The goal of newborn screening (NBS)1 for inherited disorders of metabolism is the early detection and confirmation of disease, thus enabling early medical intervention, treatment, and improved outcomes (1). Important characteristics of a screening method include analytical specificity and sensitivity, coupled with rapid, high throughput and timely reporting of abnormal results. Routine primary screening methods are designed to identify as many abnormal infants as possible, with diagnostic sensitivity favored over specificity for disorder detection. This approach not only increases the numbers of false-positive test results, thus adding to the cost of operating NBS programs, but also places unnecessarily increased stress, anxiety, and possibly parent–child dysfunction on families (2). As the number of disorders in the NBS test panels grows, however, so does the overall number of false-positive results, which has increased severalfold per true case (3). One solution to this problem is to use improved methods or to couple primary screening methods with second-tier tests that improve selectivity.

The use of tandem mass spectrometry (MS/MS) for detecting phenylketonuria is an example of an NBS method that improves detection as a primary screen while also being more selective than older, classic NBS methods such as fluorometry. In one study, MS/MS analysis of newborn spot samples of dried blood collected ≤24 h after birth was compared with fluorometric analysis of the same samples. Because of this early time of collection, the decision level for an increased phenylalanine concentration was lowered by the public health laboratory using fluorometry to ensure that no infants with phenylketonuria were missed. MS/MS analysis of the identical samples demonstrated that disease detection could be sustained while improving selectivity (4). The ability to measure multiple analytes in the same analysis enabled the calculation of the phenylalanine/tyrosine molar ratio, which reduced false-positive rates a 100-fold. This screen for phenylketonuria was the first instance of a new paradigm in NBS, in which both current screens could be improved and new screens could be added for other disorders, such as fatty acid oxidation defects and organic acidemias (5). The ability of MS/MS to improve efficacy without the need for collecting a second sample reduces the false-positive rate.

The next level in improving diagnostic specificity without reducing diagnostic sensitivity is to develop a second, linked test that is more specific than the original method. The linked second test has a lower sample throughput but measures additional metabolites that either strongly support the presumption of a true positive case or refute the notion that the patient has the disorder. These coupled screening tests use the original blood spot sample after the initial primary screening test has made a presumptive positive identification; such tests are known as “second-tier tests.” These tests should not be confused with testing that is repeated with a new sample collected at a later date or with the diagnostic testing. Second-tier tests are not typically used for primary screening because of the low sample throughput and the cost, complexity, or analysis time.

An earlier application of a second-tier test that several screening laboratories presently use for the detection of congenital adrenal hyperplasia cases is the liquid chromatography–MS/MS test designed to improve the selectivity for measuring 17α-hydroxyprogesterone and associated steroid hormones in the metabolic pathway (6). In this application, MS/MS is used to measure additional metabolites (e.g., cortisol, androstenedione) to yield a better interpretation of the original analysis. Although this method would have better utility as a primary screen, several limitations presently prevent this application. Therefore, the primary screening of congenital adrenal hyperplasia is done with less selective methods, with a larger capture of presumptive positive cases being followed by a second-tier analysis. This second-tier test, when applied, has markedly reduced the false-positive report rate.

As NBS and technologies expand, a broader range of biomarkers (metabolites, proteins, DNA/RNA) will be used in NBS. Changes in the concentrations of many metabolites can identify a large array of diseases, an

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2 Nonstandard abbreviations: NBS, newborn screening; MS/MS, tandem mass spectrometry; Tyr I, tyrosinemia type I.
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approach that might be called a single-metabolite, multiple-disease biomarker model. For example, propionylcarnitine as measured by MS/MS is a primary marker for methylmalonic and propionic acidemias (7); algorithms and criteria have been developed to detect these diseases reliably. Other metabolic disorders, environmental or nutritional factors, and other disease processes may produce milder increases in the propionylcarnitine concentration. Lowering the cutoff concentration for abnormal results may pick up new diseases but with a loss in specificity, possibly diminishing the justification for screening. This is a classic dilemma in NBS methodologies, as has been demonstrated in other assays, such as tyrosinemia types I, II, and III. Detection of tyrosinemia type I (Tyr I) has always been a challenge because the tyrosine concentration may be increased only slightly, whereas the moderate increases of tyrosine found in Tyr II and Tyr III are easier to detect. Adjusting the cutoff concentration was not satisfactory because the number of false positives became quite large and diagnostic sensitivity was still relatively poor. A second-tier test, an assay for succinylacetone that was more specific for screening Tyr I, was developed and gradually evolved through research efforts to a method suitable for a primary screening application. Presently, many NBS laboratories are adopting succinylacetone analysis as a routine primary screen.

In this issue of Clinical Chemistry, Turgeon et al. (8) describe a method that could potentially expand the list of disorders detectable by MS/MS while screening for biomarkers presently included in the American College of Medical Genetics recommended panel. This method involves lowering the cutoff concentrations for several of these biomarkers and following up the results with a second-tier test. Specifically, this new algorithm lowers the cutoff concentrations for propionylcarnitine and methionine to capture additional disorders of propionate, methionine, and cobalamin metabolism. The new second-tier method uses aliquots (i.e., paper punches) taken from the initial NBS sample and detects total homocysteine, methylmalonic acid, and methylcitric acid. The authors report that the use of these methods and associated algorithms led to the identification of 13 affected infants who would not have otherwise been detected.

In this application, the second-tier tests were not used primarily to reduce false-positive rates or to enhance the performance of the original method. Instead, the authors lowered the cutoff concentration criteria to capture these disorders, which are not routinely detected by most NBS programs, thus reducing the risk of false-negative results. The availability of the new second-tier tests allows for better capturing of presumptive positive samples without increasing the false-positive rate. The primary screen is still MS/MS, but the increased burden from lowering the cutoff values requires another panel of screening tests to reduce the false-positive rate to a reasonable level. In one sense, these new methods are actually part of the primary screens and not additive second-tier tests. The concentrations of the key (primary) markers are not increased significantly for these disorders and therefore require additional testing—the second tier. The second-tier tests are often quite specific but prohibitively complex, time-consuming, or expensive for routine population-based screening. The question is whether it is better to develop newer, simpler primary screening tests (as was done for Tyr I) or to develop a new screening model that relies on an expanded battery of second-level testing. It is likely that such second-tier testing will require another level of laboratory sophistication and resources, so that most public health screening laboratories will have to rapidly distribute samples to designated regional test centers for the process to be effective, be cost reasonable, and yield a substantial laboratory work load for quality assurance. New operational systems will be required to meet the efficient turnaround times necessary for data that are critical for NBS programs, but more newborns will be saved from the adverse outcomes of additional disorders.

Clearly, for second-tier tests to become widely accepted, there must be mechanisms to cover the additional costs and infrastructure changes for recognized regional centers. More importantly, however, these second-tier algorithms will introduce early identification of more disorders and thereby reduce the anxiety and stress to families caused by repeated sample collection and testing. With new innovative techniques and technologies, the NBS spectra will slowly expand to cover hundreds of treatable metabolic disorders. The increased numbers of biomarkers and disorders will also require a higher level of interpretative skills and will increase interactions with metabolic specialists, ideally in full-service regional metabolic centers.

Perhaps the most striking aspect of the second-tier test relates not only to its ability to reduce false-positive rates derived from screening for the primary metabolite of a disorder but also to its capacity to expand the number of disorders by using the same screen for the same metabolite as a secondary metabolite for other diseases. Secondary metabolites have lower decision concentrations but are likely to detect other related disorders. The second-tier test is used to selectivity identify which of these other disorders are present while sustaining a lower false-positive report rate. Without a doubt, NBS is making another advance in the level of screening and quality with the expansion and intro-
duction of new roles for routine second-tier testing of the initial sample spot of dried blood.

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