gray matter during childhood, which decreases before adulthood (23). From the electrophysiologic point of view, electroencephalographic findings become similar to those in adults early in the second decade of life (24).

The time window of maximal plateau and decline of LCMRgce in cerebral cortex and the consolidation of adult electroencephalographic patterns observed in older children are closely associated with the variations in serum concentrations of S100B in healthy individuals. Considering that the S100B protein exerts tropic effects on neural cells, we believe that it could play an important role during the early phase of human brain development, which is reflected in its serum concentrations.

In conclusion, we have shown a negative correlation between blood S100B and age in the first 20 years of life; after age 20, S100B does not appear to vary with age. Therefore, in studies involving measurement of blood S100B in pediatric and adolescent patients, it is important to establish age-matched reference values. Further work will be required to clarify the role of S100B during neural development.

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References

“Reconstituted” α-Thalassemia Genomic Samples as Positive Controls for the Molecular Diagnostic Laboratory, Wen Wang, Arnold S-C. Tan, and Samuel S. Chong Department of Pediatrics and Obstetrics and Gynecology, National University of Singapore, Singapore 119074, Singapore; Molecular Diagnosis Center, Department of Laboratory Medicine, National University Hospital, Singapore 119074, Singapore; 3 Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; * address correspondence to this author at: Department of Pediatrics, National University of Singapore, Level 4, Main Building, National University Hospital, 5 Lower Kent Ridge Rd., Singapore 119074, Singapore; fax 65-6779-7486, e-mail paecs@nus.edu.sg)

We and others recently described strategies for multiplex-PCR analysis of deletional determinants of α-thalassemia (1–3), culminating in the development of a single-tube assay for simultaneous screening of seven common deletions (4). Since then, several molecular diagnostic laboratories wanting to set up the test have requested DNA samples carrying these deletions for use as validation and positive controls. These requests have led to a critical shortage of our limited stocks of genomic samples, especially those with the rarer deletions, something that has caused our inability to fulfill all requests. The ideal solution to limited genomic DNA is to establish immortal lymphoblastoid cell lines from peripheral-blood leukocytes of patients by Epstein–Barr virus transformation (5, 6). To do so, however, requires that patients be contacted again to provide renewed consent and a fresh aliquot of blood for transformation, something that may
be inconvenient or impractical for third-party referral laboratories to implement.

We have devised an alternative strategy for creating a renewable resource of positive control samples of known \( \alpha \)-thalassemia genotype, derived from existing patient DNA samples. Briefly, genomic DNA of known \( \alpha \)-thalassemia genotype was initially used as a template to amplify each deletion junction fragment individually by PCR. The relevant primer pairs and thermo-cycling conditions were as described previously (4). Junction fragments of seven \( \alpha \)-thalassemia deletions were PCR-amplified separately from patient DNA, gel-purified with the GFX\textsuperscript{TM} reagent set (Amersham Pharmacia Biotech), and ligated to a pBluescript (Stratagene) T-vector (Fig. 1A). Each 5-\( \mu \)L ligation reaction contained 15 ng of T-vector and an amount of PCR product, which led to a final vector-to-insert molar ratio of 1:3, and 1 U of T\(_4\) DNA ligase in 1X supplied ligation buffer (Fermentas). Ligation reactions were incubated at 16 °C for 16 h, and products were transformed into DH5\( \alpha \)\textsuperscript{TM} competent cells (Life Technologies). Recombinant clones containing each junction fragment were identified by PCR amplification with appropriate insert- and vector-specific primers, and verified recombinant plasmid DNA was isolated by standard techniques (7).

To generate a reconstituted genomic DNA sample heterozygous for an \( \alpha \)-thalassemia deletion, recombinant plasmid DNA containing the relevant deletion junction fragment was mixed with genomic DNA extracted from either a lymphoblastoid cell line or peripheral-blood lymphocytes that were previously determined to be homozygous normal (nondeleted) at the \( \alpha \)-globin locus. Multiplex-PCR amplifications were performed as described (4), and one-fifth of each product was resolved across a 1% agarose, 1X Tris-borate-EDTA gel at 15 V/cm for 1 h. Beginning with equimolar mixes of haploid genome equivalents and supercoiled plasmid constructs, we empirically adjusted the molar ratios such that the multiplex-PCR results for each of the reconstituted heterozygous positive-control samples closely resembled those obtained from actual heterozygous patient samples. We further readjusted the ratios of plasmid constructs to normal genomic DNA such that the signal intensities of the amplified deletion junction fragments relative to the normal \( \alpha 2 \) fragment were weaker coming from reconstituted positive controls than from actual heterozygous patients (Table 1). The higher stringency for detection of deletion junction fragments from reconstituted positive controls compared with actual samples was intended to ensure that a negative diagnostic test result was real when positive amplification was observed from reconstituted positive controls. Conversely, false-negative diagnostic results that could occur because of suboptimal PCR conditions would not be expected to be missed because the reconstituted positive controls would be the first to fail under the same conditions. As shown in Fig. 1B, the reconstituted samples yielded amplification results resembling those from actual heterozygous DNA samples, but with weaker ratios of deletion junction fragment to normal \( \alpha 2 \) fragment signal intensities.

Interestingly, a molar excess of plasmid construct to haploid genome equivalents is still required in each reconstituted positive control (Table 1), although actual heterozygous patient samples contained an equal ratio of

![Fig. 1. Cloning of deletion junction fragments and multiplex-PCR analysis of reconstituted positive control samples.](https://academic.oup.com/clinchem/article-abstract/48/6/952/5641696)
normal and deletion alleles. This disparity in template requirement could be attributable to lower amplification efficiencies from supercoiled DNA resulting from stearic hindrances and/or rapid complementary-strand reannealing during the primer-annealing step. This hypothesis is supported by our observation of stronger signal intensities of the deletion junction fragments when plasmid constructs were linearized before mixing with genomic DNA (data not shown). Although prelinearization of the plasmid constructs enables molar ratios to be closer to parity, supercoiled plasmids may be the preferred form for long-term storage as mixtures with genomic DNA because they are less susceptible than linearized plasmids to trace amounts of nucleases.

Another approach to reconstituting positive-control samples, an approach we initially used before cloning all the junction fragments, is to mix the isolated PCR-amplified deletion junction fragments directly with genomic DNA. Although this method works equally well and obviates the need for cloning, all the other optimization steps are still necessary. Furthermore, the amount of plasmid DNA that can be obtained from a single bacterial maxi-preparation far exceeds the amount of the deletion junction fragment that can be obtained by PCR, yielding sufficient stable DNA to generate innumerable positive controls indefinitely without the need for periodic re-PCR and fragment isolation.

We also assessed the feasibility of using as positive controls the diluted multiplex-PCR products of patients who were positive for deletions. In a test using multiplex-PCR product from a $-\alpha^{3,7}$ deletion carrier diluted over more than three different orders of magnitude, however, we were unable to generate results anywhere resembling the original multiplex-PCR result, with only the normal $\alpha_2$ fragment amplifying strongly (data not shown). This observation was not surprising considering that the relative ratios of $-\alpha^{3,7}$, $\alpha_2$, and LIS1 copies in a genomic-DNA sample are unlikely to be maintained in the amplified product.

Furthermore, we also assessed the feasibility of generating a single-tube “multipositive control” by mixing all seven plasmid constructs together with normal genomic DNA, in the amounts indicated in Table 1. All nine fragments (seven deletion junction fragments plus the $\alpha_2$ and LIS1 control fragments) amplified successfully from the reconstituted sample (Fig. 1C). The signal intensities of the various deletion junction fragments relative to the normal $\alpha_2$ fragment were also comparable with the corresponding relative intensities observed in the individually reconstituted positive controls. Use of a multipositive control sample such as this would reduce the number of positive control reactions necessary in a diagnostic test by sevenfold. It remains to be seen whether such a reconstituted genomic sample containing multiple deletion alleles will find wide acceptance as a sufficiently “authentic” positive control for use in a diagnostic-test setting.

In summary, we have successfully used a strategy to create “reconstituted” genomic DNA samples heterozygous for each of the seven deletions screened for in our multiplex-PCR assay, and these renewable reagents are now available on request. Genomic DNA samples of known mutational genotype are necessary reagents in the molecular diagnostic laboratory, serving a critical role as positive controls during the testing of clinical samples. Our reconstitution strategy represents a convenient, rapid, and inexpensive alternative to cell immortalization techniques for generating renewable positive controls for $\alpha$-thalassemia multiplex-PCR testing and should be generally applicable to other inherited disorders where immortalized cell lines are not readily available.

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References

3. Chong SS, Boehm CD, Cutting GR, Higgs DR. Simplified multiplex-PCR

### Table 1. Generation of heterozygous $\alpha$-thalassemia genomic samples: optimization of mixtures of normal genomic DNA with plasmid constructs carrying deletion junction fragments.

<table>
<thead>
<tr>
<th>Deletion</th>
<th>Junction fragment, bp</th>
<th>Construct size, bp</th>
<th>Molecular weight of construct</th>
<th>Construct added per 100 ng of gDNA, $^{a,b}$ pg</th>
<th>Molar ratio (deletion:$\alpha^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-\alpha^{3,7}$</td>
<td>2029</td>
<td>4990</td>
<td>$3.29 \times 10^6$</td>
<td>0.566</td>
<td>$-3.5:1$</td>
</tr>
<tr>
<td>$-\alpha^{4,2}$</td>
<td>1628</td>
<td>4589</td>
<td>$3.03 \times 10^6$</td>
<td>0.459</td>
<td>$-3:1$</td>
</tr>
<tr>
<td>$-\alpha^{SEA}$</td>
<td>1249</td>
<td>4314</td>
<td>$2.84 \times 10^6$</td>
<td>0.714</td>
<td>$-5:1$</td>
</tr>
<tr>
<td>$-\alpha^{THAI}$</td>
<td>1153</td>
<td>4114</td>
<td>$2.72 \times 10^6$</td>
<td>1.176</td>
<td>$-8.5:1$</td>
</tr>
<tr>
<td>$-(\alpha)^{2.5}$</td>
<td>1007</td>
<td>3968</td>
<td>$2.62 \times 10^6$</td>
<td>0.227</td>
<td>$-1.5:1$</td>
</tr>
<tr>
<td>$-\alpha^{MED}$</td>
<td>807</td>
<td>3768</td>
<td>$2.48 \times 10^6$</td>
<td>0.271</td>
<td>$-2:1$</td>
</tr>
<tr>
<td>$-\alpha^{FIL}$</td>
<td>546</td>
<td>3507</td>
<td>$2.31 \times 10^6$</td>
<td>0.750</td>
<td>$-6.5:1$</td>
</tr>
</tbody>
</table>

$^{a}$ One hundred nanograms of human genomic DNA contain $\sim 3 \times 10^4$ haploid genome-equivalents. Normal human genomic DNA contains 1 copy of the $\alpha_2$ globin gene per haploid genome.

$^{b}$ gDNA, genomic DNA.

$^{c}$ Ratio of $\alpha_2$ globin gene copies to deletion junction copies in reconstituted genomic samples necessary to achieve results similar to actual heterozygous DNA samples.
cyte proliferation specific cell-cycle regulatory protein. The inhibition of everolimus is mammalian target of rapamycin (mTOR), a such as interleukin 2, the primary target of sirolimus and activation of T lymphocytes through inhibition of cytokines effective immunosuppressive regimens are undergoing agents sirolimus and everolimus, new, potentially more With the introduction of the novel immunosuppressive K. Klein G, Henle W, Henle G. The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV. Int J Cancer 1971;8:443–90. 7. Sambrook J, Fritsch EF, Maniatis T, eds. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989:1.1-1.110. Rapid Liquid Chromatography–Tandem Mass Spectrometry Routine Method for Simultaneous Determination of Sirolimus, Everolimus, Tacrolimus, and Cyclosporin A in Whole Blood, Frank Streit, Victor William Armstrong, and Michael Oellerich (Department of Clinical Chemistry, George-August University Goettingen, 37075 Goettingen, Germany; * address correspondence to this author at: Abteilung Klinische Chemie, Zentrum Innere Medizin, Georg-August-Universität Göttingen, Robert-Koch-Straße 40, 37075 Göttingen; fax 49-551-398551, e-mail varmstro@med.uni-goettingen.de) With the introduction of the novel immunosuppressive agents sirolimus and everolimus, new, potentially more effective immunosuppressive regimens are undergoing clinical evaluation. Whereas the calcineurin inhibitors cyclosporin A (CsA) and tacrolimus suppress early activation of T lymphocytes through inhibition of cytokines such as interleukin 2, the primary target of sirolimus and everolimus is mammalian target of rapamycin (mTOR), a specific cell-cycle regulatory protein. The inhibition of mTOR leads to suppression of cytokine-driven T-lymphocyte proliferation (1). Because of their distinct modes of action, a calcineurin inhibitor and an mTOR inhibitor act synergistically to block acute allograft rejection (2–4). Current evidence (5) suggests that drug monitoring is necessary, not only for the calcineurin inhibitors, but also for the mTOR inhibitors. In contrast to the calcineurin inhibitors, however, there are currently no commercially available immunoassays for the latter two drugs. There is a need, therefore, for assays that can simultaneously quantify both the calcineurin and the mTOR inhibitors. Furthermore, a major shortcoming of the commercial immunoassays for CsA and tacrolimus is their cross-reactivity with unpredictable amounts of both active and inactive metabolites of the parent drugs in patient samples. In the case of CsA, no immunoassay has completely fulfilled the criteria recommended by a consensus panel (6).

New developments in immunosuppression protocols require that assays for calcineurin inhibitors provide broader dynamic ranges. On the one hand, C2 monitoring has been proposed for optimizing dosage of the CsA microemulsion (7), thereby necessitating quantification of CsA at concentrations up to 2500 µg/L: the lack of validated dilution protocols can be a problem for C2 monitoring (8). On the other hand, lower target ranges for CsA (5) and tacrolimus (9) are being investigated for long-term maintenance therapy to minimize the adverse side effects, in particular nephrotoxicity, of these drugs. Current immunoassays exhibit high coefficients of variation at drug concentrations below the present therapeutic ranges.

These facts stimulated us to develop a rapid, sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method with a broad dynamic range for the simultaneous determination of sirolimus, everolimus, tacrolimus, and CsA in whole blood.

Sirolimus was a kind gift of Wyeth-Ayerst Research (Princeton, NJ). CsA, cyclosporin D (CsD), and everolimus were kind gifts of Novartis (Basel, Switzerland), and tacrolimus was a kind gift of Fujisawa (Osaka, Japan). HPLC-grade methanol and analytical grade ammonium acetate and ZnSO4 were obtained from Merck. Ascomycin was purchased from Sigma-Aldrich. The calibrators and in-house controls were prepared in drug-free whole blood from stock solutions of the respective drugs in methanol. The final calibrator concentrations for sirolimus, everolimus, and tacrolimus were 0.25, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100 µg/L; for CsA, the concentrations were 5.0, 25, 50, 100, 250, 500, 1000, and 2500 µg/L. The concentrations of the in-house controls were 3, 12, and 30 µg/L for sirolimus, everolimus, and tacrolimus and 60, 200, 600, and 2000 µg/L for CsA. Controls were aliquoted and kept frozen at −20 °C until use. Two internal standards were selected on the basis of the similarity of their chemical structures to those of the analytes (i.e., ascomycin for the determination of sirolimus, everolimus, and tacrolimus and CsD for the determination of CsA).

For sample preparation, 100 µL of calibrator, quality control, or patient sample (EDTA blood) was vortex-mixed for 30 s with 200 µL of a mixture of methanol and 0.3 mol/L ZnSO4 (70:30, by volume) containing the internal standards ascomycin (50 µg/L) and CsD (250 µg/L) in 1.5-mL polypropylene tubes. After centrifugation for 5 min at 4000g, the supernatants were decanted; after recentrifugation for 1 min at 4000g, they were then placed in a series 200 autosampler (Perkin-Elmer) for injection into the LC-MS/MS system.

The column was an Aqua Perfect (150 mm × 3.0 mm; 5-µm) C18-reversed phase column (MZ-Analysetechnik) maintained at 65 °C with a Du Pont column oven. The LC-MS/MS system further consisted of a Series 200 binary pump from Perkin-Elmer, an M480 pump (Dionex), and a 10-port Rheodyne valve. Supernatants (150 µL) were injected onto the column with the series 200 autoinjector fitted with a 200-µL sample loop. The column was washed for 1 min (flow rate, 1500 µL/min) with methanol and 30 mmol/L ammonium acetate solution (80:20, by volume), followed by a 2.5-min elution step (flow rate, 800 µL/min) with methanol and 30 mmol/L ammonium acetate solution (97:3, by volume), and a 0.5-min step with methanol and 30 mmol/L ammonium