Allelic discrimination of *cis-trans* relationships by digital polymerase chain reaction: *GJB2* (p.V27I/p.E114G) and *CFTR* (p.R117H/5T)

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Purpose: To distinguish the *cis-trans* relationship of two sequence changes and to arrive at an accurate molecular diagnosis for autosomal recessive disorders, methods such as Sanger sequencing cannot differentiate whether sequence changes are in *cis* or *trans*. In addition, most techniques theoretically appropriate for allelic discrimination depend on the specific identified sequence changes for assay design, need extensive optimization, or may not be suitable. We developed a method that does not fully depend on the specific nucleotide changes. It enables efficient assay design and practical implementation of allelic discrimination. **Methods:** Digital polymerase chain reaction (PCR) was used to separate and amplify alleles. Sanger sequencing was subsequently used to identify sequence changes. **Results:** We developed a cost-effective digital PCR method for allelic discrimination of short amplicons and demonstrated it with p.Val27Ile and p.Glu114Gly in *GJB2* as an example. We also successfully developed a long-range digital PCR approach to determine the *cis-trans* relationship of p.Arg117His and 5T in the *CFTR* gene. **Conclusion:** Digital PCR for allelic discrimination can be clinically implemented to determine the allelic configuration of relatively common sequence changes which frequently appear together and have clinical ramifications, such as the combination of p.Val27Ile and p.Glu114Gly in the *GJB2* gene and p.Arg117His and 5T in *CFTR*. ***Genet Med*** **2011:13(12):1025–1031.**

Key Words: *digital PCR, allelic discrimination, long-range digital PCR, CFTR*, *GJB2*

In autosomal recessive single gene disorders, the allelic configuration of identified sequence changes plays an important role in arriving at the correct molecular diagnosis. Depending on whether two changes are on the same (in *cis*) or on opposite (in *trans*) chromosomes, a patient, respectively, is considered to be a carrier or to have a genotype congruent with being affected. It is also possible, however, that two sequence changes in *trans* are interpreted as benign, whereas the *cis* configuration of these same sequence variants would amount to a single pathogenic allele due to small but additive effects of the two (i.e., rendering the patient a carrier if the other allele is unaffected). Such a dilemma can only be resolved by determining whether the sequence changes are in *cis* or in *trans*, before reporting the results to clinicians and patients. Most commonly used test methods, such as Sanger sequencing, cannot usually by themselves differentiate whether two sequence changes identified in a patient sample are in *cis* or in *trans*. As a result, allelic differentiation can only be accomplished through additional testing.

Allelic discrimination can be accomplished by several strategies. Testing of family members, ideally the parents of a proband, can clarify the allelic relationship of two sequence variants, because the proband inherited one copy of a gene from each parent. It may be difficult, however, to obtain samples from family members and to receive approval from insurance companies for the testing of unaffected individuals. Direct analysis of the sample from the proband is a more viable solution. Not only is it more likely that the cost would be covered because the proband is the individual to whom the testing directly applies, but time and cost could also be reduced. Restriction enzyme digestion[1] and allele specific polymerase chain reaction (PCR)[2,3] are examples of methods that have been used to determine allelic configurations. These methods, respectively, detect and separate two alleles by restriction digestion and by selective amplification of the specific sequence changes. Drawbacks of these approaches include the possibility that a restriction enzyme may not be available for the sequence change of interest, and that designing optimal primers for allele specific PCR can be challenging in certain gene regions. Using methods that do not fully depend on specific sequence change(s) has the advantage of assay design flexibility. Cloning of PCR products containing both mutations is one such alternative, but cloning is very labor intensive and not practical in the molecular diagnostic setting. Another approach is based on digital PCR, which can clonally amplify each copy of the template through partitioning of the input templates into multiple PCR reactions, by which each allele is separated. Although it was initially developed for quantitation of target templates[4,5] and later used to detect mutations in tumor samples,[6] amplification of single copy templates can easily be adapted to enable determination of *cis-trans* relationships. This has been suggested before[6] but was never commonly applied to molecular testing because of the original need for expensive probes for amplification detection. We now report the application of efficient digital PCR methods that can be practically implemented for clinical diagnosis: one for shorter amplicons (p.Val27Ile/p.Glu114Gly in *GJB2*) and one more challenging, involving long-range digital PCR (p.Arg117His/5T in *CFTR*).

Mutation analysis of the *GJB2* gene in hearing loss (DFNB1A: autosomal recessive nonsyndromic congenital deafness, locus 1A; MIM ID #220290) and of *CFTR* in cystic fibrosis (MIM ID #219700) are illustrations of molecular tests for autosomal recessive disorders. Up to 50% of individuals with inherited nonsyndromic autosomal recessive hearing loss have mutations in *GJB2*.[7,8] These occur throughout the gene. The sequence changes that we used in our study, p.Val27Ile and p.Glu114Gly, occur most often in *cis*,[3] with evidence of a high population frequency of standalone p.Val27Ile alleles and a very low frequency of separate p.Glu114Gly alleles.[9,10] The coding region of the gene

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spans only one exon and the two changes are relatively close. The clinical significance of the combination of the two variants, however, is still controversial, because different in vitro model systems were used in contradictory functional studies.[3,11,12] One of the postulated hypotheses was that both variants were benign if they were in *trans*, but pathogenic if present in *cis*.[3] It was speculated that the *cis* configuration may have incomplete clinical penetrance, similar to another *GJB2* variant, p.Val37Ile.[13]

The long-range digital PCR approach was applied to cystic fibrosis, the most common life-limiting autosomal recessive disorder in the Caucasian population, caused by mutations in the *CFTR* gene. The American College of Medical Genetics and the American College of Obstetrics and Gynecology recommended 23 *CFTR* mutations for cystic fibrosis carrier screening.[14,15] p.Arg117His is one of these. When this change is identified, reflex testing of the poly T track in intron 8 must be completed because presence of the 5T allele (in contrast to the more common 7T and 9T alleles) reduces splicing efficiency of exon 9, and when present in *cis* with p.Arg117His culminates in what is considered a *CFTR* mutation that could result in cystic fibrosis if paired with a pathogenic mutation on the opposite gene.[16–20] p.Arg117His and the poly T track are separated by several exons and introns, spanning a distance of 17.6 kb.

MATERIALS AND METHODS

Genomic DNA samples

Patient samples were de-identified and collected under the Stanford University Institutional Review Board on Human Subjects Research approval with waived consent. Patients P1 to P9 had received *GJB2* testing by Sanger sequencing. P1 to P8 were heterozygous for both p.Val27Ile and p.Glu114Gly. P9 was heterozygous for p.Val37Ile and 299_300delAT. Patient P10 who underwent *CFTR* testing was heterozygous for p.Arg117His and 5T at the poly T site of intron 8. Genomic DNA was extracted from peripheral blood using the PUREGENE® Blood Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's instructions. The gDNA concentration was measured by NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Cell line NA13591 with a *CFTR* genotype of p.Phe508del/p.Arg117His/5T (p.Phe508del on one allele and p.Arg117His and 5T on the opposite allele) was purchased from Coriell cell repositories (Coriell Institute for Medical Research, Camden, NJ). Sequencing was completed on an ABI 3130 instrument (Applied Biosystems, Foster City, CA).

Allelic discrimination by restriction enzyme digestion

A 431-base pair (bp) fragment of *GJB2* that contains p.Val27Ile and p.Glu114Gly was amplified using gDNA from patients P1 to P8, who underwent molecular testing of *GJB2* by sequencing. PCR reactions were carried out in a 25-μL reaction with 1× AmpliTaq 360 buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 300 nM each of the primers (forward primer: 5′-AGAGTAGAAGATGGATTGGGGC-3′ and reverse primer: 5′-AGATGCTGCTTGTGTAGGTCCA-3′), 0.1 u/μL of AmpliTaq Gold® 360 polymerase (Applied Biosystems), and 8 ng of gDNA template. PCR was performed on a PTC-200 DNA Engine Cycler (MJ Research, Waltham, MA) at 95°C for 10 minutes, followed by 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, with an extension cycle of 72°C for 7 minutes. Ten microliters of the PCR reaction was used for 30 μL of restriction enzyme digestions. First, HypCHIII (New England Biolabs, Ipswich, MA) was digested for 30 minutes at 37°C, followed by 20 minutes at 80°C to inactivate the enzyme. Then, ApoI (New England Biolabs) was added into the reaction for 1 hour at 50°C. Fifteen microliters of each digestion reaction was run on a 3% agarose gel.

Real-time PCR quantification of gDNA

All the gDNA samples to be used for digital PCR were quantified by real-time PCR analysis using TaqMan® probes for RNaseP (Applied Biosystems). The human gDNA control provided in the RNase P quantification kit was used to generate the standard curve (0.6, 1.2, 3, 6, and 12 ng). Real-time PCR was performed in a 10-μL reaction, containing 1× TaqMan® Gene Expression Master Mix, 1× RNase P primer-probes (Applied Biosystems) and gDNA template. PCR conditions were followed as suggested by the manufacturer.

Allelic discrimination of p.Val27Ile and p.Glu114Gly by digital PCR

For each digital PCR, an average of 0.5 copy of gDNA per well was distributed into 45 wells. Each well on a 384-well plate contained 1× MeltDoctor™ HRM Master Mix and 300 nM of each primer that was the same as described earlier in "Allelic discrimination by restriction enzyme digestion." PCR was carried out on a 7900HT real-time PCR system (Applied Biosystems) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, with the addition of a dissociation curve after the last cycle. Amplification of the digital PCR products was directly visualized under UV light (AlphaImager, Alpha Innotech, San Leandro, CA), by comparing the assay wells to no template control (NTC) wells. For wells with amplification, PCR products were diluted 1:20, and 1 μL was used directly for Sanger sequencing (Applied Biosystems). To determine the allelic status of the variants, five wells with amplification were chosen for sequencing because for a heterozygous variant, each well that contains one copy of the template would have a one in two chance to either be the variant or to be the wild-type (WT) sequence. Sequencing five wells would have a probability of $(1/2)^5$ to obtain results from only one of the two alleles, thus the probability of having the sequencing results from these five wells reflecting the two alleles of interest would be about 97% (i.e., $1 - (1/2)^5$). The same digital PCR conditions were also applied for the allelic discrimination of p.Val37Ile and p.His100fs (c.299_300delAT) in *GJB2* because both pairs of sequence variants are within the same amplicon.

Allelic discrimination of p.Arg117His/5T by long-range digital PCR

Long-range digital PCR was performed on a PTC-200 DNA Engine Cycler (MJ Research) in 40 wells. Seven of the 40 wells contained NTC mix and the rest had digital PCR mix. Two positive controls were included in the reaction, with the addition of 0.5 ng of the test gDNA into the NTC mix and the digital PCR mix. This was to make sure that the NTC and digital PCR mix had all the components for long-range digital PCR amplification, except gDNA. Each NTC PCR well contained 50 μL of 1× LongAmp Taq reaction buffer, 300 μM dNTPs, 1.5 mM of $MgSO_4$, 400 nM of each primer (forward: 5′-AGTCTCCTCTAAAGATGAAAAGTCTTGTGTT-3′ and reverse: 5′-AGAGACATGGACACCAAATTAAGTTCTTAATAGT-3′) and 0.2 u/μL of the LongAmp Taq DNA polymerase (NEB, Ipswich, MA). Each digital PCR mix contained all the components of the NTC and an average 0.5 copy of the test gDNA. The digital long-range PCR was performed at 94°C for 30 seconds, followed by 10 cycles of 94°C for 10 seconds, 62°C

for 30 seconds, and 65°C for 20 minutes. This was followed by 35 cycles of 94°C for 10 seconds, 62°C for 30 seconds, and 65°C for 20 minutes, with a 20-second increase of extension in each cycle. Two microliters of the PCR reaction in each well were run on a 0.4% agarose gel with a GeneRuler high range DNA ladder (Fermentas, Glen Burnie, MD). First, electrophoresis was performed without Ethidium bromide at 50 V for 2 hours and then at 30V for an additional half an hour with the addition of Ethidium bromide. The image was taken using an AlphaImager (Alpha Innotech, San Leandro, CA). After a 1:10,000 dilution of the first round PCR products, eight wells with successful long-range amplification were chosen for the second round of PCR. The second round of long-range PCR was also performed in a 50-μL reaction under the same conditions, except with 1 μL of the diluted first round digital PCR products as DNA template, 2 mM of $MgSO_4$, a 63°C annealing temperature, and 41 cycles of 94°C for 10 seconds, 62°C for 30 seconds, and 65°C for 20 minutes, with a 20-second increase of extension in each cycle. Two microliters of the eight wells were run on a 0.4% agarose gel. Five wells with the best amplification were chosen for sequencing with four sequencing primers, CF-117-F-S (5′-TGTTGAAATTCTCAGGGTATTTTA-3′) and CF-117-R-S (5′-TCCTGCCATTTATTTAATAGGCATATTA-3′) for p.Arg117His, and CF-pT-F-S (5′-TGTAATGGATCATGGGCCATGT-3′) and CF-pT-R-S (5′-TTGCCTGCTCCAGTGGATCCA-3′) for 5T/7T/9T. As described earlier, the probability of having results from these five wells reflecting the two alleles of interest was approximately 97% (i.e., $1 - (1/2)^5$).

RESULTS

Restriction enzyme digestion for allelic discrimination of p.Val27Ile and p.Glu114Gly in *GJB2*

Restriction enzyme analysis was used to determine the allelic configuration of each patient sample heterozygous for both p.Val27Ile and p.Glu114Gly, as detected by Sanger sequencing. As indicated in Figure 1A, WT *GJB2* contains the unique restriction enzyme digestion sites of HpyCH4III and ApoI at amino acids position p.Val27 and p.Glu114, respectively. With the sequence changes of the Val to Ile at amino acid position 27, and the Glu to Gly at amino acid position 114, the corresponding restriction enzyme digestion sites were abolished. Therefore, restriction digestion of HpyCH4III and ApoI would yield 264-, 87-, and 80-bp fragments for a WT allele, 344- and 87-bp fragments for a heterozygous p.Val27Ile allele, 351- and 80-bp fragments for the heterozygous p.Glu114Gly allele, and a 431-bp fragment for a heterozygous p.Val27Ile and p.Glu114Gly allele. Different restriction enzyme digestion patterns are generated depending on the *cis-trans* relationship of the heterozygous p.Val27Ile and p.Glu114Gly. Thus, the *trans* configuration would generate fragments of 344 and 87 bp (allele p.Val27Ile), as well as fragments of 351 and 80 bp (allele p.Glu114Gly), whereas the *cis* configuration would demonstrate a 431-bp fragment for allele p.Val27Ile and p.Glu114Gly, and fragments of 264, 87, and 80 bp, reflecting the opposite WT allele. Figure 1B demonstrates that all patients (P1 to P8) generated the 431, 264, and 87 with 80-bp fragments, representing a WT allele and another allele with both p.Val27Ile and p.Glu114Gly in *cis*. The faint band between 431 and 264 bp in patients P1 to P8 may be

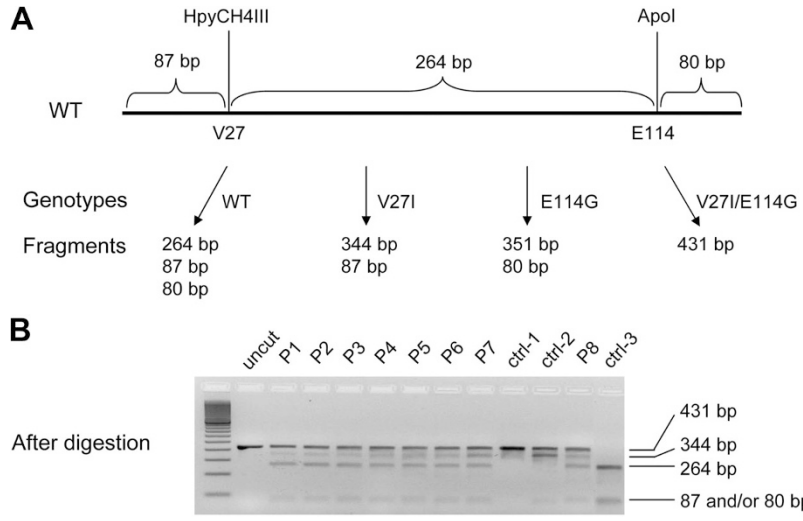

Fig. 1. Restriction enzyme digestion to determine the *cis-trans* relationship of p.Val27Ile and p.Glu114Gly in *GJB2*. A, Restriction map of the 431-bp fragment that contains p.Val27Ile and p.Glu114Gly. The WT allele has a unique restriction pattern created by HpyCH4III on p.Val27Ile and ApoI on p.Glu114Gly. The size of each restriction fragment is indicated. After HpyCH4III and ApoI digestion, the WT allele generates 264-, 87-, and 80-bp fragments; the p.Val27Ile (V27I) allele generates 344- and 87-bp fragments; the p.Glu114Gly (E114G) allele produces 351- and 80-bp fragments; alleles with both p.Val27Ile (V27I) and p.Glu114Gly (E114G) abolish both restriction enzyme sites and therefore have the full-length PCR products of 431 bp. B, Restriction enzyme digestion of the 431-bp PCR fragments from patient samples. P1 to P8 were heterozygous for p.Val27Ile and p.Glu114Gly by Sanger sequencing. Ctrl-1 was homozygous for p.Val27Ile and p.Glu114Gly, presenting with only a 431-bp fragment; ctrl-2 was homozygous for p.Val27Ile and heterozygous for p.Glu114Gly, generating fragments of 431, 344, and 87 bp; ctrl-3 was WT for both alleles and generated 264-, 87-, and 80-bp fragments. Uncut represents the 431-bp fragment without restriction digestion. The weak band in between 431 and 264 bp in P1 to P8, and ctrl-1 was explained by star activity of the restriction enzyme.

attributed to star activity of the restriction enzymes because the homozygous p.Val27Ile/p.Glu114Gly (ctrl-1) had the same band, which was not indicated by the restriction map of these sequences.

Digital PCR to determine the allelic configuration of p.Val27Ile and p.Glu114Gly in *GJB2*

After real-time PCR quantitation of the gDNA, digital PCR of each sample was performed in 45 wells with an average of 0.5 copy gDNA in each well. Five wells with amplification signal (i.e., strong fluorescent signal under UV light, Fig. 2A) were sequenced to determine the allelic location of p.Val27Ile and p.Glu114Gly in our samples (Fig. 2B). For example, digital PCR of P1 gDNA generated seven amplified wells, B9, B12, C7, C12, D6, D8, and D11 (Fig. 2A). Sequencing of five wells, B12, C7, C12, D6, and D8, indicated that two wells (B12 and C7) contained only WT allele (p.Val27/p.Glu114), whereas three wells (C12, D6, and D8) carried both sequence changes on the same allele (p.Ile27/p.Gly114). Therefore, heterozygous p.Val27Ile and p.Glu114Gly are in *cis* for P1. Digital PCR of the other seven patient samples, P2 to P8, generated the same results as P1 (data not shown).

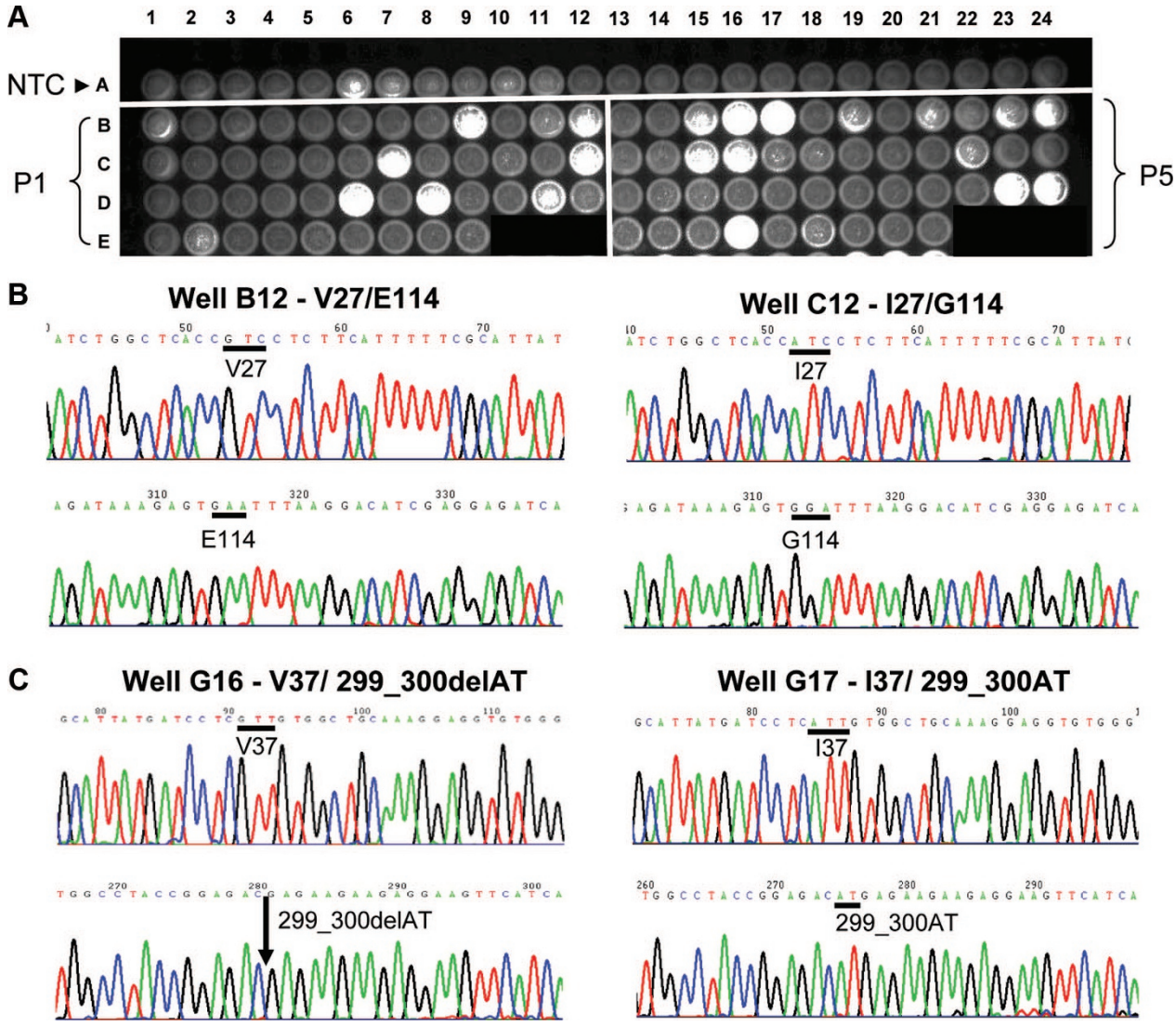

Fig. 2. Digital PCR to determine the allelic configuration of two sequence changes in *GJB2*. A, Example of a plate image of the digital PCR results. Only two patients, P1 and P5, were included in the example although digital PCR of all 8 patients were performed on a 384-well plate. Each patient covered 45 wells. Row A was for the NTC only. From column 1 to 12, rows B to E were used for P1; from column 13 to 24, rows B to E were used for P5. Wells with PCR amplification indicated strong fluorescent signal (white color image), whereas wells with no amplification had a dim background signal. B, Example of the sequencing results of well B12 and C12 for P1. Sequencing indicated that well B12 contained template that had the WT allele with p.Val27 (V27) and p.Glu114 (E114); well C12 contained template that had an allele with both p.Ile27 (I27) and p.Gly114 (G114). C, Example of the sequencing results of wells G16 and G17 for P9. Sequencing indicated that well G16 contained template that had only p.His100fs (c. 299_300delAT); well G17 contained template that had an allele with p.Ile37 (I37). Thus, these mutations are on opposite chromosomes.

Digital PCR to determine the allelic configuration of p.Val37Ile and p.His100fs (c.299_300delAT) in *GJB2*

Because digital PCR can separate two alleles of the same gene into different PCR reactions, it is also possible to test whether any other two mutations detected in the same 431-bp fragment are in *cis* or *trans*. Patient P9 was heterozygous for p.Val37Ile and p.His100fs (c.299_300delAT) which were both previously detected by Sanger sequencing. With the combination of a missense (p.Val37Ile) and a frameshift (p.His100fs, c.299_300delAT) mutation in the same amplicon, analysis of the electropherogram could determine that these two mutations were in *trans* (data not shown). After digital PCR of P9, sequences derived from two separate digital PCR wells (G16 and G17) are included in Figure 2C. Well G16 contains p.Val37 and p.His100fs (c. 299_300delAT), and well G17 has sequences for p.Ile37 and WT between nucleotides 299 and 300. The digital PCR result was consistent with the conclusion, based on Sanger sequencing, that p.Val37Ile and p.His100fs (c. 299_300delAT) were in *trans*.

Long-range digital PCR to determine the allelic configuration of p.Arg117His and 5T in *CFTR*

The distance between p.Arg117His and the poly T track (5T/7T/9T) of *CFTR* is about 17.6 kb (Fig. 3A). Digital PCR primers (CF-F and CF-R) were designed to include both p.Arg117His and the poly T track in the same amplicon. To be able to generate reproducible sequencing results from the digital PCR products, two rounds of PCR were performed to increase specificity. First, long-range digital PCR was performed to separate the two alleles in different PCR reactions. Then, the second round of PCR was performed with a higher annealing temperature, using a 1:10,000 dilution of the first round digital PCR products. Because Sanger sequencing from one direction cannot sequence through an 18-kb fragment, two sequencing primers were designed for each sequence change, CF-117-F-S and CF-117-R-S for p.Arg117His and CF-pT-F-S and CF-pT-R-S to capture the poly T track. To improve the specificity of the sequencing reactions, both sets of primers were nested primers of the long-range PCR primers.

Subsequently, to optimize the long-range digital PCR conditions for allelic discrimination, a gDNA sample (NA13591 from Coriell cell repositories) with known *CFTR* sequence changes was tested. The gDNA was derived from a patient who had one allele with p.Phe508del/9T and another allele with p.Arg117His/5T. The first round of the digital PCR was performed in 40 wells, seven of which were reserved for NTCs. Eight digital PCR wells with amplification of the expected product size were chosen for the second round of PCR (data not shown). Five of the second round PCR products with good amplification were sequenced. Sequencing results indicated that two of the wells had a genotype of p.Arg117/9T and the rest carried p.His117/5T. As an example in Figure 3B, digital PCR well 13 contained the template with p.Arg117/9T whereas well 28 contained the template with p.His117/5T. Therefore, the long-range digital PCR successfully separated the two alleles in different long-range PCR reactions and generated correct results.

Another gDNA with unknown p.Arg117His/5T configuration from patient P10 was tested. Figure 3C shows sequences of the second round PCR products from digital PCR wells 16 and 32 of the long-range digital PCR. Of the five second round PCR products sequenced, one had p.His117 in *cis* with 7T and four had p.Arg117 in *cis* with 5T. Thus, in this case, p.His117 was in *trans* with 5T.

DISCUSSION

We used digital PCR to directly analyze the *cis-trans* relationship of pairs of heterozygous sequence changes in two genes. For short fragments such as those containing p.Val27Ile/p.Glu114Gly in the *GJB2* gene, our method is as fast and straight forward as regular sequencing analysis (Fig. 2, A and B). With the addition of the double-stranded DNA binding dye in digital PCR reactions, wells with amplification can be visualized directly under UV light, without running the PCR products of each digital well on an agarose gel and without the need to use expensive probes to detect the PCR products.[5,6] For a larger 18 kb fragment, which contained both locations of p.Arg117His and the poly T site in the *CFTR* gene, we developed a long-range digital PCR method to amplify the target sequence. Two rounds of PCR amplification were conducted for allelic typing: a first round of long-range digital PCR and a second round of long-range PCR. Thus, we have successfully developed digital PCR methods for allelic discrimination of both short and large fragments.

Two modifications of the typical digital PCR approach were critical in our methods: we added a binding dye for double-stranded DNA and performed two rounds of PCR for short and long amplicons, respectively. This simplified the assay design for short amplicons when compared with previous reports[5,6] and made the allelic typing more robust for long amplicons. Because the dye can bind to any double-stranded DNA, however, visualization of DNA amplification with this dye is not necessarily specific. It is important to design the PCR with excellent specificity and minimal primer-dimmer formation. If the digital PCR for short amplicons were to be run on real-time PCR instruments, melt curve analysis could be added to differentiate the fluorescent signal of specific products from primer dimers because they usually have a different melting temperature (T_m). Given that a conventional long-range PCR is more difficult to perform than PCR of short fragments and usually requires about 50 to 100 ng of human gDNA templates (i.e., 16,667 to 33,333 copies[21]) in each reaction,[2,22] development of our long-range digital PCR (i.e., single copy template in each reaction) approach was much more challenging. To our knowledge, this may be the first reported long-range digital PCR assay for human gDNA, which could be implemented in a clinical setting. For the long-range digital PCR method, all digital wells were run on an agarose gel after initial amplification to identify the wells with amplicons and to make sure that the long-range digital PCR generated specific PCR products of the expected size. Each template gDNA was also electrophoresed on an agarose gel to confirm that the gDNA was intact and suitable for long-range PCR amplification, that is, the size of the gDNA templates has to be greater than the targeted long-range PCR products. Because direct sequencing of the long-range digital PCR products failed to generate consistently reliable sequencing results, second round PCR reactions with a higher annealing temperature were performed before sequencing, which generated quality sequencing data. Because the templates for the second round of PCR were diluted from the selected amplified digital PCR wells, there may be a concern of cross-contamination between different wells during second round PCR. This can be addressed by examining the sequencing results. If cross-contamination ever occurs, the site that has a specific sequence change would be heterozygous. In addition, the sequencing of five different wells in one digital PCR experiment inherently builds redundancy for allelic discrimination and serves as an internal measure of quality control. Cross-contamination between different digital wells has not been observed in our hands.

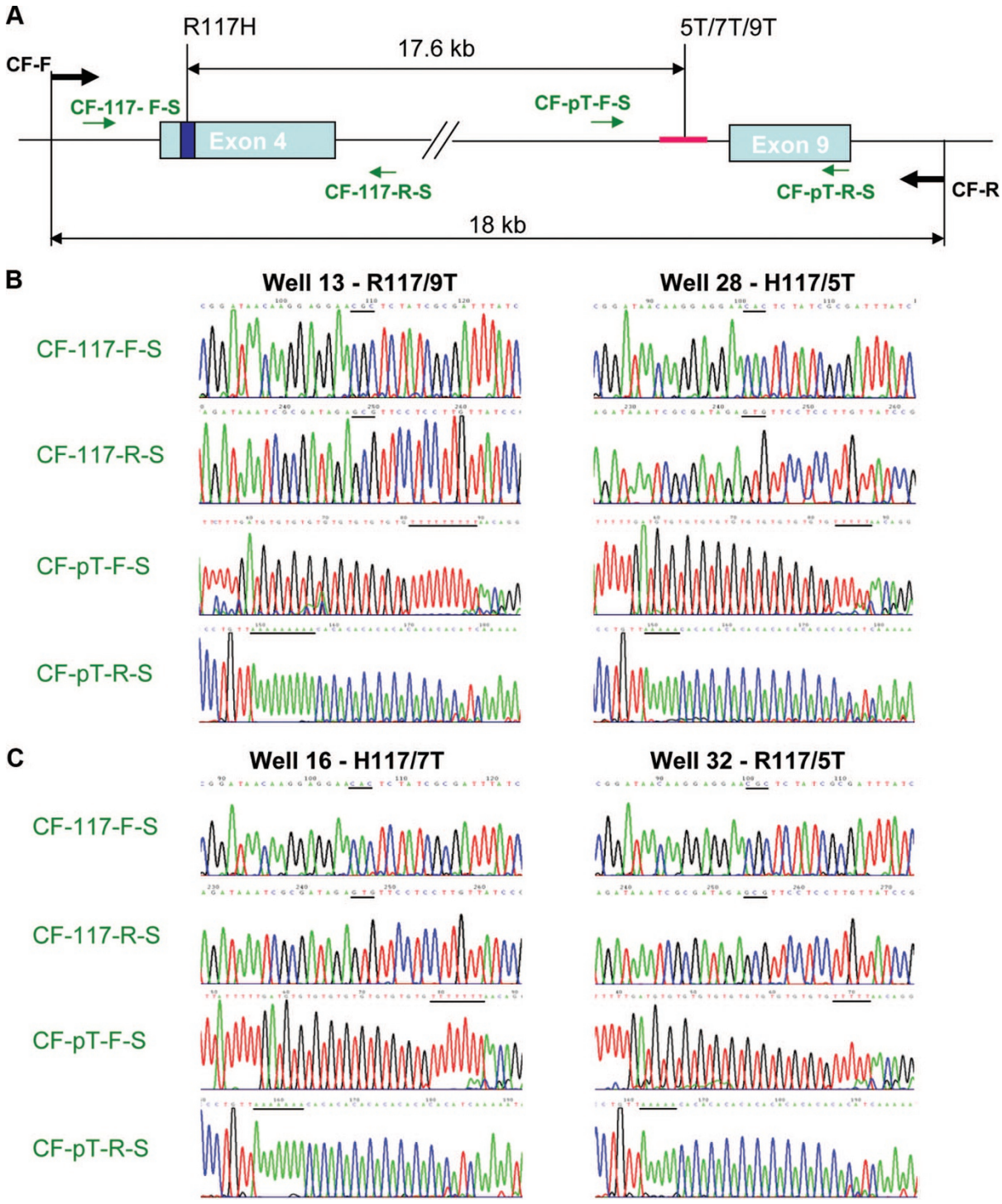

Fig. 3. Long-range digital PCR to determine whether p.Arg117His and poly T (5T/7T/9T) in *CFTR* are in *cis* or in *trans*. A, Schematic diagram of the p.Arg117His (R117H) and poly T (5T/7T/9T) allele in *CFTR*. p.Arg117His is in exon 4. The polyT region is in intron 8. Two primers were designed for the long-range 18-kb PCR fragment, CF-F and CF-R. To increase the specificity, nested primers (CF-117-F-S with CF-117-R-S for p.Arg117His and CF-pT-F-S with CF-117-R-S for the polyT region) were used in Sanger sequencing. B, Example of the sequencing results of the second round PCR after initial digital PCR of wells 13 and 28 for cell line gDNA sample NA13591. Sequencing indicated that well 13 contained a template that had both p.Arg117 (R117) and 9T; well 28 contained a template that had an allele with both p.His117 (H117) and 5T. C, Example of the sequencing results of the second round PCR after initial digital PCR for patient P10. Sequencing indicated that P10 had two alleles, one with p.His117 (H117) and 7T (well 16) and another with p.Arg117 (R117) and 5T (well 32).

To perform a successful digital PCR for allelic separation, two factors have been taken into consideration, accurate quantitation of the copy number of the individual gDNA and the total copy number of the individual gDNA added in the digital PCR. We used real-time PCR with a standard curve quantification method to quantitate the gDNA copy numbers and to achieve a much better dynamic range for quantitation than with spectrophotometer O.D. readings. To determine the number of wells for each digital PCR, we first assessed whether it is experimentally possible to pipette a low copy number of templates from an individual gDNA sample. Our evaluations using the Poisson distribution indicated that the probability of pipetting at least five copies (i.e., the level at which five copies would most likely distributed into five different digital wells, which can then be selected for sequencing) would be 97% if the intention was to pipette 10 copies into the digital PCR mix. Therefore, a minimum of 10 copies in each digital PCR experiment would generate enough amplified wells (at least five) for sequencing at a high probability (i.e., 97%). Thirty-three wells in one digital PCR, with an average of 0.5 copy in each digital well, contain a total of 16.5 (0.5×33) copies of gDNA template. If the gDNA quantitation is 40% more or less, the intended 16.5 copies would range from 10 to 23 copies, which would result in an average of 0.3 to 0.7 copies in each digital well. Within the range, an individual gDNA sample will generate enough amplified wells for allelic discrimination. Therefore, 33 wells of digital PCR would allow 40% variation in gDNA quantification. For this reason, we considered 33 wells to be our minimum number of wells for the digital PCR, with a potential 40% fluctuation in gDNA quantitation. We used 33 wells for long-range digital PCR. For the short amplicons, 33 wells would have sufficed, but we used 45 wells in our assay just because we had space on the plate.

The benefit of our digital PCR method is that it enables the direct analysis of the *cis-trans* relationship of sequence changes identified in the gDNA of a proband, without requesting samples from family members. In addition, such direct analysis can generate a valid allelic typing result when one of the sequence changes is new (not inherited from a parent) which would be inconclusive from family studies alone. As the digital PCR separates the different alleles into different amplicons, any sequence changes within the digital PCR fragment are easily identified by subsequent sequencing. In the case of poly T analysis in *CFTR*, Sanger sequencing of the digital PCR products also cleanly indicates the length of the preceding TG tract on the 5T allele, which influences the likelihood of pathogenicity of the allele.[23] Our method can be used to determine the *cis-trans* relationship of any sequence changes within the targeted digital PCR fragments (Fig. 2C). For *GJB2*, variants, p.Val27Ile and p.Glu114Gly were detected in *cis* in all the patients from our study and others[3]; however, the low frequency of p.Glu114Gly in the population[9,10] cannot exclude the possibility that a heterozygous genotype of p.Val27Ile and p.Glu114Gly could be in *trans*. Therefore, determination of the *cis-trans* relationship of the variants remains necessary for definitive clinical interpretation.

Since the initial suggestion by Vogelstein and Kinzler[6] of using digital PCR for allelic discrimination, there had not been widespread adoption toward molecular assay development, except for quantitation purposes.[24,25] This is most likely due to the great variety of possible combinations of different sequence changes for an individual patient. Developing individual digital PCR primers for routine allelic typing of each set of identified variants is not practical for clinical diagnostic laboratories. However, it would be feasible to establish and clinically implement practical digital PCR assays to determine the allelic configuration of relatively common sequence changes which frequently appear together and have known clinical ramifications, such as the combination of p.Val27Ile and p.Glu114Gly in the *GJB2* gene and p.Arg117His and 5T in *CFTR*.

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