molecular cloning and expression analysis or three calcium-binding proteins from human heart. Biochemistry 1992;31:10258–64.
13. Wiesmann M, Missler U, Gottmann D, Gehring S. Plasma S-100b protein concentration in healthy adults is age and sex independent. Clin Chem 1998;44:1056–8.
14. Wiesmann M, Wandinger KP, Missler U, Eckhoff D, Rothermundt M, Arolt V, et al. Elevated plasma levels of S-100b protein in schizophrenic patients. Biol Psychiatry 1999;45:1508–11.
15. Woertgen C, Rothoerl RD, Holzschuh M, Metz C, Brawanski A. Comparison of serial S-100 and NSE serum measurements after severe head injury. Acta Neurochir (Wien) 1997;139:1161–5.
16. Usui A, Kato K, Abe T, Murase M, Tanaka M, Takeuchi E. S-100ao protein in blood and urine during open-heart surgery. Clin Chem 1989;35:1942–4.
17. Böni R, Burg G, Doguoglu A, Ilg EC, Schäfer BW, Müller B, et al. Immunohistochemical localization of the Ca^{2+} binding S100 proteins in normal human skin and melanocytic lesions. Br J Dermatol 1997;137:39–43.
18. Cho KH, Hashimoto K, Taniguchi Y, Pietruk T, Zarbo RJ, An T. Immunohistochemical study of melanocytic nevus and malignant melanoma with monoclonal antibodies against S-100 subunits. Cancer 1990;66:765–71.
19. Barger SW, Wolchok SR, van Eldik LJ. Disulfide-linked S100b dimers and signal transduction. Biochim Biophys Acta 1992;1160:105–12.

Multiplexed Genotyping of β-Globin Variants from PCR-amplified Newborn Blood Spot DNA by Hybridization with Allele-specific Oligodeoxynucleotides Coupled to an Array of Fluorescent Microspheres, *Robert J. Colinas,* Ronald Bellisario, and Kenneth A. Pass* (Division of Genetic Disorders, Wadsworth Center, New York State Department of Health, Albany, NY 12201-0509; * author for correspondence: fax 518-402-4989, e-mail colinas@wadsworth.org)

Detection of hemoglobinopathies in newborns is critical for the identification of those infants in need of follow-up care [reviewed in Ref. *(1)*]. For example, infants homozygous for the sickle cell mutation are at greater risk for developing fatal pneumococcal infections and sepsis, which can be prevented by prophylactic antibiotic therapy. At present, the majority of newborn screening for hemoglobin (Hb) variants is done by electrophoresis, isoelectric focusing, or HPLC *(2)* using Hb extracted from dried blood spots. Nevertheless, detection of adult Hb variants often is complicated by the presence of fetal Hb in neonatal blood. Alternative approaches to these protein-based methodologies have been developed that directly detect the presence of hemoglobinopathy-associated mutations in newborn DNA *(3)*, and some have been adapted to use blood spots *(4, 5)*. Using the S and E mutations in the β-globin gene as examples, we have developed a multiplexed, high-throughput methodology that uses an array of allele-specific fluorescent beads and the Luminex^{100} analyzer (Luminex Corporation, Austin, TX; www.luminexcorp.com). This methodology distinguishes between the S and E alleles and their wild-type counterparts, HbA and non-E, of the β-globin gene in each specimen, making it possible to determine the genotype at each locus.

The methodology is based on the principle that fluorescent microspheres with unique fluorescent profiles, called classifications, can be cross-linked to different analyte-specific reagents and used to create a fluorescence-based array capable of simultaneously assaying multiple analytes in each sample *(6)*. The bead classifications were obtained separately from the Luminex Corporation with surface carboxyl groups for chemical cross-linking to different analyte-specific reagents, which in our studies were 5′-amino-modified oligodeoxynucleotides. As indicated above, each bead classification has a unique spectral address based on its 658 nm/712 nm emission ratio when excited by the 635 nm laser in the Luminex^{100} instrument. The Luminex software uses this spectral profile to assign beads to their classifications, and each classification occupies a known position on a dot plot of 658 nm vs 712 nm fluorescence. Thus, multiple bead classifications can be combined in one sample, and the Luminex software processes the fluorescent signals to generate an array of bead classifications on the dot plot of 658 nm/712 nm fluorescence. Determination of the amounts of the different analytes bound to each bead classification is accomplished by coincident excitation of the beads with the 532 nm laser in the Luminex^{100} instrument. Thus, labeling bead-bound analytes with a fluorescent reporter molecule such as phycoerythrin, which emits at 575 nm when excited at 532 nm, produces a third fluorescent signal that allows the amounts of analytes bound to the beads to be quantified. Thus, in each sample, the amounts of multiple analytes can be determined from the emissions of a single fluorescent reporter molecule because the analyte specificity and position of each bead classification in the array is known.

The following oligodeoxyribonucleotides (ODNs) used in our studies were purchased from Oligos Etc, and the positions of the S and E mutations are indicated in bold: 5′-amino-modified 18mer ODNs for coupling to beads were HbA, GCAGACTTCTCCTCAGGA; HbS, GCAGACTTCTCC**A**CAGGA; non-E, CAGGGCCTCACCACCAAC; HbE, CAGGGCCT**T**ACCACCAAC; nonsense (NS), GCATACTCCGTCTCAAGG. 5′-Biotinylated ODNs complementary to HbA, HbS, non-E, HbE, and NS were also used to quality control coupling efficiency of 5′-amino ODNs to the beads.

The five 5′-amino-ODNs were coupled in separate reactions to five different bead classifications. More specifically, 2.5×10^6 beads were coupled to 0.5 nmol of ODN with freshly made 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl, using the protocol provided by the Luminex Corporation.

PCR was performed after treatment of 1-mm blood-spot punches with methanol and boiling water as described *(5)*. A 327-bp region of the β-globin gene between nucleotides 62010 and 62336 of the GenBank sequence (Accession No. U01317.1) was amplified using Qiagen 10× PCR buffer and Taq polymerase with a 5′-biotinylated PCR primer (ACGGCTGTCATCACTTAG) and an unmodified reverse PCR primer (TCCCACATGCCCAGTTTC). Amplification conditions were 5 min at 95 °C and 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by extension for 5 min at 72 °C. PCR amplification was verified by agarose gel electrophoresis using an aliquot of each reaction. Both the S and E loci are present in the

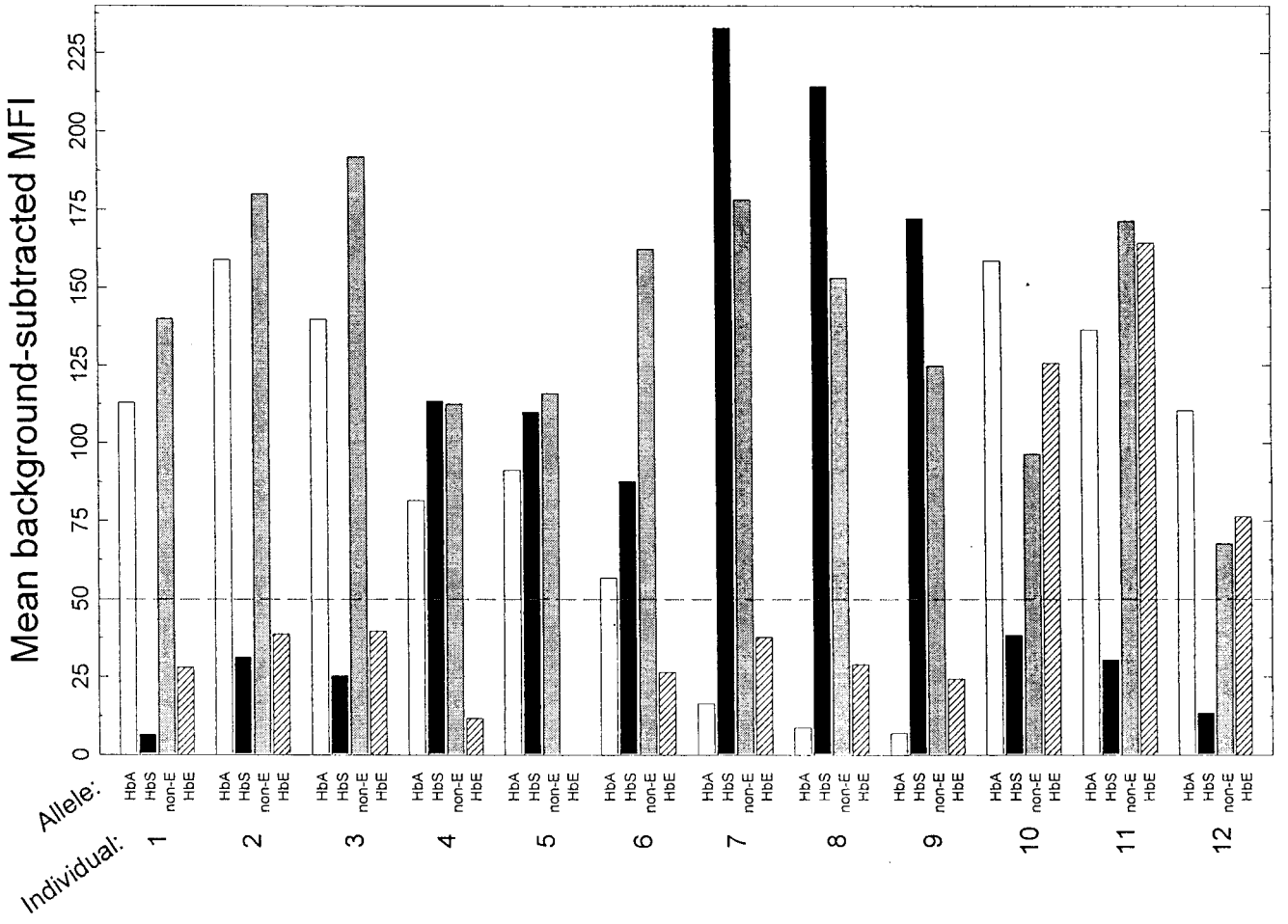

Fig. 1. Five bead classifications, each coupled to one of four β-globin allele-specific ODNs or a NS ODN, were hybridized to biotinylated PCR-amplified newborn dried blood spot DNAs, labeled with streptavidin-phycoerythrin, and analyzed using the Luminex^{100}.

Mean background-subtracted MFIs were calculated, and representative results from 12 individuals are shown. □, HbA; ■, HbS; ▨, non-E; ▨, HbE. The *dashed line* indicates the MFI value below which samples were considered negative.

region of the β-globin gene that was amplified and are separated by 60 nucleotides.

Hybridization of the biotinylated PCR products to the five different ODN-coupled bead classifications was performed in tetramethylammonium chloride (TMAC) buffer (1× is 3 mol/L TMAC, 1 g/L sodium dodecyl sulfate, 50 mmol/L Tris-HCl, pH 8.0, 4 mmol/L EDTA, pH 8.0) as follows. Duplicate samples containing 5 μL of each PCR were diluted to 17 μL with water and denatured at 98 °C for 10 min. To each sample, 33 μL of 1.5× TMAC containing 5000 of each of the five ODN-coupled bead classifications was added while at 98 °C. The samples were mixed and immediately transferred to a 45 °C heating block. The samples were hybridized at 45 °C for 30 min and diluted to 150 μL with 100 μL of 1× TMAC (45 °C). The beads were pelleted at 8000*g* for 1 min, and the supernatant was removed. The beads were labeled for 7 min with 50 μL of freshly made 0.5 mg/L streptavidin-phycoerythrin (at 45 °C; Molecular Probes) in 1× TMAC. The samples were again diluted with 100 μL of 1× TMAC (at 45 °C), the beads were pelleted, and the supernatants were removed. Each sample was resuspended in 100 μL of 1× TMAC at 45 °C, and the samples were analyzed using the Luminex^{100} instrument set in Multiplex Analysis mode to exclude multimers of beads and collect median fluorescent intensities (MFIs) on 100 events per bead classification. In each sample, the background MFI obtained from the NS ODN-coupled beads was subtracted from the MFIs of the four β-globin allele-specific MFIs, and the mean background-subtracted MFIs were calculated for duplicate samples. The intersample variation of duplicate background values (as MFIs) was 70–130.

To test this genotyping methodology, blood spots from 12 individuals with known β-globin genotypes were screened. Representative data from these experiments are shown in Fig. 1. From these results, it appears that mean background-subtracted MFIs <50 can be considered negative. However, further experimentation is required to confirm this observation. The data show that individuals

Table 1. Interexperimental variation in background-subtracted MFIs.[a]

	Allele[c]			
Individual[b]	HbA	HbS	Non-E	HbE
1	116.5 (19.2)	14.1 (9.3)	135.9 (19.9)	27.5 (4.5)
4	82.2 (1.2)	121.8 (7.6)	116.6 (8.2)	17.0 (6.5)
7	8.6 (6.8)	211.9 (22.8)	138.8 (34.4)	28.8 (8.3)
10	149.0 (11.1)	36.1 (5.5)	87.3 (9.9)	115.6 (12.8)

[a] Five classifications of fluorescent beads were coupled separately to NS ODN or one of the four indicated β-globin allele-specific ODNs. Five thousand beads from each group were combined and hybridized in duplicate to denatured biotinylated PCR products amplified from newborn dried blood spots. Streptavidin-phycoerythrin was added to each sample, and the background-subtracted MFIs for each bead classification were determined using the Luminex^{100} analyzer.

[b] Numerical designations correspond to those individuals shown in Fig. 1 and have the following genotypes: 1, A/A, non-E/non-E; 4, A/S, non-E/non-E; 7, S/S, non-E/non-E; 10, A/A, non-E/E.

[c] The data shown represent the means (SD) of background-subtracted MFIs obtained from three experimental repetitions.

1, 2, and 3 were homozygous for both HbA and non-E; individuals 4, 5, and 6 were HbA/S heterozygotes and homozygous for non-E; individuals 7, 8, and 9 were homozygous for both HbS and non-E; and individuals 10, 11, and 12 were HbA homozygotes and non-E/HbE heterozygotes. These data also demonstrate an additional advantage of this multiplexed fluorescent bead array-based approach. Because it is very rare for an individual to be negative for all four of the β-globin alleles being assessed in this study, the majority of samples will yield positive background-subtracted MFIs for at least one β-globin allele, which will also serve as an internal positive control. Those samples negative for all of the alleles being assessed would most likely represent PCR failures and require retesting.

The interexperimental variation for this genotyping methodology was also assessed. As shown in Table 1, the standard deviations calculated from the background-subtracted MFIs from the three experiments did not interfere with the ability to correctly designate the β-globin genotype of these individuals. These data suggest that the results from each experiment are correct and that the retesting rate should be low.

In conclusion, we have shown that this multiplexed fluorescent bead array-based genotyping methodology can be used to accurately determine the allelic composition at two β-globin loci, using dried blood spots from newborns. The array of fluorescent beads is flexible and could be expanded to include additional β-globin mutations or other clinically important genetic loci. This method would also be adaptable to automation to meet the high throughput requirements of a large newborn screening program.

This research was also supported by the Wadsworth Center Genomics Core Facility. We thank Dr. Michelle Caggana (Division of Genetic Disorders, Wadsworth Center) for providing laboratory workspace and access to equipment and supplies.

References

1. Weatherall DJ, Clegg JB, Higgs DR, Wood WG. The hemoglobinopathies. In: Scriver CR, Beaudet AL, Sly WS, Valle D., eds. The metabolic and molecular bases of inherited disease, 7th ed. New York: McGraw-Hill, 1995:3417–82.
2. Eastman JW, Wong R, Liao CL, Morales DR. Automated HPLC screening of newborns for sickle cell anemia and other hemoglobinopathies. Clin Chem 1996;42:704–10.
3. Conner BJ, Reyes AA, Morin C, Itakura K, Teplitz RL, Wallace RB. Detection of sickle β^S-globin allele by hybridization with synthetic oligonucleotides. Proc Natl Acad Sci U S A 1983;80:278–82.
4. Jinks DC, Minter M, Tarver DA, Vanderford M, Hejtmancik JF, McCabe ERB. Molecular genetic diagnosis of sickle disease using dried blood specimens on blotters used for newborn screening. Hum Genet 1989;81:363–6.
5. Caggana M, Conroy JM, Pass KA. Rapid, efficient method for multiplex amplification from filter paper. Hum Mutat 1998;11:404–9.
6. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR Jr. Advanced multiplexed analysis with the FlowMetrix™ system Clin Chem 1997;43:1749–56.

S100B Protein Concentrations in Cord Blood: Correlations with Gestational Age in Term and Preterm Deliveries, *Diego Gazzolo,[1] Paola Vinesi,[2] Emanuela Marinoni,[3] Romolo Di Iorio,[3] Mauro Marras,[4] Mario Lituania,[4] Pierluigi Bruschettini,[1] and Fabrizio Michetti[2*]* (Departments of [1] Pediatrics and [4] Obstetrics and Gynecology, Giannina Gaslini Children's University Hospital, I-16147 Genoa, Italy; [2] Institute of Anatomy, Catholic University, I-00168 Rome, Italy; [3] Laboratory of Perinatal Medicine and Molecular Biology, University "La Sapienza", I-00124 Rome, Italy; * address correspondence to this author at: Institute of Anatomy, Catholic University, Largo Francesco Vito 1, I-00168 Rome, Italy; fax 39-063051343, e-mail fabrizio.michetti@rm.unicatt.it)

S100B is an acidic calcium-binding protein of the EF-hand family concentrated in the nervous system, where it is located mainly in glial cells *(1)*. Although many hypotheses have been formulated, its biological role is still debated, but its appearance in biological fluids has been shown to be a reliable index of brain distress *(2)*.

We investigated the relationship between S100B concentrations in cord blood and gestational age in the third trimester of pregnancy in normal preterm and term deliveries.

We investigated 58 women with consecutive singleton physiological pregnancies (30 at term and 28 preterm), with normal flow velocimetry waveforms in the uteroplacental vessels, whose delivery was between 27 and 42 weeks of gestation. Gestational age was determined by clinical data and by a first-trimester ultrasound scan. Appropriate growth was defined by the presence of ultrasonographic signs (when biparietal diameter and abdominal circumference were between the 10th and 90th centiles) according to the normograms of Campbell and Thoms *(3)* and by postnatal confirmation of a birth weight between the 10th and 90th centiles according to our