tant to understand the effects of these Hb^{VAR} on the various methods.

Grant/funding support: This work was supported by a Samsung Biomedical Research Institute grant (SBRI C-A6-403-2).
Financial disclosures: None declared.

References

1. Bry L, Chen PC, Sacks DB. Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin. Clin Chem 2001;47:153–63.
2. Schnedl WJ, Liebminger A, Roller RE, Lipp RW, Krejs GJ. Hemoglobin variants and determination of glycated hemoglobin (HbA1c). Diabetes Metab Res Rev 2001;17:94–8.
3. Sacks DB. Hemoglobin variants and hemoglobin A1c analysis: problem solved? Clin Chem 2003;49:1245–7.
4. Weykamp CW, Penders TJ, Muskiet FA, van der Slik W. Influence of hemoglobin variants and derivatives on glycohemoglobin determinations, as investigated by 102 laboratories using 16 methods. Clin Chem 1993;39:1717–23.
5. Roberts WL, De BK, Brown D, Hanbury CM, Hoyer JD, John WG, et al. Effects of hemoglobin C and S traits on eight glycohemoglobin methods. Clin Chem 2002;48:383–5.
6. Lee ST, Kim MS, Choi DY, Kim SK, Ki CS. Incidence of variant hemoglobin (Hb) and increased fetal Hb concentrations and their effect on Hb A1c measurement in a Korean population. Clin Chem 2006;52:1445–6.
7. Hardison RC, Chui DH, Giardine B, Riemer C, Patrinos GP, Anagnou N, et al. HbVar: a relational database of human hemoglobin variants and thalassemia mutations at the globin gene server. Hum Mutat 2002;19:225–33.
8. Blackwell RQ, Ro IH, Liu CS, Yang HJ, Wang CC, Huang JT. Hemoglobin variant found in Koreans, Chinese, and North American Indians: alpha-2 beta-2 22 Glu Ala. Am J Phys Anthropol 1969;30:389–91.
9. Blackwell RW, Liu CS, Yang HJ, Wang CC, Huang JT. Hemoglobin variant common to Chinese and North American Indians: alpha-2-beta-22 Glu-Ala. Science 1968;161:381–2.
10. Li HJ, Zhao XN, Qin F, Li HW, Li L, He XJ, et al. Abnormal hemoglobins in the Silk Road region of China. Hum Genet 1990;86:231–5.
11. Jeppsson JO, Kobold U, Barr J, Finke A, Hoelzel W, Hoshino T, et al. Approved IFCC reference method for the measurement of HbA1c in human blood. Clin Chem Lab Med 2002;40:78–89.
12. Hoelzel W, Weykamp C, Jeppsson JO, Miedema K, Barr JR, Goodall I, et al. IFCC reference system for measurement of hemoglobin A1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison study. Clin Chem 2004;50:166–74.
13. Martin RF. General Deming regression for estimating systematic bias and its confidence interval in method-comparison studies. Clin Chem 2000;46:100–4.
14. Roberts WL, Safar-Pour S, De BK, Rohlfing CL, Weykamp CW, Little RR. Effects of hemoglobin C and S traits on glycohemoglobin measurements by eleven methods. Clin Chem 2005;51:776–8.
15. Ogawa K, Bando T, Ogawa M, Miyazaki A, Nakanishi T, Shimizu A. Hemoglobin variant HbG-Coushatta (beta-22 Glu –> Ala) found by dissociation of blood glucose from values of HbA1C measured by HPLC. Intern Med 2003;42:781–7.
16. Nakanishi T, Miyazaki A, Shimizu A, Yamaguchi A, Nishimura S. Assessment of the effect of hemoglobin variants on routine HbA1c measurements by electrospray ionization mass spectrometry. Clin Chim Acta 2002;323:89–101.
17. Ohba Y, Miyaji T, Murakami M, Kadowaki S, Fujita T, Oimomi M, et al. Hb Himeji or beta 140 (H18) Ala—Asp. A slightly unstable hemoglobin with increased beta N-terminal glycation. Hemoglobin 1986;10:109–25.

Previously published online at DOI: 10.1373/clinchem.2007.093963

Mass Spectrometry–Based Detection of Hemoglobin E Mutation by Allele-Specific Base Extension Reaction,

Jason C.H. Tsang,[1] *Pimlak Charoenkwan,*[2] *Katherine C.K. Chow,*[1] *Yongjie Jin,*[3] *Chanane Wanapirak,*[2] *Torpong Sanguansermsri,*[2] *Y.M. Dennis Lo,*[1,4] *and Rossa W.K. Chiu*[1,4*]
([1] Department of Chemical Pathology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR; [2] Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand; [3] Centre for Emerging Infectious Diseases and [4] Centre for Research into Circulating Fetal Nucleic Acids, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR; * address correspondence to this author at: Department of Chemical Pathology, Rm. 38063, 1/F, Clinical Sciences Building, Prince of Wales Hospital, 30-32 Ngan Shing St., Shatin, Hong Kong SAR; fax 852 2636 5090, e-mail rossachiu@cuhk.edu.hk)

Background: **The specific detection of a minor population of mutant DNA molecules requires methods of high specificity and sensitivity. While the single-allele base extension reaction (SABER) was shown to be useful for the detection of certain beta-thalassemia mutations, we encountered problems with false positivity during development of SABER for the noninvasive prenatal diagnosis of the hemoglobin E (HbE) disease. Systematic optimization resulted in an alternative protocol, the allele-specific base extension reaction (ASBER).**
Methods: **An artificial model was established by mixing genomic DNA of HbE carriers and normal individuals. Effects of terminator concentration and annealing temperature on the nonspecificity of SABER were then studied. The use of a single relevant terminator and the other 3 types of dideoxynucleotide as competing terminators were also compared in the development of the ASBER protocol. Thirteen cases of HbE-susceptible pregnancies were tested to compare the SABER and the ASBER protocols.**
Results: **Decreasing the single relevant terminator concentration and increasing the annealing temperature in SABER were found to improve specificity. The use of the other 3 types of dideoxynucleotide as competing terminators was shown to offer better detection sensitivity than a single terminator in ASBER. Genotyping results were all correctly determined by ASBER, except one false-negative detection (sensitivity: 80%, specificity: 100%).**
Conclusions: **An alternative mass spectrometry–based protocol for noninvasive prenatal diagnosis, ASBER, has been successfully developed to allow the detection of a minor DNA population with a point mutation.**

The discovery of circulating fetal nucleic acids in maternal plasma has opened up exciting possibilities for noninvasive prenatal diagnosis *(1, 2)*. The recent development of

the mass spectrometry-based single-allele base extension reaction (SABER) protocol has enabled sensitive differentiation of fetal-specific alleles down to a single-nucleotide level *(3–5)*. In this report, we intended to develop a mass spectrometry–based method for the noninvasive prenatal diagnosis of the hemoglobin E (HbE) mutation. Unexpectedly, the lack of specificity of SABER for the HbE mutation was discovered during assay development, and systematic optimization on an artificial model has been carried out. This development has resulted in an alternative protocol, the allele-specific base extension reaction (ASBER).

HbE disease is an autosomal recessive hemoglobinopathy caused by a (GAG→AAG) missense mutation in codon 26 of the β-globin gene *(6)*. It is the most common thalassemic hemoglobinopathy in Southeast Asia *(7)*. Although homozygotes of HbE are mildly affected by the mutation, compound heterozygotes of the HbE and the β-thalassemia mutation will result in severe anemia (HbE/β-thalassemia). Therefore, detection of the HbE mutant allele is critically important in the prenatal diagnosis of thalassemia major in Southeast Asia. In noninvasive prenatal diagnosis of HbE, the absence of the paternally transmitted fetal HbE mutant allele in the plasma of a pregnant female carrier of a β-thalassemic mutation negates the fetal inheritance of HbE/β-thalassemia. Theoretically, this suggests that 50% of invasive procedures are unnecessary and can thus be avoided.

All samples in this study were collected with informed consent, and approval was granted by the institutional ethics committee. Venous blood (6 mL) was collected into EDTA tubes from each couple referred for prenatal diagnosis. Plasma and buffy coat were harvested from blood samples after a 1st centrifugation at 1600*g* for 10 min and a further microcentrifugation of plasma aliquot at 16 000*g* for 10 min as previously described *(8)*. DNA was extracted from buffy coat and 800 μL of plasma by a Nucleon Blood DNA Extraction Kit (GE Healthcare) and a QIAamp Blood Mini Kit (Qiagen) with elution volume of 50 μL H_2O, respectively, according to the manufacturers' recommendations.

The principle of standard homogenous MassEXTEND (Sequenom) and SABER protocols have been described previously *(3)*. HotStar *Taq* polymerase (Qiagen) was used in the PCR at a final volume of 25 μL, containing 10 μL of plasma DNA and 0.2 μmol/L PCR primers (Integrated DNA Technologies). The thermal profile was 95 °C for 15 min for hot start, 45 cycles of denaturing at 95 °C for 20 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, followed by a final incubation at 72 °C for 3 min. PCR products were then treated with shrimp alkaline phosphatase (Sequenom) for 40 min at 37 °C to remove unreacted dNTPs. Base extension reaction was carried out with thermosequenase (Sequenom) on 10 μL of shrimp alkaline phosphatase–treated PCR product in a final reaction volume of 14 μL, with 1.54 μmol/L of the extension primer (Integrated DNA Technologies) and a terminator mix of dideoxy/deoxynucleotides, each at 64 μmol/L. The thermal profile consisted of 94 °C for 2 min, followed by a rapid thermocycling for 75 cycles at 94 °C, 52 °C, and 74 °C, all for 5 s. Products were then analyzed by the MassARRAY™ Analyzer Compact Mass Spectrometer (Brucker), a MALDI-TOF system. Details of the PCR primers, termination mix, and extension primers are listed in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol53/issue12.

Thirteen HbE-negative pregnant participants (gestational age: 16–22 weeks) with male partners being HbE carriers were recruited. Genotypes of the couples and the fetuses were confirmed by analysis of buffy coat and cord blood samples, respectively, by the standard homogenous MassEXTEND protocol. Five of the fetuses were shown to be HbE positive.

Genotyping results of the SABER protocol on maternal plasma showed nonspecificity (see Fig. 1 in the Data Supplement). All 8 informative-negative pregnancies were misclassified as positive. To evaluate the important analytical parameters for the nonspecificity of SABER, an artificial model was established. Genomic DNA of a male HbE carrier was mixed with wild-type maternal genomic DNA to mimic the plasma DNA of an affected pregnancy. Direct dilution of wild-type maternal genomic DNA was used to mimic plasma DNA of normal pregnancy. Systematic evaluation revealed that the terminator concentration and the annealing temperature in the base

Table 1. Comparing the percentage of false-positive detection and true-positive detection of SABER at different terminator concentrations (A) and annealing temperatures (B).

A. Effect of annealing temperature in the base extension step on the percentage of positive call.

	52 °C*	54 °C	56 °C	58 °C	60 °C	62 °C	64 °C	66 °C	68 °C	70 °C
True positive	100%	100%	100%	100%	100%	100%	87.5%	100%	87.5%	62.5%
False positive	100%	100%	100%	87.5%	100%	75%	12.5%	0%	0%	0%

B. Effect of terminator concentration on the percentage of positive call.

	Standard	2×	8×	20×	50×	100×
True positive	100%	100%	100%	87.5%	100%	100%
False positive	100%	100%	62.5%	12.5%	0%	0%

[a] The percentage of positive call was calculated similarly as described in Fig. 1B.

extension reaction were critical for the reduction of false-positive detection in SABER. A 50-fold dilution of the standard terminator concentration and annealing temperature at 66 °C were shown to be optimal for both specific and sensitive detection of the HbE mutation in the artificial model (Table 1).

We explored the development of an alternative protocol, ASBER. The 3′ end of the extension primer for ASBER was engineered to be complementary to the fetal mutant allele. Hence, primer extension of the maternal wild-type allele would be inhibited by 3′-primer-template mismatch (Fig. 1A). Initially, the use of a single relevant terminator (ddA only) at standard concentration also revealed non-specificity (data not shown). Thus, terminator dilution and the introduction of competing terminators (ddA, ddC, ddG, and ddT, each at 64 μmol/L) in the ASBER protocol were evaluated. Both the use of competing terminators and 20-fold dilution of the relevant terminator were found to provide specific genotyping results. However, the use of competing terminators appears to offer better detection sensitivity on the artificial model with different percentage mix of mutant DNA (Fig. 1B).

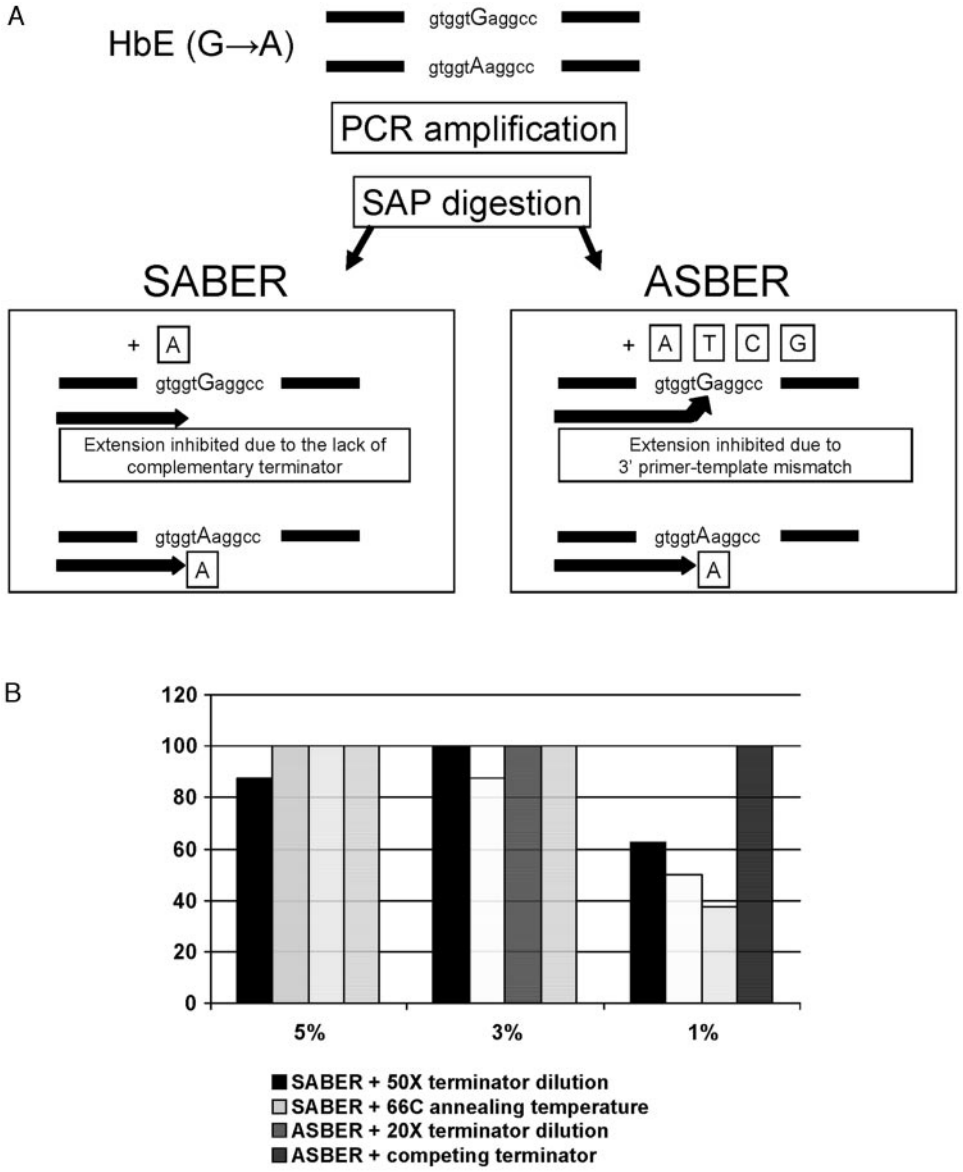

Fig. 1. Comparison between the SABER and ASBER protocols.

(A), Schematic diagram comparing the principles of the SABER and ASBER protocols. The site of the point mutation is indicated in *capital letters*. The *boxed letter* indicates the type of dideoxynucleotide terminator in the base extension reaction. *(B)*, grouped bar chart comparing detection sensitivity of different protocol designs on artificial plasma model with different concentrations. Eight separate PCRs were performed for a particular concentration of artificial mutant and wild-type allele mix followed by 8 separate base extension reactions. The base extension products were then analyzed in duplicate by the MassARRAY™ system. A reaction was scored positive if 1 of the duplicates was called by the TyperAnalyzer software (Sequenom). The sensitivity was then calculated as the percentage of positive detection out of the 8 separate PCRs.

The design of ASBER with competing terminators was adopted to reanalyze the 13 maternal plasma samples, and the fetal genotypes were all correctly determined except 1 case of false-negative result.

In this study, we have shown that for certain mutational context, SABER might generate nonspecific results due to incorporation of the single terminator in the extension reaction mixture despite it being noncomplement to the template PCR product. Although the mechanism behind this nonspecificity or misincorporation is still unclear, we hypothesize that the intrinsic specificity of SABER is a subtle balance between the fidelity of the polymerase and the degree of excess of the single relevant terminator in the primer extension reaction mixture *(9)*. The lower the polymerase fidelity and the higher the terminator concentration promote misincorporation. Based on this reasoning, optimization can be achieved by adjusting the terminator concentration as described above (Table 1A). Others have shown that the polymerase fidelity could be improved by using a proofreading polymerase *(9)*. However, this method did not offer any improvement in the specific detection of the trace amount of fetal DNA in maternal plasma in our preliminary findings (data not shown). On the other hand, we showed that nonspecificity of SABER was reduced by increasing the annealing temperature for base extension (Table 1B). This result might be because the environment becomes less favorable for misincorporation of the noncomplementary terminator.

To overcome the intrinsic tendency of terminator misincorporation in SABER, we explored and developed ASBER based on the principle of allele-specific primer extension, which has been reported to offer successful mass spectrometry–based mutation genotyping *(10, 11)*. However, such design has not been applied to specific fetal allele detection in noninvasive prenatal diagnosis. Compared with previous studies *(10, 11)*, specific priming of the fetal-specific allele in noninvasive prenatal diagnosis is less favorable due to the overwhelming background of maternal allele and the lack of priming competition from alternative allele-specific primer. Yet we expect better specificity in ASBER than the conventional allele-specific PCR *(12)*, because the ASBER extension products would not serve as templates for further amplification. Thus, a misprimed ASBER product, if it occurs, would not be exponentially propagated. Nevertheless, false-positive detection is also detectable in ASBER with the single relevant terminator and therefore required optimization. False-positive detection was not observed in ASBER with competing terminators. This finding further suggested that an overdominance of any single terminator in the extension mixture promotes misincorporation. Further comparison of detection sensitivity of the 2 ASBER protocols shows that improving specificity by reduction of the fractional concentration of a terminator (i.e., inclusion of 3 other types of dideoxynucleotide as competing terminators) is better than dilution of the relevant terminator (Fig. 1B).

Reanalysis of the 13 pregnancies at risk for HbE/β-thalassemia with the ASBER protocol showed substantial improvement in specificity compared with the SABER protocol, with 1 false-negative result. The diagnostic performance of the HbE ASBER assay needs to be confirmed with larger-scale studies.

In summary, we have successfully developed ASBER, which confers advantages in terms of specificity and sensitivity over SABER for the detection of the HbE mutation. Using this approach, noninvasive prenatal diagnosis of the HbE mutation has been achieved with a specificity of 100% (8 of 8) and a sensitivity of 80% (4 of 5). To further improve the sensitivity of the assay, potential fetal allele enrichment steps—for example, PCR clamping by PNA probe and size fractionation—can be included *(13)*. We speculate that the application of the ASBER protocol can be extended to other fields of circulating nucleic acids, for instance, detection of circulating tumor-specific DNA in cancer patients such as *KRAS* point mutations *(14)* and detection of circulating donor-specific DNA in transplant recipients *(15)*.

Grant/funding support: This work was supported by an Earmarked Research Grant (CUHK4395/03M) from the Research Grants Council of the Hong Kong Special Administrative Region, China. Y.M.D.L. is supported by the Chair Professorship Scheme of the Li Ka Shing Foundation.
Financial disclosures: Y.M.D.L. and R.W.K.C. hold patents and have filed patent applications on aspects of the use of fetal nucleic acids in maternal plasma for noninvasive prenatal diagnosis, a proportion of which has been licensed to Sequenom, Inc. Y.M.D.L. is a consultant for Sequenom Inc.

References

1. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Lo YMD, Chiu RWK. Prenatal diagnosis: progress through plasma nucleic acids. Nat Rev Genet 2007;8:71–7.
3. Ding C, Chiu RWK, Lau TK, Leung TN, Chan LC, Chan AY, et al. MS analysis of single-nucleotide differences in circulating nucleic acids: application to noninvasive prenatal diagnosis. Proc Natl Acad Sci U S A 2004;101:10762–7.
4. Li Y, Page-Christiaens GC, Gille JJ, Holzgreve W, Hahn S. Non-invasive prenatal detection of achondroplasia in size-fractionated cell-free DNA by MALDI-TOF MS assay. Prenat Diagn 2007;27:11–7.
5. Li Y, Wenzel F, Holzgreve W, Hahn S. Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation. Electrophoresis 2006;27:3889–96.
6. Orkin SH, Kazazian HH Jr, Antonarakis SE, Ostrer H, Goff SC, Sexton JP. Abnormal RNA processing due to the exon mutation of beta E-globin gene. Nature 1982;300:768–9.
7. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin 1987;11:65–88.
8. Chiu RWK, Poon LL, Lau TK, Leung TN, Wong EM, Lo YMD. Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. Clin Chem 2001;47:1607–13.
9. Di Giusto D, King GC. Single base extension (SBE) with proofreading polymerases and phosphorothioate primers: improved fidelity in single-substrate assays. Nucleic Acids Res 2003;31:e7.
10. Blievernicht JK, Schaeffeler E, Klein K, Eichelbaum M, Schwab M, Zanger UM. MALDI-TOF mass spectrometry for multiplex genotyping of CYP2B6 single-nucleotide polymorphisms. Clin Chem 2007;53:24–33.

11. Higgins GS, Little DP, Koster H. Competitive oligonucleotide single-base extension combined with mass spectrometric detection for mutation screening. Biotechniques 1997;23:710–4.
12. Wu DY, Ugozzoli L, Pal BK, Wallace RB. Allele-specific enzymatic amplification of beta-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci U S A 1989;86:2757–60.
13. Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. JAMA 2005;293:843–9.
14. Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Chen XQ, et al. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology 1997;112:1114–20.
15. Lo YMD, Tein MS, Pang CC, Yeung CK, Tong KL, Hjelm NM. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. Lancet 1998;351:1329–30.

Previously published online at DOI: 10.1373/clinchem.2007.095133

Development and Validation of an Automated Thawing and Mixing Workcell, *Charles D. Hawker,[1,2*] William L. Roberts,[1,2] Antonio DaSilva,[3] Gordon D. Stam,[1] William E. Owen,[4] DeVirl Curtis,[1] Byung-Sang Choi,[5†] and Terry A. Ring[5] ([1] ARUP Laboratories, Salt Lake City, UT; [2] Department of Pathology, School of Medicine, University of Utah, Salt Lake City, UT; [3] Motoman, Inc., Irvine, CA; [4] ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; [5] Department of Chemical and Fuels Engineering, College of Engineering, University of Utah, Salt Lake City, UT; * address correspondence to this author at: ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108; fax 801-584-5261, e-mail hawkercd@aruplab.com); † Current address: Department of Materials Science and Engineering, Gwangju Institute of Science and Technology (GIST), Republic of Korea)*

Background: **Working toward a goal of total laboratory automation, we are automating manual activities in our highest volume laboratory section. Because half of all specimens arriving in this laboratory section are frozen, we began by developing an automated workcell for thawing frozen specimens and mixing the thawed specimens to remove concentration gradients resulting from freezing and thawing.**

Methods: **We developed an initial robotic workcell that removed specimens from the transport system's conveyor, blew high-velocity room temperature air at the tubes, mixed them, and replaced them on the conveyor. Aliquots of citrated plasma were frozen with thermocouples immersed in the tubes, and thawing times and temperatures were monitored. Completeness of mixing of thawed specimens was studied by careful removal of small aliquots from the uppermost layer of the upright tubes without disturbing tube contents and analysis of total protein and electrolytes.**

Results: **High velocity ambient air aimed directly at tubes ranging from 12 × 75 to 16 × 100 mm brought specimens to room temperature in a maximum of 23 min. Adequate mixing of the specimens by the workcell's robot required only 2 approximate 126° movements from an upright starting point, a surprising observation, because laboratorians are usually trained to mix 10 or 20 times. We also observed that, in a frozen overfilled tube, resulting analyte concentrations will be lower because more concentrated solutes leak from the tube.**

Conclusions: **A high-throughput, automated thawing and mixing workcell was successfully built, validated, and installed on our automated transport and sorting system.**

Our laboratory is a high-volume esoteric reference laboratory, accepting approximately 25–30 000 specimens per day. One of our largest laboratory sections, the Automated Core Laboratory, receives approximately 25% of that daily volume, performing more than 140 different chemistry, immunoassay, and specific protein tests, emphasizing cancer antigens, endocrine testing, and urine chemistry, but not routine serum chemistry. More than half of these 6–7000 specimens per day are frozen. The laboratory has been thawing them manually at room temperature (to prevent degradation of labile analytes) by blowing air from an ordinary electric fan at batches of tubes, a process requiring more than 1 h to assure complete thawing. The specimens were then mixed by manually inverting the tubes 10 times, before decapping for the various analyses.

Our long-term objective for this laboratory section is to interface analyzers to our automated transport and sorting system to achieve total laboratory automation and to automate other manual activities such as inspecting for adequate specimen volume and for the presence of interfering substances as indicated by hemolysis, lipemia, and icterus. The development of some form of automated workcell for rapidly thawing specimens at room temperature and mixing the thawed specimens was a 1st step toward our overall automation objective. We are not aware that such a robotic system has previously been built or described.

Our design intention with the workcell was to leave the specimen tubes in their track carriers (see Supplemental Fig. 1 that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol53/issue12). Therefore, we sought to aim the thawing air directly at the tubes in the carrier through a slit normally used for reading bar codes. We developed an experimental apparatus with which we could evaluate a variety of nozzle designs that would direct air into the carrier slit (Supplemental Fig. 2). This design included thermocouples to monitor the thawing times of the tubes. Initially, thawing of frozen water was evaluated, but later, when the conditions of air flow and nozzle design had been established, we tested the system with specimens of out-of-date blood bank plasma. These studies (shown in Supplemental Fig. 3) indicated that a nozzle shaped as a small brass plug with a 2-mm orifice on a beveled edge (Supplemental Fig. 4) aimed at the bottom of the specimen