioskan Flash microplate reader was programmed to perform the assay. The required timing of the measurements and dispensing actions were synchronized via the following assay protocol (see Figure 2):

STEP 1. Dispensing step using Dispenser 1. Dispensing volume 135 µl, dispensing speed "Medium," dispensing position "L1," tip priming "Automatic." Dispenses 135 µl of Reagent 1 into each well.

STEP 2. Incubation step. Incubation time 5 min, temperature 37 °C, select "Leave the instrument at this temperature" and "Wait until the instrument has reached the target or higher temperature." Incubates the plate for five minutes and keeps the instrument at the same temperature during the following steps.

STEP 3. Well loop step. Well interval 600 ms, execution block type: Plate. Executes well interval (well-to-well timing) at exactly 0.6 seconds. When the second dispensing step and kinetic measurement are done using this well interval, the timing between these actions is the same although the dispensing of the well takes longer time than reading the absorbance. Well interval "0.6 s" enables dispense and measurement of all 96 wells within one minute.

STEP 4. Dispensing step using Dispenser 2 under the well loop. Dispensing volume 45 µl, dispensing speed "Medium", dispensing position "L2," tip priming "Automatic," step duration one minute. Dispenses 45 µl of Reagent 2 into each well using 0.6 second dispensing interval from defined in well loop step. One-minute step duration is used to synchronize the dispensing and first kinetic reading. First well is measured exactly one minute after it was dispensed.

STEP 5. Kinetic loop step under the well loop. Readings 2, kinetic interval 1 min. Sets the instrument to perform two readings with one-minute interval time between the readings. All readings use 0.6 second-well interval from well loop step.

STEP 6. Photometric measurement step under the kinetic loop. Wavelength 405 nm, bandwidth 5 nm, measurement time 100 ms.

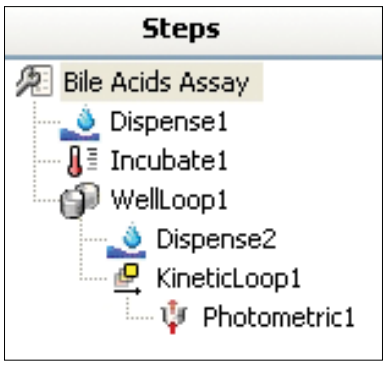

Figure 2. Step structure of the assay protocol used in the measurements. The time differences between dispense and photometric reading are all kept to exactly one minute.

Result

After calculating the averages from the absorbance values obtained for each duplicate, the ΔA_{405}/min values were calculated by subtracting A_{405} nm after 1 min (absorbance recorded 1 min after Reagent 2 addition) from A_{405}nm at 2 min (absorbance recorded 2 min after Reagent 2 addition):

$$\Delta A_{405}/\text{min} = A_{405\text{nm}} \text{ at } 2\,\text{min} - A_{405\text{nm}} \text{ at } 1\,\text{min}$$

Bile acids concentration levels in each serum and quality control samples were calculated by the following equation:

$$\frac{\text{Sample } \Delta A_{405}/\text{min} - \text{Blank } \Delta A_{405}/\text{min}}{\text{Standard } \Delta A_{405}/\text{min} - \text{Blank } \Delta A_{405}/\text{min}} \text{ x Standard (umol/l)}$$

Results are shown in Table I. All quality control samples show bile acids concentration levels well within the acceptable range. Serum samples 1 and 2 showed normal bile acids concentration levels, while bile acids concentration in sample 3 was above the normality cut-off (10.0 µmol/l), evidencing a probable pathological condition.

	Absorbance (1 min)			Absorbance (2 min)			ΔAbs/min (405 nm)	Bile acids [µmol/l]
	Replicate1	Replicate2	Mean	Replicate1	Replicate2	Mean		
Blank	0.185	0.202	0.194	0.186	0.204	0.195	0.002	
Standard	0.238	0.242	0.240	0.272	0.276	0.274	0.034	
CTR1 (10.6 ± 1.9)	0.210	0.211	0.211	0.219	0.220	0.220	0.009	11.5
CTR2 (30.3 ± 5.1)	0.223	0.224	0.224	0.247	0.247	0.247	0.024	33.8
CTR3 (108.5 ± 18.4)	0.281	0.283	0.282	0.352	0.358	0.355	0.073	110.0
Sample1	0.162	0.166	0.164	0.164	0.169	0.167	0.003	1.5
Sample2	0.188	0.174	0.181	0.191	0.178	0.185	0.004	3.1
Sample3	0.249	0.247	0.248	0.324	0.317	0.321	0.073	109.2

Table I. Result summary from the bile acids assay

Conclusion

The TBA assay can easily be adapted to a microplate format using 96-well plates. This option dramatically reduces reagent consumption, thus significantly lowering assay costs. This also increases throughput since up to 43 samples can be analyzed in a single run.

The Varioskan Flash enables all additions of the reagents and readings to be performed automatically at precise timing while maintaining the microtiter plate at a controlled temperature. These features are crucial to obtain reliable results when measuring enzyme reaction kinetics.

Further information

For further information about the Varioskan Flash multimode reader, please refer to the following web pages:

www.thermo.com/readingroom
www.thermo.com/varioskan

For more information about the Diazyme Total Bile Acid Assay kit, please refer to the following web page:

www.diazyme.com/products/reagents/DZ042A.php

References

Goldstein D.E., et al, Diabetes Care. 27(7), 1761 (2004).
United Kingdom Prospective study, 1998, Lancet 352:837.

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