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Quantitative Beutler Test for Newborn Mass Screening of Galactosemia Using a Fluorometric Microplate Reader

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Background: **The Beutler enzyme spot test is an effective assay for newborn mass screening of galactosemia, but it is qualitative and relies on visual interpretation. We describe a quantitative, instrumental modification of the assay.**

Methods: **We modified the macroscopic visual Beutler enzyme spot test by adding extraction of blood components from filter paper, deproteinization with acetone-methanol, and quantification and recording by a fluorescent microplate reader and personal computer. All handling was performed in microplates. The measurement time was 90 min.**

Results: **Fluorescence intensity (FI) of healthy controls correlated with hematocrit and galactose-1-phosphate uridyltransferase (GALT) activity. Patients with GALT deficiency were distinguished clearly from healthy subjects and heterozygous carriers by FI. FI decreased to 75% of the initial activity after storage at 25 °C for 3 days and to 40% after storage at 37 °C for 7 days. Screening of 46 742 newborns yielded 1 false-positive result (in a heterozygous carrier), 1 patient with glucose-6-phosphate dehydrogenase deficiency, and no apparent false negatives as judged by concurrent measurements of galactose and galactose-1-phosphate.**

Conclusions: **The quantitative Beutler test can provide precise GALT activity in newborn mass screening, and can take into consideration the influence of high temperature and humidity, duration between sampling and testing, and anemia. This method is clinically useful, simple, automated, and highly reliable for newborn mass screening of galactosemia.**

Classic galactosemia is caused by enzymatic deficiency of galactose-1-phosphate uridyltransferase (GALT),[3] one of the three galactose metabolic enzymes, and is the major inborn error of galactose metabolism. Mass screening for galactosemia in newborn infants commenced with screening for phenylketonuria in 1977 in Japan. The screening presently includes almost all live births in Japan. Two methods of screening for galactosemia are available, one involving the assessment of GALT activity (Beutler enzyme spot test) *(1)* and the other involving the measurement of blood concentrations of galactose and galactose-1-phosphate (Gal-1-P) *(2)*. In the Beutler enzyme spot test, GALT enzyme activity is monitored with the aid of phosphoglucomutase and glucose-6-phosphate dehydrogenase (G6PD) and visualization of the fluorescence of reduced $NADP^+$ under ultraviolet light. Mass screening for galactosemia by the Beutler enzyme spot test and/or microbiological Paigen test has indicated a relatively low incidence of this disorder in Japan: 1 in 1 000 000 compared with 1 in 40 000–60 000 in Caucasian populations *(3)*.

Classic galactosemia causes severe and rapidly progressive symptoms in the neonate, including jaundice, cataracts, hepatomegaly, failure to thrive, and even neonatal death if not treated during the early stages *(4)*. The Beutler enzyme spot test is an effective screening test for galactosemia, offering the following advantages: immediate detection of classic galactosemia, and diagnosis even when the infants is fed a non-milk formula. However, it also has disadvantages, including false-positive results after deterioration of the enzyme activity by exposure to high temperature and humidity, dependence on visual evaluation, and quenching by hemoglobin.

In the present study, we describe a quantitative Beutler test modified by separating the enzyme extraction and

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[3] Nonstandard abbreviations: GALT, galactose-1-phosphate uridyltransferase; Gal-1-P, galactose-1-phosphate; G6PD, glucose-6-phosphate dehydrogenase; FI, fluorescence intensity; and RBC, red blood cell.

reaction, and quantifying and recording the results by a fluorescent microplate reader and personal computer.

Materials and Methods

MATERIALS

The galactosemia test (fluorescent test for GALT deficiency), supplied by Roche Diagnostics (Boehringer Mannheim), consisted of 0.25 mol/L Tris-acetate buffer (pH 8.0), 1.8 mmol/L Gal-1-P, 0.32 mmol/L UDP-glucose, 0.66 mmol/L $NADP^+$, 0.135 mmol/L EDTA, and 1.3 g/L saponin. Nunc microwell plates with 0.275-mL wells with conical bottoms (cat. no. 245128) were supplied by Nalge Nunc International. Microstrip wells (cat. no. 9502157), combi frames (cat. no. 9503017), and strip retainers (cat. no. 9503047) were obtained from Labosystem. Seals for the microplates were supplied by Eiken Chemical.

METHODS

A 3.2-mm blood disc from a dried-blood newborn screening filter paper and 50 μL of galactosemia test reagent were placed in each well of a microplate with conical bottoms. The plate was allowed to stand for 1 min, followed by a 5-min centrifugation at 2010*g* by a plate centrifuge (himac CT5DL; Hitachikoki). After the plate was sealed and shaken for 1 min, it was incubated for 1 h at 37 °C for the enzyme reaction. The plate was then shaken for 1 min, and 100 μL of acetone-methanol mixture (1:1, by volume) was added to each well to precipitate the hemoglobin and protein. The plate was sealed again and centrifuged for 15 min at 2010*g*. In the next step, 35 μL of the supernatant was added to a microstrip well containing 230 μL of distilled water and shaken continuously for 1 min. Fluorescence intensity (FI) was measured at an emission wavelength of 360 nm (excitation wavelength, 450 nm) using an MTP-100F fluorescent microplate reader (Corona Electric), and the data were automatically digitized into a personal computer for analysis.

SAMPLES

Dried blood filter paper specimens collected for routine newborn mass screening at maternity hospitals in Osaka City were used in these studies. These filter papers were examined for (*a*) distribution of GALT activity as determined by FI in 1494 newborns in November 1995, (*b*) pilot mass screening of 15 233 newborns conducted from May to November 1996, and (*c*) full-scale mass screening of 46 742 newborns conducted from August 1997 to March 1999. The measurement of GALT activity in newborn mass screening in our institution proceeded (*a*) from the first routine screening test, (*b*) to retesting of specimens with abnormal first test results, (*c*) to obtaining and testing of a second dried blood filter paper specimen from subjects whose initial specimen had repeatedly abnormal results, and (*d*) to examination of subjects with abnormal results in the repeat specimens by a medical specialist. Resampling of blood and the close examination were requested with low GALT activity and high galactose and/or Gal-1-P concentrations; the cutoff values in our institution were 1.17 mmol/L (7 mg/dL) for Gal-1-P plus galactose and/or 0.17 mmol/L (3 mg/dL) for galactose. The entire study protocol was approved by the Institutional Review Board of the Osaka City Environment and Public Health Association.

STATISTICS

All data are expressed as the mean ± SD. Simple linear regression analysis was used to examine the relationship between FI and hematocrit, and correlation coefficients and probabilities were determined according standard statistical methods.

Results

EXTRACTION FROM DRIED BLOOD SPOTS

The recovery of blood from dried spots was determined by measuring hemoglobin concentrations in the extracts. A total of 80 dried blood spots were examined and extracted twice with microplate centrifugation. The mean hemoglobin content was 323 ± 70 μg/disc by the first extraction and 5.3 ± 5.3 μg/disc by the second extraction. These results indicated that the majority of hemoglobin (98.5% ± 1.7%) was extracted by the first extraction process. The dried blood spots became white after the first extraction.

EFFECT OF REACTION TIME OF GALT ENZYME

In the first series of experiments, we examined the effect of reaction time (0–180 min) on GALT velocity using dried blood spots obtained from healthy subjects, carriers, and patients (n = 3 for each group). The FI in healthy subjects was almost proportional to the incubation time (0–120 min; Fig. 1). FI values at 60-min reaction time were markedly different among patients, carriers, and healthy controls (mean ± SD, 121 ± 6, 352 ± 124, and 818 ± 158, respectively).

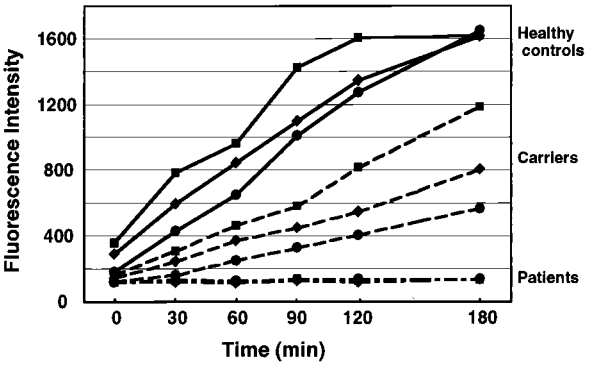

Fig. 1. Time course of quantitative Beutler test.

The effects of reaction time on FI values were examined in dried blood spots obtained from three healthy control subjects (———), three carriers (– – – –), and three patients with GALT deficiency (– - – - –).

PRECISION AND REPRODUCIBILITY

The relationship between FI and the hematocrit of dried blood spots was examined. Blood samples with hematocrit values of 20%, 30%, and 55% were prepared by diluting red blood cells (RBCs) with the plasma, and then spotting the diluted RBCs on filter papers (n = 12). There was a significant relationship between hematocrit and FI values (r^2 = 0.98; P = 0.0001; Fig. 2). These results demonstrated that FI reflected hematocrit values as well as the amount of GALT enzyme, and that the FI of dried blood spots from subjects with anemia was markedly lower than for the controls.

To examine reproducibility, dried blood spots on filter paper were prepared from the blood of healthy controls adjusted to a hematocrit of 55% (n = 24). The mean FI was 766 ± 68, and the CV was 8.9%. Dried blood spots for use as blanks were prepared by adding 0.3 U of alkaline phosphatase (to decompose the Gal-1-P and phosphorylated substrates) to 1 mL of blood from healthy controls adjusted to a hematocrit of 55% (n = 19). The mean FI was 112 ± 5.8, and the CV was 5.2%. Thus, the procedure showed satisfactory reproducibility in samples of healthy controls and in blank samples in which GALT activity was considered to be 0.

EFFECTS OF DIFFERENT TEMPERATURES AND STORAGE DURATION

The effects of storage temperature and duration on FI values for dried blood spots from seven healthy control subjects are shown in Fig. 3. The dried blood spots stored at 4 °C were relatively stable, i.e., GALT activity remained steady. FI values were >80% of the initial intensity during the first week of storage and were 66% ± 3% of the initial reading after storage for 30 days. Storage at 25 or 37 °C was associated with a clear deterioration of FI values relative to the initial values. For example, FI values decreased to 75% and 40% of the initial intensity when the samples were stored at 25 °C for 3 days and 37 °C for 7 days, respectively, a period and temperature representative of that between blood sampling and testing in the summer.

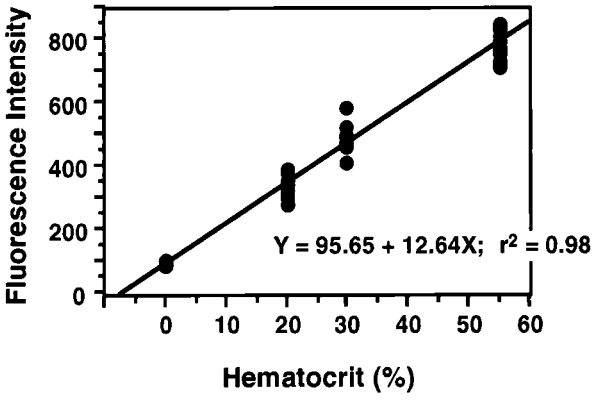

Fig. 2. Correlation of FI and hematocrit by quantitative Beutler test.

Dried blood spots with hematocrits of 20%, 30%, or 55% (n = 12 for each hematocrit) were prepared from healthy controls. FI values were measured by the quantitative Beutler test.

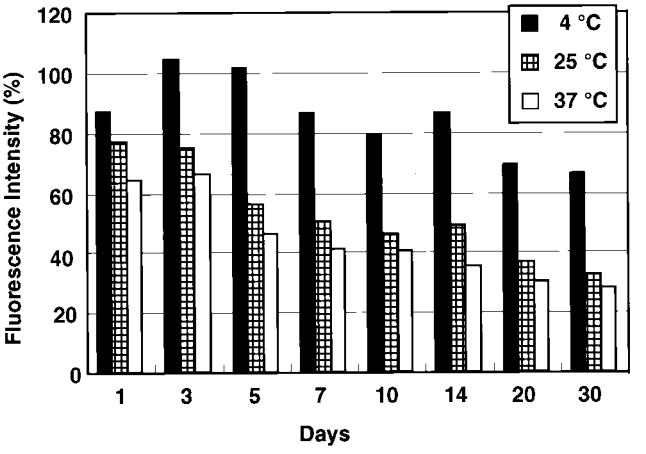

Fig. 3. Effects of temperature and duration of storage on FI values by the quantitative Beutler test.

The day of preparation of dried blood spots from seven healthy controls was set as *day 0*. The FI values on day 0 were set as 100% and examined on days 1, 3, 5, 7, 10, 14, 20, and 30 under the following conditions: at 4 °C and 32% humidity (■), at 25 °C and 43% humidity (⊞), and at 37 °C and 55% humidity (□).

DISTRIBUTION OF FI IN DRIED BLOOD SPOTS FROM NEWBORN MASS SCREENING

We examined the distribution of FI in dried blood spots of 1494 newborns collected by routine newborn mass screening. There were no new cases of GALT deficiency in the screened population. The distribution of FI was not significantly different from a gaussian distribution, as determined by the Kolmogorov–Smirnov fit test (P = 0.659; Fig. 4). FI values were 139–1128 (mean ± SD, 541 ± 132; median, 538). The FI at the third percentile was 296. The cutoff value in the pilot mass screening was set at 300.

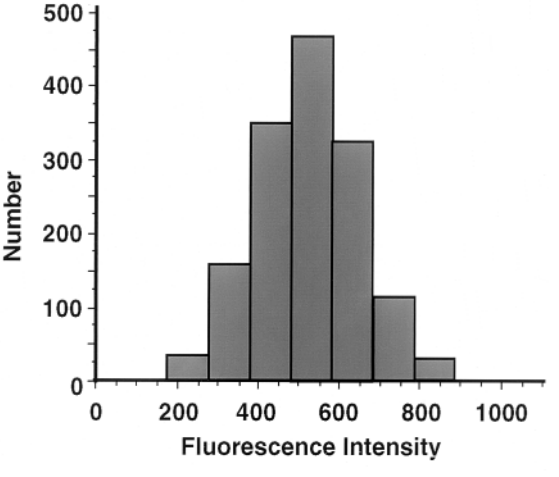

Fig. 4. Histogram of FI values for dried blood spots by the quantitative Beutler test.

The 1494 dried blood spots were collected by routine newborn mass screening. FI values ranged from 139 to 1128, with a mean value of 541 ± 132.

Table 1. Results of newborn mass screening for GALT.

	First screening of dried blood spot		Resampling of dried blood spot		No. of patients requiring close examination (%)[a]
	No. of subjects	No. retested (%)[a]	No. of requests (%)[a]	No. of examinations (%)[a]	
Beutler enzyme spot test					
90-min incubation (1-point method), 1986–1989	129 622	478 (0.37)	164 (0.13)	147 (0.11)	8 (0.006)
40- and 80-min incubation (2-point method), 1990–1995	176 634	384 (0.22)	125 (0.07)	121 (0.07)	9 (0.005)
Quantitative Beutler test					
Pirot mass screening, May–Nov 1996	15 233	396 (2.6)	49 (0.32)	49 (0.3)	7 (0.045)
Full-scale mass screening, Aug 1997–Mar 1999	46 742	765 (1.6)	235 (0.5)	235 (0.5)	1 (0.002)

[a] Percentage of examinations relative to the total number of subjects in the first newborn mass screening.

NEWBORN MASS SCREENING

Table 1 shows the results of newborn mass screening by the visual Beutler enzyme spot test and the quantitative Beutler test, and compares the proportions of samples designated for retesting, resampling, and close examinations. From 1986 to 1989, the method used by our institutions for the Beutler enzyme spot test entailed only one general visual determination after a 90-min incubation; from 1990 to 1995, this was changed to visual determinations at 40 and 80 min of incubation.

In the pilot mass screening, which involved a quantitative Beutler spot test of 15 233 newborns, with the cutoff value of the first screening test and retest set at 300, retest was performed in 396 newborns (2.6%), yielding FI values of 107–325 on retesting. Forty-nine subjects (0.32%) were requested to undergo resampling of dried blood spots because of FI values ≤250 and Gal-1-P and galactose of ≥1.17 mmol/L (7 mg/dL). Among these subjects, seven were requested for close examination. Six were found to be heterozygous carriers of GALT deficiency (G/N), and one was a compound heterozygote (G/D), based on the results of GALT activity using the consumption test *(5)*. Table 1 shows the results of the full-scale mass screening of 46 742 newborns. The cutoff FI value of the first test and retest was lowered to 250. Resampling was requested when the FI value was ≤250 and Gal-1-P and galactose were ≥1.17 mmol/L (7 mg/dL), whereas close examination was requested when the FI value was ≤200 and Gal-1-P and galactose were≥1.17 mmol/L (7 mg/dL). The full-scale mass screening by quantitative Beutler test based on the cutoff points described above did not substantially reduce the proportion of retesting and resampling of subjects. However, the proportion requiring close examination was reduced to only one subject, who was found to be a heterozygous carrier of GALT deficiency (G/N).

One boy was identified to have G6PD deficiency. The boy had a FI value of 141, which was in the same range as those for complete GALT-deficient patients, and his Gal-1-P concentration was 0.08 mmol/L (1.5 mg/dL). Thus, the Beutler test identified one other RBC enzyme deficiency. G6PD activity in this boy was reduced to 0 unit/g hemoglobin (reference range, 6.33–7.91 units/g hemoglobin). The child did not have a hemolytic diathesis under routine conditions.

Discussion

Measurement of GALT activity using the reduction of methylene blue for newborn mass screening was first reported by Beutler et al. *(6)*. Several methods have been reported subsequently, including the visual evaluation method of NADPH fluorescence (Beutler enzyme spot test) *(1)*, automated analyzer method *(7, 8)*, and quantitative methods for GALT using a fluorescence detector *(9–11)*. Several mass-screening kits are also available commercially. The quantitative Beutler test in the present study has the following features: (*a*) The GALT enzyme was extracted from dried blood spots on filter paper by centrifugation. The extraction efficiency reached almost 100% by centrifugation. In addition, the reaction time was short (1 h). A more accurate quantitative measurement was achieved by separating enzyme extraction from reaction. (*b*) The influence of quenching by hemoglobin was reduced in our test. In our quantitative Beutler test, we eliminated protein and hemoglobin by adding a mixture of acetone and methanol (at a ratio of 1:1), followed by centrifugation *(12)*. The influence of quenching by hemoglobin was further reduced by diluting the sample 6.6-fold with distilled water. (*c*) The use of a microplate centrifuge and fluorescence microplate reader allowed the use of 96-well microplates, shortening the total measurement time to 1.5 h. (*d*) Direct input of FI values to a data processor allowed automatic quantification and recording and eliminated the need for visual evaluation as in the Beutler spot test. (*e*) The running costs of our Beutler test were comparable to those of the visual evaluation method.

Shih et al. *(13)* identified an increased frequency of false positives in the Beutler spot test during the summer season. The deterioration of the GALT enzyme in dried blood spots was attributed to high humidity and high temperature, and was recognized as a weak point of Beutler enzyme spot tests *(8, 11)*. In our experiments, FI values decreased markedly when samples were stored at 25 or 37 °C for 3–7 days, respectively (Fig. 3). Furthermore, these conditions are fully conceivable in Osaka,

Japan, where the mean temperatures in July and August are 27.6 and 29.6 °C, respectively, and the mean humidity is 71% in July and 66% in August. In fact, the rate of resampling of dried blood spots was the highest between May and August (data not shown). Thus, it is important to take into consideration the number of days from blood sampling to testing when interpreting the results of samples showing low FI values. In contrast, the quantitative Beutler test described in our study allows prediction of the influence of high temperature and humidity because GALT activity is reported in a numerical value.

When carrying out newborn mass screening using the quantitative Beutler test, the false-negative results are a matter of highest importance. Therefore, the cutoff value for retesting in the pilot mass screening was set to 300, based on the third percentile for FI (Fig. 4), although the FI values in patients with GALT deficiency were much lower, 112 ± 4.7. In the pilot mass screening, seven subjects were requested to have close examination by medical specialists, which identified six heterozygous carriers and one compound heterozygous carrier of G/D. However, in the pilot study, the percentages of individuals requiring retesting, resampling, or close examination were higher than those reported in previous studies using the Beutler enzyme spot test because of the high cutoff value for FI (Table 1). Therefore, in the full-scale newborn mass screening, the cutoff value for retesting and resampling of dried blood spots was set at 250 and that for close examination at 200. With these cutoff values, we could not substantially reduce the proportions that required retesting and resampling. However, close examination was required for only one heterozygous carrier. We simulated the retesting and resampling rates for a cutoff of 200 in a screening of 46 742 subjects. In this simulation, the proportions of retesting and resampling were reduced from 765 (1.6%) to 168 (0.39%) and from 235 (0.5%) to 79 (0.17%), respectively, which are similar to the results obtained with the visual Beutler enzyme spot test. We recently have set the cutoff values to 200 in newborn mass screening.

The Beutler enzyme spot test utilizes the phosphoglucomutase, G6PD, and 6-phosphogluconate dehydrogenase present naturally in RBCs as the enzyme reactions subsequent to the GALT enzyme. GALT activity in the Beutler enzyme spot test is determined by the fluorescence of NADPH converted from $NADP^+$ in the G6PD reaction. On the basis of this principle, it is suspected that G6PD deficiency appears as a positive result. In fact, the quantitative Beutler test could detect a patient with G6PD deficiency showing almost null G6PD activity with the same FI values as those given by patients with GALT deficiency. On the other hand, Mediterranean G6PD patients with an activity 2–7% of the activity of healthy subjects and G6PD A patients with an activity 8–20% of that of healthy subjects showed almost normal values in a mass screening test for GALT activity, as reported by Frazier and Summer *(8)*. These results are attributable to the fact that the enzyme reaction rates of phosphoglucomutase, G6PD, and 6-phosphogluconate dehydrogenase are 7- to 20-fold higher than GALT. The above findings suggest that, although our quantitative Beutler test may not detect partial G6PD deficiency, this test can detect complete deficiency of G6PD with a normal Gal-1-P concentration and low FI value similar to those of patients with GALT deficiency.

These results demonstrate that because it uses quantitative analysis, the quantitative Beutler test can take into account the influence of high temperature and humidity, the interval between sampling to test, and anemia in the dried blood spot. Thus, the quantitative Beutler test is a clinically useful, simple, automated, and highly reliable test for mass screening of newborns for galactosemia.

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References

1. Beutler E, Baluda MC. A simple spot screening test for galactosemia. J Lab Clin Med 1966;68:137–41.
2. Paigen K, Pacholec F, Levy HL. A new method of screening for inherited disorders of galactose metabolism. J Lab Clin Med 1982;99:895–907.
3. Aoki K, Wada Y. Outcome of the patients detected by newborn screening in Japan. Acta Paediatr Jpn 1988;30:429–34.
4. Segal S, Berry GT. Disorders of galactose metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease, 7th ed., Vol. 1. New York: McGraw-Hill, 1995:967–1000.
5. Anderson EP, Kalckar HM, Kurahashi K, Isselbacher KJ. A specific enzymatic assay for the diagnosis of congenital galactosemia. J Lab Clin Med 1957;50:469–77.
6. Beutler E, Baluda M, Donnell GE. A new method for the detection of galactosemia and its carrier state. J Lab Clin Med 1964;64: 695–705.
7. Hochella NJ, Hill JB. Fluorometric screening procedure for galactosemia utilizing the autoanalyzer. Clin Chem 1969;15:949–60.
8. Frazier P, Summer G. Automated fluorometric micromethod for detection of transferase deficiency galactosemia. J Lab Clin Med 1974;83:334–8.
9. Beutler E, Mitchell M. New rapid for the estimation of red cell galactose-1-phosphate uridyl transferase activity. J Lab Clin Med 1968;72:527–32.
10. Rhode H, Elei E, Taube I, Podskarbi T, Horn A. Newborn screening for galactosemia: ultramicro assay for galactose-1-phosphate-uridyltransferase activity. Clin Chim Acta 1998;274:71–87.
11. Frazier DM, Clemons EH, Kirkman HN. Minimizing false positive diagnoses in newborn screening for galactosemia. Biochem Med Metab Biol 1992;48:199–211.
12. Yamaguchi A, Fukushi M, Mizushima Y, Shimizu Y, Takasugi N, Arashima S, et al. Microassay for screening newborns for galactosemia with use of a fluorometric microplate reader. Clin Chem 1989;35:1962–4.
13. Shih VE, Levy H I, Karolkewicz V, Houghton S, Efron ML, Isselbacher KJ, et al. Galactosemia screening of newborns in Massachusetts. N Engl J Med 1971;284:753–7.