Digital PCR for the molecular detection of fetal chromosomal aneuploidy

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Trisomy 21 is the most common reason that women opt for prenatal diagnosis. Conventional prenatal diagnostic methods involve the sampling of fetal materials by invasive procedures such as amniocentesis. Screening by ultrasonography and biochemical markers have been used to risk-stratify pregnant women before definitive invasive diagnostic procedures. However, these screening methods generally target epiphenomena, such as nuchal translucency, associated with trisomy 21. It would be ideal if noninvasive genetic methods were available for the direct detection of the core pathology of trisomy 21. Here we outline an approach using digital PCR for the noninvasive detection of fetal trisomy 21 by analysis of fetal nucleic acids in maternal plasma. First, we demonstrate the use of digital PCR to determine the allelic imbalance of a SNP on PLAC4 mRNA, a placenta-expressed transcript on chromosome 21, in the maternal plasma of women bearing trisomy 21 fetuses. We named this the digital RNA SNP strategy. Second, we developed a nonpolymorphism-based method for the noninvasive prenatal detection of trisomy 21. We named this the digital relative chromosome dosage (RCD) method. Digital RCD involves the direct assessment of whether the total copy number of chromosome 21 in a sample containing fetal DNA is overrepresented with respect to a reference chromosome. Even without elaborate instrumentation, digital RCD allows the detection of trisomy 21 in samples containing 25% fetal DNA. We applied the sequential probability ratio test to interpret the digital PCR data. Computer simulation and empirical validation confirmed the high accuracy of the disease classification algorithm.

The detection of fetal trisomy 21 (T21) is an important indication for prenatal diagnosis. The sampling of fetal materials by amniocentesis and chorionic villus sampling are invasive, with a finite risk of fetal loss (1). A variety of screening methods, such as ultrasound, have been investigated (2). However, these screening methods typically target T21-related epiphenomena instead of the core chromosomal abnormality and thus have suboptimal diagnostic accuracy and disadvantages, such as being highly influenced by gestational age.

The discovery of cell-free fetal DNA in maternal plasma in 1997 offered new possibilities for noninvasive prenatal diagnosis (3, 4). This method has been readily applied to sex-linked (5) and certain single-gene (6, 7) disorders, but its use for fetal chromosomal aneuploidies has been a challenge (4). First, fetal nucleic acids coexist in maternal plasma with a high background of maternal nucleic acids that can often interfere with analysis (8). Second, fetal nucleic acids circulate in maternal plasma in a cell-free form, making it difficult to derive chromosome dosage information. Significant developments have recently been made (9–11). One approach focuses on the detection of nucleic acid species that are fetal-specific, including DNA fragments with a placenta-specific DNA methylation pattern (10, 12) and RNA molecules expressed by the placenta (9). Because circulating fetal nucleic acids are mainly derived from the placenta, the problem of maternal background interference can be overcome by targeting such molecules in maternal plasma (4). Dosage of chromosome 21 (chr21) is then inferred from the ratios of polymorphic alleles in the placenta-derived DNA/RNA molecules. However, the dependence on genetic polymorphisms limits the use of these approaches to heterozygous fetuses.

It would be ideal if a noninvasive test for fetal T21 detection based on circulating fetal nucleic acid analysis were not dependent on the use of genetic polymorphisms. Theoretically, even with the small fractional concentration of fetal DNA (8), a T21 fetus would contribute an additional dose of chr21 sequences per genome equivalent (GE) of fetal DNA released into maternal plasma. For example, a maternal plasma sample from a euploid pregnancy containing 50 GE per milliliter of total DNA with 5 GE per milliliter of DNA contributed by the fetus (i.e., 10% fetal DNA) should contain a total of 100 copies (90 maternal copies plus 10 fetal copies) of chr21 sequences per milliliter of maternal plasma. For a T21 pregnancy, each fetal GE would contribute three copies of chr21, resulting in a total of 105 copies (90 maternal copies plus 15 fetal copies) of chr21 sequences per milliliter of maternal plasma. At 10% fetal DNA concentration, the amount of chr21-derived sequences in the maternal plasma of a T21 pregnancy would therefore be 1.05 times that of a euploid case. If an analytical approach could be developed to determine this small degree of quantitative difference, a polymorphism-independent test for noninvasive prenatal diagnosis of fetal T21 would be achieved.

Gene dosage assessment requiring 2-fold discrimination power can readily be attained with quantitative PCR (13). Through DNA quantification of a chr21 locus and a reference locus in amniocyte cultures, Zimmermann et al. (14) were able to detect the 1.5-fold increase in chr21 DNA sequences in T21 fetuses. Because a 2-fold difference in DNA template concentration constitutes a difference of only one threshold cycle (CT), the discrimination of a 1.5-fold difference has been the limit of conventional real-time PCR. To achieve finer degrees of quantitative discrimination, alternative strategies are needed. Here, we explore the use of digital PCR (15) for this purpose.


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Abbreviations: T21, trisomy 21; chr, chromosome; RCD, relative chromosome dosage; SPRT, sequential probability ratio test.

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Digital PCR involves multiple PCR analyses on extremely dilute nucleic acids such that most positive amplifications reflect the signal from a single template molecule (15), permitting the counting of individual template molecules. The proportion of positive amplifications among the total number of PCR analyses allows an estimation of the template concentration in the original nondiluted sample. This technique has been proposed to allow the detection of a variety of genetic phenomena (15), including the detection of loss of heterozygosity (LOH) in tumor samples (16) and plasma of cancer patients (17). Because template molecule quantification by digital PCR does not rely on dose–response relationships between reporter dyes and nucleic acid concentrations, its analytical precision should theoretically be superior to that of real-time PCR. To test whether this approach is precise enough to detect fetal chromosomal aneuploidies in maternal plasma, we first assessed whether digital PCR could measure the allelic ratio of PLAC4 mRNA in maternal plasma (9), thereby distinguishing T21 from euploid fetuses. This is referred to as the digital RNA SNP method. We then evaluated whether the increased precision of digital PCR would allow the detection of fetal chromosomal aneuploidies without depending on genetic polymorphisms. We call this digital relative chromosome dosage (RCD) analysis.

Results
Principles of Digital PCR. The first step in digital PCR is the dilution of the extracted nucleic acids to a concentration such that, on average, one template molecule is present per reaction well. PCR is then set up so that a multitude of such single-molecule PCRs is analyzed per sample. We used 96-well and 384-well reaction plates and distributed each diluted nucleic acid sample to the reaction wells of one or more plates. Under these conditions, the actual number of template molecules distributed to each reaction well followed the Poisson distribution. Thus, an individual reaction well could contain zero, one, or more template molecules. The expected proportion of wells with no template molecule is given by $e^{-m}$, where $m$ is the average concentration of template molecules per well. For example, at an average concentration of one template molecule per well, the expected proportion of wells with no template molecule is given by $e^{-1}$, i.e., 0.37 (37%). The remaining 63% of wells will contain one or more template molecules. Typically, the number of positive and informative wells in a digital PCR run would then be counted. The definition of informative wells and the manner by which the digital PCR data are interpreted depend on the application (15) and are described below.

Principles of Digital RNA SNP. Digital RNA SNP is a digital version of our previously reported approach (9) for T21 detection by determining an imbalance in the ratio of polymorphic alleles of an A/G SNP, rs8130833, located on PLAC4. For a heterozygous euploid fetus, the A and G alleles should be equally represented in the fetal genome (1:1), whereas, in T21, an additional copy of one of the SNP alleles would give a 2:1 ratio. Digital RNA SNP analysis aims to determine whether the amounts of the two PLAC4 alleles in the sample are equal or otherwise. Thus, both the A and G PLAC4 alleles are the target templates. The analytical steps are schematically shown in Fig. 1.

After digital real-time PCR analysis of the PLAC4 SNP alleles in 384-well plates, the number of informative wells was counted. An informative well is defined as one that was only positive for the A or G allele but not both (Fig. 1). For a euploid case, we expect an equal number of A-positive and G-positive wells (Fig. 1). However, when template molecules from a T21 fetus are analyzed, the number of wells containing just one allele should be higher than the number containing just the other allele (Fig. 1). In short, allelic imbalance is expected for T21. The same degree of imbalance would be expected if this approach were applied to the analysis of placental DNA, placental RNA, and maternal plasma RNA [PLAC4 mRNA in maternal plasma being completely fetal in origin (9)].

The allele with the higher number of counts is referred to as the overrepresented allele, and its proportion among all informative wells, $P_i$, was calculated (Fig. 1). The sequential probability ratio test (SPRT) (16, 18) (see below) was applied to determine whether the $P_i$ indicated the degree of allelic imbalance that would be expected for a T21 sample. Alternatively, the SPRT analysis may indicate that the available data are not yet adequate for disease classification. When classification was not achieved, additional 384-well plates were analyzed until the aggregated data became classifiable by SPRT.

Principles of Digital RCD. We determined chromosome dosage by digital PCR analysis of a nonpolymorphic chr21 locus relative to one located on a reference chromosome, chr1. We aimed to differentiate a change in the ratio of chr21 to chr1 from 2:2 in the genome of a euploid fetus to 3:2 in a T21 fetus (Fig. 1). Here, an informative well is defined as one that is positive for either the chr21 or chr1 locus but not both. For a euploid fetus, the number of informative wells positive for either locus should be approximately equal (Fig. 1). For a T21 fetus, there should be an overrepresentation of wells positive for chr21 (Fig. 1). The degree of overrepresentation would depend on the fractional fetal DNA concentration in the sample. For example, when placental DNA is analyzed, the theoretical RCD ratio in the fetal genome should be 3:2, i.e., a 1.5-fold difference. However, as described earlier, the theoretical
RCD ratio would decrease to 1.05 when analyzing a maternal plasma sample containing 10% fetal DNA. The $P_r$ was calculated by dividing the number of wells positive only for the chr21 locus by the total number of informative wells (Fig. 1). The $P_r$ was subjected to SPRT analysis (16, 18) for disease classification. If the data were unclassifiable, one or more additional 384-well plates were analyzed.

Assessment of Allelic or Chromosomal Imbalance by Digital PCR. To determine whether the analyzed sample is from a T21 case, the observed RNA SNP or RCD ratio would be compared with that expected for a T21 case. The theoretical RNA SNP ratio is 2:1, and the RCD ratio is 3:2 for a pure T21 sample. However, due to the Poisson distribution, the exact ratios are not the same as those in the fetal genome. Furthermore, template concentration is a key variable in the Poisson equation. Thus, the exact ratios are dependent on the template concentration used in a particular experiment. Because the total number of template molecules for a given volume of sample from a T21 subject would be greater than that for a euploid case, we standardize our definition of the level of diluted template concentration as the average number of reference template molecules per reaction well, $m_r$. For digital RNA SNP analysis, the reference template would be the allele that was not overrepresented, whereas the reference template for digital RCD analysis would be the chr1 locus. Thus, the dilution of one target template molecule of any type per well for the digital PCR analysis of a euploid case equates to an $m_r$ of 0.5.

The basis for the difference between the theoretical and expected degree of allelic or chromosomal imbalance and the calculations to determine the latter for a range of $m_r$ values are shown in supporting information (SI) Tables 3 and 4. In digital RNA SNP analysis of a T21 sample, when the $m_r$ value was 0.5, the digital RNA SNP ratio (namely, the ratio of wells containing just the overrepresented allele with respect to wells containing just the reference allele) was 2.65 (SI Table 3). In digital RCD analysis of a specimen composed of 100% fetal DNA, when the $m_r$ value was 0.5, the digital RCD ratio (namely, the ratio of wells positive solely for the chr21 locus with respect to those positive solely for the chr1 locus) was 1.7 (SI Table 4). As the fractional fetal DNA concentration decreases, the digital RCD ratio decreases for the same $m_r$ (SI Table 4). As shown in SI Tables 3 and 4, the extent of allelic or chromosomal overrepresentation increases with $m_r$. However, the percentage of informative wells approaches its maximum near an $m_r$ value of 0.5 and decreases gradually with further increase in $m_r$. In practice, the decline in the proportion of informative wells could be compensated by increasing the total number of wells analyzed if the amount of specimen template molecules is not limiting, with an associated increase in reagent costs. Hence, optimal digital PCR performance is a tradeoff between the template concentration and total number of wells tested per sample.

### SPRT Analysis

To determine whether an observed degree of overrepresentation of a $PLAC4$ allele in digital RNA SNP, or the chr21 locus in digital RCD, is statistically significant, a SPRT-based approach was used (16, 18). SPRT is a method that allows testing of a hypothesis as data accumulate. SPRT has been used to interpret digital PCR data for loss of heterozygosity (LOH) in tumor samples (16, 18). In T21 detection, the null hypothesis is that there is no allelic or chromosomal imbalance (i.e., T21 is not detected). The alternative hypothesis is that allelic or chromosomal imbalance exists (i.e., T21 is detected). Operationally, SPRT can be performed with a pair of SPRT curves that are constructed to define the probabilistic boundaries for accepting or rejecting the null hypothesis (Fig. 2A and SI Materials and Methods). These curves show the required proportion of informative wells positive for the overrepresented allele or chr21, $P_r$ (y axis, Fig. 2A), for a given total number of informative wells ($x$ axis, Fig. 2A) needed for classification. Samples with data points that are above the top curve are classified as trisomic (Fig. 2A). Samples with data points that are below the bottom curve are classified as euploid. Samples with data points in between the two curves are unclassifiable and would require an increased total number of informative counts before classification. SPRT thus offers the advantage that a smaller amount of testing is required for a given level of confidence than other statistical methods. This feature is of particular relevance to the analysis of plasma nucleic acids in which the number of available template molecules is limited.

As discussed above, the exact degree of allelic or chromosomal imbalance depends on the actual template concentration per experiment. We therefore constructed a series of SPRT curves for a range of $m_r$ values (SI Materials and Methods). Each set of digital PCR data should be interpreted with the curves relevant to the $m_r$ of that particular run. Thus, in practice, after digital RNA SNP or digital RCD analysis, $m_r$ and $P_r$ are calculated (Fig. 1). $m_r$ is calculated by using the Poisson equation and the proportion of wells negative for the reference template (SI Materials and Methods). $P_r$ is the proportion of informative wells positive just for the overrepresented template. The experimentally derived $P_r$ is interpreted with the relevant SPRT curves selected by the corresponding $m_r$. This is in contrast to the previously reported use of SPRT for molecular detection of loss of heterozygosity (LOH) by digital PCR, where a fixed set of curves was used (16). Because the expected degrees of allelic or chromosomal imbalance for the digital RNA SNP and RCD approaches are different (2:1 for the former and 3:2 for the latter), different series of SPRT curves are needed. Fig. 2B illustrates the degree of differences in the SPRT curves for $m_r$ values of 0.1, 0.5, and 1.0 for digital RNA SNP analysis. Compared with the use of a fixed set of SPRT curves in previous studies (SI Materials and Methods) (16, 18), the proportion of
Computer simulation of classification accuracies of digital PCR detection of T21. Computer simulation was performed to estimate the accuracy of diagnosing T21 by using the SPRT approach. Separate simulations were performed for different values of three parameters, namely, reference template concentration ($m_r$), number of informative counts, and projected degree of allelic or chromosomal imbalance ($P_r$). For digital RNA SNP, simulations of a 384-well experiment with $m_r$ values of 0.1–2.0 were performed. At each $m_r$ value, we simulated the scenario whereby 5,000 euploid and 5,000 T21 fetuses were tested (SI Materials and Methods). The SPRT curves appropriate for the given $m_r$ were used to classify the 10,000 fetuses. The percentages of fetuses correctly and incorrectly classified as euploid or aneuploid and those unclassifiable for the given informative counts were determined (SI Table 7). The accuracies for diagnosing euploid and aneuploid cases are both 100%, for $m_r$ values between 0.5 and 2.0. When the $m_r$ value was 0.1, only 57% and 88% of euploid and T21 fetuses could be accurately classified by using 384 wells. Simulation results, using an illustrative repetition number of 300 times, are shown in SI Fig. 4.

Table 1. Digital RNA SNP analysis in placental tissues of euploid and T21 pregnancies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>A only</th>
<th>G only</th>
<th>AG</th>
<th>All negative</th>
<th>$m_r$</th>
<th>$P_r$</th>
<th>Unclassifiable region</th>
<th>Classification</th>
</tr>
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<td>85</td>
<td>83</td>
<td>126</td>
<td>90</td>
<td>0.79</td>
<td>0.51</td>
<td>0.63–0.65</td>
<td>Euploid</td>
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<td>N710</td>
<td>AG</td>
<td>102</td>
<td>83</td>
<td>73</td>
<td>126</td>
<td>0.52</td>
<td>0.55</td>
<td>0.61–0.63</td>
<td>Euploid</td>
</tr>
<tr>
<td>N435</td>
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<td>157</td>
<td>130</td>
<td>48</td>
<td>0.63</td>
<td>0.76</td>
<td>0.62–0.64</td>
<td>T21</td>
</tr>
<tr>
<td>N981</td>
<td>AAG</td>
<td>135</td>
<td>69</td>
<td>82</td>
<td>98</td>
<td>0.50</td>
<td>0.66</td>
<td>0.61–0.63</td>
<td>T21</td>
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Placental RNA

<table>
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<th>Sample</th>
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<th>G only</th>
<th>AG</th>
<th>All negative</th>
<th>$m_r$</th>
<th>$P_r$</th>
<th>Unclassifiable region</th>
<th>Classification</th>
</tr>
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<td>V533</td>
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<td>93</td>
<td>71</td>
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<td>0.56</td>
<td>0.53</td>
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<td>V943</td>
<td>AG</td>
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<td>100</td>
<td>74</td>
<td>121</td>
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<td>0.61–0.63</td>
<td>Euploid</td>
</tr>
<tr>
<td>N435</td>
<td>AGG</td>
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<td>138</td>
<td>95</td>
<td>99</td>
<td>0.48</td>
<td>0.73</td>
<td>0.61–0.63</td>
<td>T21</td>
</tr>
<tr>
<td>T215</td>
<td>AAG</td>
<td>146</td>
<td>58</td>
<td>138</td>
<td>42</td>
<td>0.71</td>
<td>0.72</td>
<td>0.62–0.64</td>
<td>T21</td>
</tr>
</tbody>
</table>

*The no. of wells for all samples was 384. Genotypes were determined by mass spectrometric assay. The $m_r$ value indicates the average no. of reference molecules per reaction well. The $P_r$ values were calculated by using the following equation: no. of wells positive for the overrepresented allele/no. of wells positive for A only + no. of wells positive for G only. The unclassifiable region for the corresponding $m_r$ is shown. “Euploid” was assigned when the $P_r$ was below the unclassifiable region; “T21” was assigned when the $P_r$ was above the unclassifiable region.

Table 2. Digital RNA SNP analysis of maternal plasma from euploid and T21 pregnancies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>A only</th>
<th>G only</th>
<th>AG</th>
<th>All negative</th>
<th>$m_r$</th>
<th>$P_r$</th>
<th>Unclassifiable region</th>
<th>Classification</th>
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<td>97</td>
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<td>65</td>
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<td>92</td>
<td>31</td>
<td>192</td>
<td>0.30</td>
<td>0.582</td>
<td>0.59–0.62</td>
<td>Euploid</td>
</tr>
<tr>
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<td>0.54–0.64</td>
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<td>85</td>
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<td>0.59–0.62</td>
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<tr>
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<td>17</td>
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<td>0.21</td>
<td>0.559</td>
<td>0.58–0.62</td>
<td>Euploid</td>
</tr>
<tr>
<td>M2525P</td>
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<td>53</td>
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<td>0.675</td>
<td>0.58–0.61</td>
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<td>130</td>
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<td>0.691</td>
<td>0.59–0.62</td>
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*The number of wells for all samples except M2272P was 384. The number of wells for sample M2272P was 768. Genotypes were determined by mass spectrometric assay. The $m_r$ value indicates the average no. of reference molecules per reaction well. The $P_r$ values were calculated by using the following equation: no. of wells positive for the overrepresented allele/no. of wells positive for A only + no. of wells positive for G only. The unclassifiable region for the corresponding $m_r$ is shown. “Euploid” was assigned when the $P_r$ was below the unclassifiable region; “T21” was assigned when the $P_r$ was above the unclassifiable region.

Computer simulations for digital RCD analysis for a pure (100%) fetal DNA sample were similarly performed (SI Table 8 and SI Fig. 5). The extent of chr21 overrepresentation in digital RCD analysis depends on the fractional concentration of fetal DNA in the tested specimen. Because the fractional fetal DNA concentration becomes lower, the degree of chr21 overrepresentation diminishes, and thus a larger number of informative wells for accurate disease classification is required. Hence, simulations were further performed for fetal DNA concentrations of 50%, 25%, and 10% for a total well number ranging from 384 to 7,680 wells at an $m_r$ value of 0.5 (SI Table 9). The performance of digital RCD is better for cases with a higher fetal DNA fractional concentration. At a fetal DNA concentration of 25% and with a total number of 7,680 PCR analyses, 97% of both euploid and aneuploid cases would be classifiable with no incorrect classification. The remaining 3% of cases require further analyses until classification can be achieved.

Validation of T21 Detection When Using Digital RNA SNP for PLAC4.

The practical feasibility of digital RNA SNP was demonstrated by using the rs8130833 SNP on the PLAC4 gene (SI Materials and Methods) (9). Placental DNA and RNA samples from two euploid and two T21 heterozygous placentas were analyzed. The placental DNA samples were analyzed with the omission of the reverse transcription step, thus essentially converting the procedure to digital DNA SNP analysis. We diluted the samples, aiming for approximately one allele of any type per well, and confirmed this
Our experimental and simulation data show that digital RNA SNP is an effective and accurate method for T21 detection. Because PLAC4 mRNA in maternal plasma is derived purely from the fetus, for 12 of the 13 maternal plasma samples tested, only one 384-well digital PCR experiment was required for correct classification. This homogenous, real-time PCR-based approach thus offers an alternative to the previously described mass spectrometry-based approach for RNA SNP analysis (9). Apart from placental-specific mRNA transcripts, other types of fetal-specific nucleic acid species in maternal plasma could be used. One example is fetal epigenetic markers (12, 21) which have recently been used for the noninvasive prenatal detection of trisomy 18 via the epigenetic allelic ratio (EAR) approach (10). Thus, we predict that digital EAR would be a possible analytical technique.

Digital RCD was developed to overcome the requirement of heterozygosity for a polymorphism-based approach such as digital RNA SNP. Digital RCD could readily discriminate T21 and euploid placental DNA samples, thus supporting its applications to samples containing virtually pure fetal DNA, e.g., amniotic fluid and chorionic villus samples.

The application of digital RCD to DNA extracted from maternal plasma is complicated by the fact that fetal DNA constitutes only...
a minor fraction of maternal plasma DNA, with a mean fractional concentration of some 3% between weeks 11 and 17 of gestation (8). Nevertheless, we have shown that digital RCD allows aneuploidy detection even when the fetal fraction is a minor population.

With a decreasing fractional concentration of fetal DNA, e.g., during early gestation, a larger amount of informative counts is needed for digital RCD. The significance of the present work, as summarized in SI Table 9, is that we have provided a set of needed for digital RCD. The significance of the present work, as summarized in SI Table 9, is that we have provided a set of methods proposed here for noninvasive prenatal diagnosis and for other applications in which allelic or chromosome imbalance is seen.

**Materials and Methods**

**Digital RNA SNP Analysis.** A real-time PCR assay was designed to amplify PLAC4 mRNA, with the two SNP alleles being discriminated by TaqMan probes. PLAC4 mRNA concentrations were quantified in extracted RNA samples followed by dilutions to approximately one target template molecule of either type (i.e., either allele) per well. We distributed the diluted sample to 96 wells for real-time PCR analysis to confirm that a usable dilution has been achieved. When ~37% (i.e., 1/e) of the wells were shown to be negative for any amplification, we proceeded to the digital RNA SNP analysis using the same diluted sample for 384-well analyses. Details are given in the SI Materials and Methods.

**Digital RCD Analysis.** Extracted DNA was quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE) and diluted to a concentration of approximately one target template from either chr21 or chr1 per well. A real-time PCR assay was designed to amplify a paralogous sequence (19) present on both chromosomes, distinguishable by a pair of TaqMan probes. The diluted DNA sample was first analyzed by the assay using the chr1 probe only in a 96-well format to confirm whether ~37% of the wells were negative; then we proceeded to digital RCD analysis using both TaqMan probes in 384-well plates. Details are given in the SI Materials and Methods.

**Computer Simulation of Classification Accuracy.** The computer simulation was performed with Microsoft Excel 2003 software (Microsoft, Redmond, WA) and SAS 9.1 for Windows software (SAS Institute, Cary, NC). Details are given in the SI Materials and Methods.

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