of heterophile interference in tumor marker assays. This is a particular achievement considering that cancer patients often display tumor-induced activation of their immune system or may suffer from infections. Both of these conditions can lead to polyspecific antibody production. Given sufficient incubation time, these would be bound by blocking reagents, but in modern automated assays, reactions are rarely allowed to reach equilibrium and there may be insufficient time to achieve complete blocking. In addition, modern assays are often configured with several mouse monoclonal antibodies rather than a mouse monoclonal antibody and a polyclonal antibody from another species. With the increasing use of mouse monoclonal antibodies in diagnostic imaging and medical therapy for malignancies, and the resulting immunization of the recipients, the potential for interference increases significantly *(1, 7)* for assays that use multiple mouse monoclonal antibodies. Consequently, we cannot assume that assays other than those included in our study perform equally well for the same analytes. For example, one particular hCG assay continues to experience a considerable number of clinical problems attributable to heterophile interference, representing the majority of such problems reported *(11)*.

There were, however, even in our study two results that followed the typical heterophile interference pattern. In one case, a free PSA result, this would not have led to a change in patient management. By contrast, in the second case, a falsely increased hCG, clinical management might have changed depending on the clinical circumstances. hCG assays, mainly from one particular manufacturer, continue to be the single most important source of erroneous test results attributable to heterophile antibody interference *(11, 12)*. Clinical correlation is therefore particularly important when interpreting hCG results.

In most suspected cases of heterophile interference, HBT treatment is a convenient way to verify and correct the problem. However, our experience shows that for assays that use antibody complexes, specialized HBR reagents should be used. A significant number of difficult to interpret results may otherwise ensue, as might have been the case in a recent publication *(10)*. Laboratories that wish to use HBT pretreatment in suspected cases of heterophile interference therefore need to first explore the effects of pretreatment on analytical performance.

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Detecting the C282Y and H63D Mutations of the *HFE* Gene by Holliday Junction-Based Allele-Specific Genotyping Methods, *Wendy Yang,[2] Takuro Yaoi,[1,2*] Shurong Huang,[2] Qinghong Yang,[2] Sandra Hatcher,[3] Henrietta Seet,[3] and Jeffrey P. Gregg[3]* ([1] Panomics, Inc., Redwood City, CA; [2] FreshGene, Inc., Concord, CA; [3] Department of Pathology, University of California Davis Medical Center, University of California Davis MIND Institute, Sacramento, CA; * address correspondence to this author at: Panomics, Inc., 2003 East Bayshore Road, Redwood City, CA 94063; fax 650-216-9790, e-mail tyaoi@panomics.com)

Hereditary hemochromatosis, one of the most common genetic diseases in Caucasians, is characterized by excessive iron deposition secondary to hyperabsorption of dietary iron and can potentially can lead to multiorgan failure if untreated. The C282Y and H63D mutations of the *HFE* gene are the most common mutations associated with symptomatic hemochromatosis. Recently, several genotyping methods have been used to identify hemochromatosis mutations and other single-nucleotide polymorphisms (SNPs) *(1)*. There are advantages and limitations for each methodology in terms of cost and efficiency *(2)*. We report here the application of a new SNP/point mutation genotyping platform developed by our group, the Holliday junction-based allele-specific genotyping (HAS) platform *(3)*, to identify the C282Y and H63D mutations associated with hemochromatosis.

For the HAS technology, we developed two detection modalities based on differences in the physical and biochemical properties between Holliday junctions (HJs) and duplex DNA or single-stranded DNA. The junctions that form in an allele-specific manner can be detected heterogeneously through gel electrophoresis (acrylamide or agarose; Fig. 1A) or homogeneously through a fluorescence polarization (FP) competition assay *(3)*. Using the HAS genotyping platform, we developed an assay for genotyping the C282Y and H63D mutations with both gel electrophoresis and FP for detection. Five primers (one forward, two reference, and two reverse primers) were designed for each of the two mutations (see Table 1 in

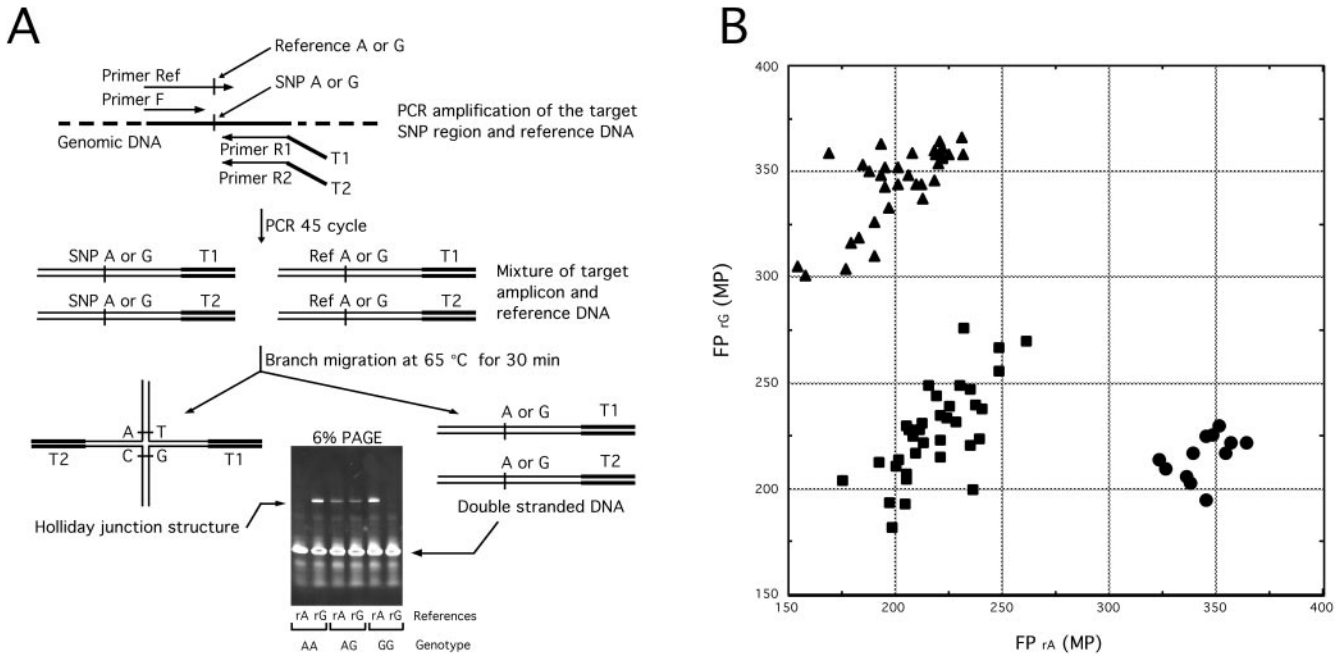

Fig. 1. Summary of the HAS genotyping technology (*A*), and scatter plots for C282Y mutation detection with FP analysis (*B*).

(*A*), SNP detection for the G/A variation is shown. If there is a mismatch at the SNP site between the target PCR amplicon and reference DNA, a stable HJ structure is formed, and the structure can be detected by gel electrophoresis or FP. (*B*), ●, homozygous AA; ■, heterozygous AG; ▲, homozygous GG. The mean (SD) ΔFP values [difference between FP for reference A (*rA*) sample and reference G (*rG*) sample] are 128 (11.9) for homozygous AA, −8.77 (15.8) for heterozygous AG, and −142 (14.7) for homozygous GG. *MP*, millipolarization units.

the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue1/). PCR amplification was performed with a PTC-200 DNA Engine thermocycler (MJ Research, Inc.). For each point mutation locus, two PCR reactions in separate tubes were carried out in parallel for each DNA sample to amplify the target DNA with each of its two DNA references. Each PCR reaction contained four primers [one forward, one reference, reverse tail I, and reverse tail II (Table 1 in the online Data Supplement)] and consisted of 45 cycles of denaturation for 15 s at 94 °C, reannealing at 58 °C for 23 s, extension at 72 °C for 45 s. The cycling was preceded by a 10-min incubation at 95 °C to activate the AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling was immediately followed by incubation at 95 °C for 2 min to denature the DNA, followed by 65 °C for 30 min to facilitate HJ formation (branch migration). The total volume of each reaction mixture was 10 μL. Each reaction mixture contained 1–2 ng of genomic DNA (final concentration, 0.1–0.2 ng/μL), 0.025 U/μL AmpliTaq Gold DNA polymerase, 200 μM each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 ng/μL bovine serum albumin, 0.75 μM desalted forward primer, and 0.25 μM desalted reference primer. For gel-based detection, 0.5 μM of each of the desalted reverse tail primers was included in the reaction mixture. For FP-based detection, 0.25 μM each of the polyacrylamide gel electrophoresis (PAGE)-purified reverse tail primers was included in the reaction mixture.

For PAGE analysis of HJ structures, 5 μL of PCR/branch migration products was mixed with 1 μL of 6× loading buffer and loaded on a 6% 12-well precast Tris-borate-EDTA polyacrylamide gel (Invitrogen, Inc.). Gels were electrophoresed at 200 V for 20 min, stained with SYBR Gold (Molecular Probes), and photographed. For each DNA sample from a patient, the two PCR reactions for each point mutation locus were run in two separate lanes on the gel (Fig. 1A). In a homozygous situation, the lane containing the target DNA and the reference DNA of the same type will not have HJ band, whereas the second lane, which contains the target DNA and the reference DNA of different type, will have a strong HJ band. In a heterozygous situation, both lanes with the two different DNA references will have a weak but visible HJ band. If the DNA is low or degraded, there will be little amplification of the target DNA, and HJs will not form in either of the two lanes.

We used a previously described FP competition assay to detect HJ structures *(3)*. In the FP competition assay, the presence and the amount of HJs are determined via their competition with a fluorescent tracer molecule for binding to RuvA, a protein that specifically binds HJs. If a HJ is present, the FP signal from labeled tracer is lower (200–250 millipolarization units) than with HJ-free condition (~350 millipolarization units). For the FP assay, 190 μmol/L wild-type *Escherichia coli* RuvA in storage buffer [20 mmol/L Tris-HCl (pH7.5), 0.1 mmol/L EDTA, 2 mmol/L 2-mercaptoethanol, 200 mmol/L NaCl, and 500 mL/L glycerol] was purchased from Dr. H. Shinagawa (Osaka University) and kept at −80 °C. We prepared aliquots of 50 μmol/L RuvA diluted in PCR buffer [10

mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 4 mmol/L $MgCl_2$, 200 mg/L bovine serum albumin] and kept them at 4 °C. We prepared the fluorescein-labeled HJ tracer by annealing four aliquots of 18-bp oligonucleotides (25 μmol/L; see Table 1C in the online Data Supplement) in PCR buffer at room temperature for 1 h. PCR/branch migration products were mixed with 10 μL of 0.67 nmol/L fluorescein-labeled tracer before addition of 4 μL of 0.125 μmol/L *E. coli* RuvA protein. After incubation at room temperature for 30 min, the FP of the samples was measured on the Analyst AD plate reader (Molecular Devices, Inc.).

To validate these assays, we obtained DNA from 80 individuals who had previously been genotyped for the C282Y and the H63D mutations by PCR with restriction fragment length polymorphism (PCR-RFLP) analysis *(4)* at the University of California (UC) Davis Medical Center Diagnostic Molecular Pathology Laboratory. DNA samples were anonymized before HAS genotyping, and the studies were approved by the UC Davis Institutional Review Board. We genotyped 80 genomic DNA samples with the newly developed HAS genotyping one-step thermocycling protocol, which allows for PCR and branch migration in a single tube. On the basis of PAGE results for the C282Y mutation (Fig. 1 in the online Data Supplement), we identified 33 samples as homozygous wild-type (G/G), 35 as heterozygous (G/A), and 12 as mutant homozygous (A/A); these results were 100% concordant with the genotyping results obtained by PCR-RFLP analysis. All DNA samples were also genotyped by the FP competition assay (Fig. 1B). Scatter plots of the results of the FP analysis showed clear differentiation of the three versions of the genotypes (AA, AG, and GG). The ΔFP values (difference between FP for reference A sample and reference G sample) gave unequivocal genotyping results for all 80 samples. The FP genotyping results were 100% concordant with both the results obtained by the PAGE-based HAS method (Fig. 1 in the online Data Supplement) and PCR-RFLP results. For the H63D mutation, both PAGE and FP genotyping indicated that 31 samples were homozygous wild type (C/C), 41 were heterozygous (C/G), and 8 were homozygous mutant (G/G; data not shown). These results were 100% concordant with PCR-RFLP genotyping results obtained at the UC Davis Medical Center.

These results demonstrate that the HAS genotyping methods can successfully detect the *HFE* C282Y and H63D mutations. For smaller molecular diagnostic laboratories, the commonly used method for detection of hereditary hemochromatosis-associated mutations is RFLP analysis after gel electrophoresis because of its relative technical simplicity and minimal instrumentation *(2, 5–7)*. From our experience, the gel electrophoresis-based HAS genotyping method has the same desirable attributes of gel-based RFLP analysis in terms of technical simplicity and low cost. Gel electrophoresis-based HAS genotyping is very robust: it works equally well on different thermocyclers, using universal assay conditions, for >95% of all point mutations/SNPs *(3)*, and only desalted and nonlabeled primers are required. Furthermore, gel electrophoresis-based HAS genotyping has advantages over traditional gel-based RFLP analysis. Specifically, it is less time-consuming and more cost-effective because it eliminates the restriction enzyme digestion step. For larger molecular diagnostic laboratories that handle a high volume of tests, homogeneous assay formats may be a better choice because they are more easily automated. Homogeneous testing methodologies typically are based on light emission or quenching and have an initial capital requirement for instruments (i.e., real-time PCR instrument, LightCycler, or fluorescence reader). We have developed a FP-based, homogeneous assay for detecting hereditary hemochromatosis mutations that is fast and easily automated, involves only a very small amount of labeled tracer, requires no enzymes/substrates, and can be carried out under a universal set of assay conditions for genotyping different point mutations. One drawback of many current homogeneous methods (i.e., Invader, TaqMan, FP-IDT assay) *(5–10)* is that they require expensive labeled primers and substrates, which increases the total cost of reagents. Some recently developed homogeneous genotyping technologies have overcome this drawback and do not require labeled primers or substrates, such as melting point-shift genotyping *(11–13)*. Compared with HAS genotyping, which requires five primers, melting point-shift genotyping has the advantage of requiring only two *(12, 13)* or three *(11)* primers. However, HAS genotyping has an advantage over melting point-shift genotyping in that it uses a set of universal assay conditions for genotyping different point mutations or SNPs. The HAS genotyping technology thus allows multiple point mutations or SNPs to be tested in one programmed operation (on a plate or on a microfluidic chip). This has become increasingly important as laboratories search for ways to test for multiple markers, rather than a single marker, in one clinical sample.

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Identification by Mass Spectrometry of a Hemoglobin Variant with an Elongated β-Globin Chain, *Philippe Lacan,[1] Michel Becchi,[2] Isabelle Zanella-Cleon,[2] Martine Aubry,[1] Denis Quinsat,[3] Nicole Couprie,[4] and Alain Francina[1*]* ([1] Unité de Pathologie Moléculaire, Fédération de Biochimie et de Biologie Spécialisée, Hôpital Edouard Herriot, Lyon, France; [2] Institut de Biologie et Chimie des Protéines (CNRS-UMR 5086), Lyon, France; [3] Laboratoire Marcel Mérieux, Lyon, France; [4] Service de Médecine Interne, Centre Hospitalier, Antibes, France; * address correspondence to this author at: Unité de Pathologie Moléculaire, Fédération de Biochimie et de Biologie Spécialisée, Hôpital Edouard Herriot, 69437 Lyon Cedex 03, France; fax 33-472110598, e-mail alain.francina@chu-lyon.fr)

Among more than 800 hemoglobin (Hb) variants currently described in the HBVar database of the Globin Gene Server *(1)*, variants with elongated chains are very rare. Standard protein techniques such as ion-exchange HPLC and isoelectric focusing (IEF) on polyacrylamide gel can detect many Hb variants *(2)*, but correct identification of single mutated, inserted, or deleted amino acid residues requires more sophisticated techniques, such as electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) *(3–5)*. The interpretation of DNA sequencing in the presence of inserted nucleotide sequences in the heterozygous state can be difficult and requires direct and reverse sequencing. Protein analysis by MS can be used to check results from DNA sequencing and also can detect posttranslational changes such as acetylation (NH_2 terminus), deamidation, methionine oxidation, Hb addition products, or artifacts. ESI-MS and MALDI-TOF MS combined with specific tryptic digestion for peptide mass mapping are rapid and sensitive techniques to confirm inserted amino acid residues. We applied these techniques to the identification of a novel Hb variant with a five-amino acid insertion in the β-globin chain. These techniques allowed us to confirm the DNA sequencing results.

Standard Hb analysis was performed by ion-exchange HPLC, IEF on polyacrylamide gel, and reversed-phase (RP)-HPLC of globin chains *(6, 7)*. RP-HPLC was performed with a Vydac C_4 analytical column (The Separations Group) with CH_3CN–H_2O containing 1 mL/L trifluoroacetic acid (TFA) as the mobile phase *(7)*. The same RP-HPLC system was also used for isolation of globin chains for MS studies. Electrospray experiments were performed on a SCIEX API 165 instrument (Applied Biosystems). Solutions were introduced by direct infusion at a flow rate of 5 μL/min. Mass spectra were acquired at a 50-V orifice value in the positive ion mode, and the scan range was set at *m/z* 650-2150 Th (Thompson).

For ESI-MS samples, we prepared a stock solution by diluting the whole blood 50-fold with water and desalting the stock solution by mixing with ~5 mg of AG 50W-X8 cation-exchange resin (Bio-Rad). We diluted 20 μL of the desalted stock solution by adding 40 μL of CH_3OH–H_2O (50:50 by volume) containing 1 mL/L HCOOH, and this solution was used for ESI-MS analysis. MALDI-TOF mass spectra were recorded on a Voyager DE-PRO mass spectrometer (Applied Biosystems) in the 700-5000 Da mass range. Use of a delayed extraction source and reflector instrumentation gave sufficient resolution to detect the monoisotopic peptide masses of $[M + H]^+$ ions.

For MALDI-TOF sample preparation, we dried 0.5 mL of the 2-mL β-globin chain sample collected from RP-HPLC purification in a vacuum concentrator and redissolved the residue in 90 μL of 50 mmol/L NH_4HCO_3 buffer. This solution was digested with 10 μL of trypsin solution (Promega; 0.1 g/L in 50 mmol/L NH_4HCO_3 buffer) for 5 h at 37 °C. We then dried 10 μL of the digest solution in a vacuum concentrator and redissolved it in 10 μL of 1 mL/L TFA. The matrix was a 1 mg (200 μL) solution of α-cyano-4-hydroxycinnamic acid (LaserBioLabs) in CH_3CN–H_2O (50:50 by volume) containing 1 mL/L TFA. We used the dried-droplet method for sample deposition : 1 μL of the 1 mL/L TFA sample solution and 1 μL of matrix solution were mixed on the target and allowed to dry.

DNA studies were carried out by PCR using the following primers: 5′-CAGCTACAATCCAGCTACCATTCTGCT-3′ (forward) and 5′-TAGGCAGAATCCAGATGCTCAAGGCCC-3′ (reverse) *(7)*. Direct sequencing of the PCR products was performed on a Li-Cor 4200 sequencer (ScienceTec) using the same primers labeled with an infrared dye with emission at 700 nm (forward strand) or an infrared dye with emission at 800 nm (reverse strand; MWG-Biotech). Functional studies were made on a Hemox-Analyzer (TCS Scientific Corporation) after stripping of blood hemolysate.

Routine protein analysis showed an abnormal Hb band in the IEF gel that migrated between Hb S and Hb A_2; this band accounted for 22.6% of the total Hb in ion-exchange HPLC. RP-HPLC analysis of the globin chains detected an abnormal peak 20.62 min after the β^A-globin chains (Fig. 1A). ESI-MS analysis revealed a mass of 16307.0 Da (β^X) vs 15868.0 for the β^A-globin chain (mass shift, +439.0). DNA sequencing revealed an insertion of a short nucleotide sequence, GTGTGCTGGCCC, in exon 3 of the β-globin gene. The same sequence was also found in exon 3 of