Evaluation of a New Pooling Strategy Based on Leukocyte Count for Rapid Quantification of Allele Frequencies, *Heidi Rossmann,[1*] Elena Büchler,[1] Jürgen J. Wenzel,[1] Carolin Neukirch,[1] Jean-Baptist du Prel,[2] and Karl J. Lackner*[1] ([1] Department of Clinical Chemistry and Laboratory Medicine and [2] Institute of Medical Biometry, Epidemiology, and Informatics (IMBEI), University of Mainz, Mainz, Germany; * Address correspondence to this author at: University of Mainz, Langenbeckstr. 1, 55101 Mainz, Germany; fax 49-6131-17-6404; e-mail rossmann@zentrallabor.klinik.uni-mainz.de)

Background: **Allele frequencies of single-nucleotide polymorphisms (SNPs) can be quantified from DNA pools. The conventional preparation of DNA pools requires DNA isolation and quantification for each blood sample. We hypothesized that pooling of whole blood samples according to their leukocyte count, which determines DNA content, would be as reliable as the conventional pooling method but much less tedious to perform.**

Methods: **We collected 100 whole blood samples and measured the leukocyte count. Samples were frozen until further use. After thawing, pools were generated by combining aliquots containing an equal number of leukocytes. In parallel, DNA was extracted from another aliquot, DNA concentration was measured, and DNA concentration-based pools were assembled. All original samples were genotyped directly using 4 different SNP assays to obtain the exact allele frequencies in the pool. In addition, samples of known genotypes were mixed according to the DNA concentration or the leukocyte count to generate artificial samples of known allele frequencies. We analyzed pools and mixes in triplicate by pyrosequencing and calculated allelic frequencies.**

Results: **Leukocyte and DNA pooling provided equally accurate and precise SNP frequencies comparable to published data.**

Conclusion: **DNA and leukocyte pooling are both suitable strategies to determine allele frequencies in frozen samples. The leukocyte pooling approach is much less tedious, quicker, and less expensive. It should be always considered if leukocyte counts are available.**

The demand for simple and reliable methods to determine allele frequencies in many different populations is increasing. Allele quantification in DNA pools has been successfully used for this purpose. We hypothesized that pooling of whole blood according to the leukocyte count, which determines DNA content of the sample, may be a simple alternative to conventional pooling procedures. The major advantages are that only 1 DNA isolation is necessary from each blood pool and that hematology analyzers can generate leukocyte counts with high accuracy and precision. Therefore we compared a leukocyte count-based pooling strategy with conventional DNA-pooling.

Surplus whole blood from 100 complete blood counts ordered anonymously by the hospital staff occupational medicine service was collected and then stored at −20 °C until further use. The study design was in accordance with the guidelines of the local ethics committee. Leukocyte counts were measured with an Advia 120 instrument (Bayer Diagnostics). The imprecision (CV) in serial measurements was 2.41%.

We extracted DNA from 200 μL of each blood sample (QIAamp DNA Mini Kit, Qiagen) and analyzed genotypes by pyrosequencing for the following SNPs: lactase promoter polymorphisms (LCT) A(-22018)G and T(-13910)C, factor V Leiden (FV) (G1691A) polymorphism, and the prothrombin (F2; G20210A) gene mutation (see Figure 1 and Table 1 in the Data Supplement that accompanies the online version of this technical brief at http://www.clinchem.org/content/vol53/issue5). Finally, we calculated allele frequencies in the whole population.

To prepare DNA pools, DNA concentration was determined by a Nanodrop-System (NanoDrop Technologies). The median CV of triplicate measurements was 1.69%. An equivalent of 710 ng genomic DNA of each sample was used for pooling. Three DNA pools were prepared independently to control for pipetting errors.

For the leukocyte count based pools, we pipetted an equivalent of 710 400 leukocytes from the original, thawed, well-mixed whole blood sample into lysis buffer. Four independent leukocyte pools were assembled and DNA was extracted as described above.

Each pooled DNA was analyzed in triplicate by the pyrosequencing assays described above. The areas under the curves were determined, and the respective allele frequencies (given as percentage) for the analyzed SNPs were calculated by the instrument's allele quantification software (Biotage). Because peaks caused by the dispensation of different nucleotides resulted in reproducible but not necessarily equal areas under the curve (see Fig. 1 in the online Data Supplement), calibration was required for all SNP assays. DNAs of known genotype were combined in the appropriate ratios to obtain suitable calibrators. These were analyzed in parallel with the pool samples. Calibration curves were linear (see Fig. 1A) for each SNP assay with regression coefficients (Pearson's correlation) $r > 0.997$. Actual allele frequencies were deduced from the calibration curves. Results of the allele quantification experiments for DNA and leukocyte pools compared to direct genotyping results are shown in Table 1.

To evaluate the correlation between expected and determined allele frequencies <10% and >80%, 2 samples of known genotypes were mixed based on DNA concentration and leukocyte count, generating samples of known, gradually increasing allele frequencies (mix experiments: Fig. 1C and Table 2 in the online Data Supplement).

All SNP assays used for this study are in routine use in our laboratory and were adequate for allele quantification without further optimization. Considering the correlation coefficients, pool quantification results, and published

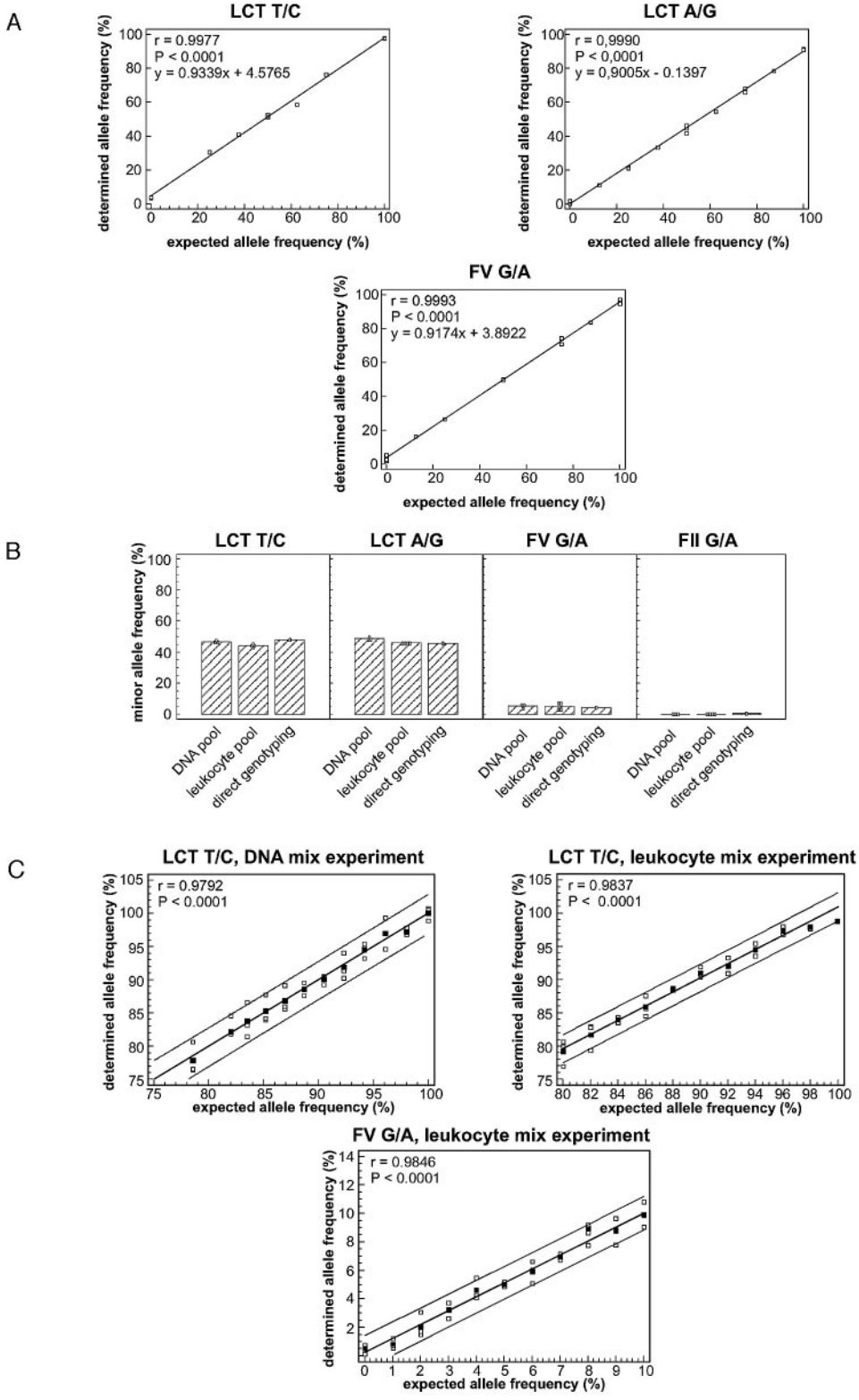

Fig. 1. Allele quantification of the lactase promoter polymorphisms (LCT) A(-22018)G and T(-13910)C, the factor V Leiden (FV) (G1691A) polymorphism, and the prothrombin (F2; G20210A) gene mutation by pyrosequencing in DNA and leukocyte pools.

Because peaks caused by the dispensation of different nucleotides during pyrosequencing result in reproducible but not necessarily equal areas under the curve, calibration was required for all SNP assays.

(*A*), calibration curves (determined allele frequencies plotted against expected allele frequencies) for exemplary SNP assays with regression coefficients and curve equations.

(*B*), quantification of allele frequencies in DNA and leukocyte pools in comparison to the results of direct genotyping. For pool quantification experiments means are shown as *hatched columns*, single pool experiments are given as *open boxes* (numerical values: see Table 1).

(*C*), comparison between expected and determined allele frequencies <10% and >80%: two samples of known genotype were mixed (LCT T/C: sample 1 C/C, sample 2 T/C; FV sample 1 G/A, sample 2 G/G) based on the DNA concentration or the leukocyte count generating samples of known, gradually increasing allele frequencies. Single LCT T/C and FV experiments (*open boxes*) are visualized in scatter diagrams with 95% prediction and regression coefficient (Pearson's correlation). Moreover, mean values are shown as *filled boxes* (numerical values: see Table 2 in the online Data Supplement).

data *(1–5)*, all evaluated SNP tests performed appropriately. Means of 3 pool/mix experiments, each measured in triplicate, showed in most cases a deviation of <1.35% (Table 1 and Fig. 1) compared to the respective target value.

Our data show that leukocyte-based pools are equivalent to DNA-based pools for determination of allele frequencies, but are much less time-consuming and costly. For this study, 100 DNA isolations were necessary

Table 1. Numeric data for allele quantification in DNA and leukocyte pools.

		Pool (100 samples) based on DNA concentration	Pool (100 samples) based on leukocyte count
SNP analysis	**Target value, %**	**Mean of 3 pools, % (SE)**	**Mean of 4 pools, % (SE)**
FV	4.0	5.3 (0.9)	5.1 (1.2)
LCT T/C	48.00	46.7 (0.4)	44.1 (0.4)
LCT A/G	45.50	48.7 (0.4)	46.0 (0.2)
FII	0.50	0.00 (0.00)	0.00 (0.00)

See Fig. 1B for graphical presentation of these data.

for the DNA pooling approach, but only 4 were necessary for the 4 leukocyte pools. We performed 100 leukocyte counts with an automated analyzer capable of measuring 120 samples per hour; 100 DNA concentrations were measured with the Nanodrop system by 1 person for 3 to 4 h. A limitation of the leukocyte pooling approach is that no individual DNA samples are available for genotyping, a shortcoming that may necessitate individual DNA preparations at a later time point. It should be noted, however, that leukocyte pooling is advantageous even when individual DNA has been prepared, because it obviates the need for time-consuming, highly accurate measurement of DNA concentrations, which is not needed for most other purposes. In summary, we believe that leukocyte pooling should always be considered if SNP analysis from pooled DNA is planned.

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Perioperative Activin A Concentrations as a Predictive Marker of Neurologic Abnormalities in Children after Open Heart Surgery, *Pasquale Florio,*[1] *Raul Felipe Abella,*[2] *Teresa de la Torre,*[2] *Alessandro Giamberti,*[2] *Stefano Luisi,*[1] *Gianfranco Butera,*[2] *Alessandro Cazzaniga,*[2] *Alessandro Frigiola,*[2] *Felice Petraglia,*[1] *and Diego Gazzolo*[3,4*] ([1] Department of Pediatrics, Obstetrics and Reproductive Medicine, University of Siena, Siena, Italy; [2] Department of Cardiac Surgery S. Donato Milanese University Hospital, San Donato Milanese, Italy; [3] Department of Pediatrics and Neuroscience, G. Gaslini Children's Hospital, Genoa, Italy; [4] Department of Maternal Fetal and Neonatal Health G. Garibaldi Hospital, Catania, Italy; * Address correspondence to this author at: Department of Maternal Fetal and Neonatal Health, G. Garibaldi Hospital, Via Palermo 636, I-95100 Catania, Italy; fax 39-95-7595208, e-mail dgazzolo@hotmail.com)

Background: **Ischemic-reperfusion injury of the brain is a major adverse event after cardiac surgery, especially when extracorporeal circuits are used. Because brain injury induces local overproduction of activin A, we measured plasma concentrations in children after open heart surgery with cardiopulmonary bypass (CPB) to investigate the potential of measuring activin A for early identification of infants at risk for brain damage.**
Methods: **We evaluated 45 infants (age <1 year) with congenital heart defects: 36 without overt neurologic injury, and 9 with neurologic injury on day 7 after the surgical procedure. Blood samples were taken before surgery, during surgery before CPB, at the end of CPB, at the end of surgery, and at 12 h after surgery. Neurologic development was assessed before surgery and on postoperative day 7.**
Results: **Activin A concentrations increased significantly during surgery (P <0.0001) to a maximum at the end of CPB. Infants who developed abnormal neurologic sequelae had concentrations significantly higher (P <0.0001, all comparisons) than patients with normal neurologic outcome at all evaluated times, but not before surgery. Activin A had a sensitivity of 100% (95% CI, 66%–100%) and a specificity of 100% (95% CI, 90%–100%) as a single marker for predicting neurologic abnormalities (area under the ROC curve, 1.0).**
Conclusions: **Activin A increases in children who experience poor neurologic outcomes after open heart surgery, and its assay may help in early identification of infants at risk for brain damage.**