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Digital PCR for Noninvasive Detection of Aneuploidy: Power Analysis Equations for Feasibility

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Noninvasive prenatal diagnostic methods · Digital PCR analysis · Trisomy 21 · Free fetal DNA

Abstract
Objective: To determine the feasibility of digital PCR analysis for noninvasive prenatal diagnosis of trisomy 21. ***Methods:*** Through power equations, we modeled the number of wells necessary to determine the feasibility of digital PCR as a practical method for trisomy 21 risk assessment. ***Results:*** The number of wells needed is a direct correlate of the ability to isolate free fetal DNA. If a 20% fetal DNA enhancement can be achieved, then 2,609 counts would be sufficient to achieve a 99% detection rate for a 1% false-positive rate and potentially feasible with readily available plates. However, if only a 2% increase is seen, then 220,816 counts will be necessary, and over 110,000 would be needed just to achieve 95% for a 5% false-positive rate – both far beyond current commercially available technology. ***Conclusion:*** There are several noninvasive prenatal diagnostic methods which may reach commercialization; all have differing potential advantages and disadvantages. Digital PCR is potentially a cheaper methodology for trisomy 21, but it is too early to determine the optimal method.

Introduction

Over the past 25 years, the search for noninvasive prenatal diagnostic methods (NIPD) has evolved from fetal cells, collected from either blood or cervical swabs, to free fetal DNA (ff DNA), free fetal RNA (ff RNA) from blood, most recently to sequencing of ff DNA [1–5]. Now, the cycle is starting over again. The varying approaches appear to have differing potentials and limitations, and no one method has emerged as the clear favorite, although Sequenom is the only company on the market in the United States.

Because of concerns about the ability to differentiate reliably between maternal and ff DNA in pregnant plasma or serum whose fetal contribution might be only a small percentage, there was obvious enthusiasm for the potential of a pregnancy-specific ff RNA analysis that should produce a 3:2 ratio between markers for chromosome 21 and a control chromosome [6]. Over the past few years, ff RNA-based methods for NIPD were developed rigorously by one university group [6, 7], but then pushed extensively by one company which claimed a 100% detection rate (DR) for a 1% false-positive rate (FPR) in investor presentations that varied from actual publications [6, 7]. Plans were publically stated to launch the test com-

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mercially without the prior completion of significant refereed published data. These claims were met with significant scrutiny by the academic community, and further analysis led to retractions, removal of leadership from the company, and SEC indictments and a guilty plea by a senior scientist [8].

Next-generation sequencing emerged as the latest generation of technology. Papers from both the Hong Kong and Stanford groups have been very encouraging and clearly do work, but there are significant hurdles to be overcome to make the technology routine [9, 10]. With hardware devices initially ranging up to USD 700,000, reagents approaching USD 1,000 a case, and limited throughput capacity, the initial approaches were research tools and not a potential clinical tool. Considerable work developed 'plexing' methods for running simultaneous patients in the same baths using patient-specific 'tags' to decrease the costs. These are becoming practical, but the tests will always be expensive. The first test using Next Gen sequencing was released by Sequenom for use as a lab-developed test in the United States in October 2011 based upon a study of 4,600 patients with a sensitivity of 98.6% with a 0.2% FPR [5]. How these statistics will hold up with expanded numbers and incorporation into screening of more low risk-patients remains to be seen. Other companies have touted, but with limited or no publications, their emerging technologies, and it can be anticipated that there may be competing technologies on the market with different countries and companies coming 'on line' at different points [11, 12].

As a potentially cheaper alternative, ff DNA analysis with enhanced digital PCR has been proposed again as potentially an optimal method. What has changed is the number of measurements by digital PCR possible which theoretically would allow for sufficient numbers of data points per case to allow differentiation between normal and abnormal populations.

To investigate the theoretical potential of this approach, here we evaluate the number of PCR reactions required to achieve reliability for clinical use.

Methods

48 × 48 and now 96 × 96 well plates have been developed for Dig-PCR (fig. 1). A 100,000-well plate is under development which if two were linked together would yield nearly 200,000 wells. We thus developed power analysis equations modeling DRs of 95 and 99% along with FPR of 1 and 5% along with proposed increased DNA chromosome 21/12 ratios of 2, 5, 10 and 20%.

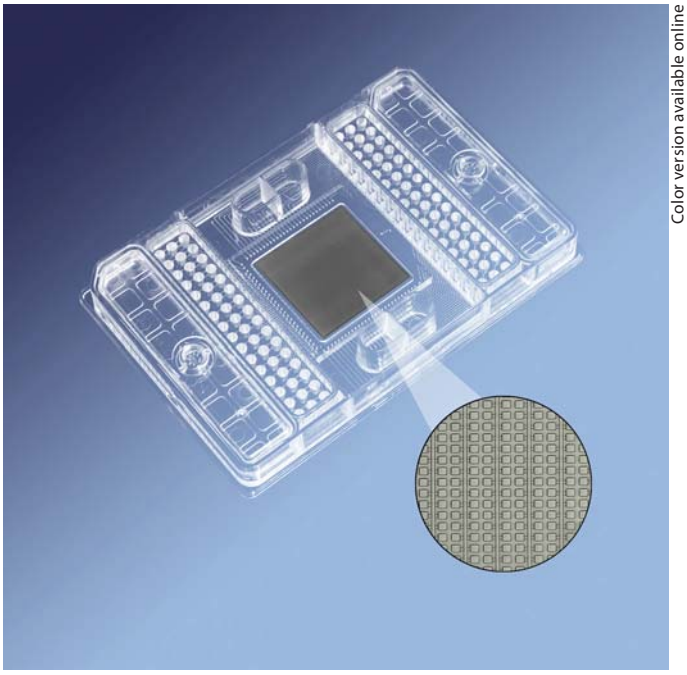

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Fig. 1. Fluidigm 96 × 96 chip containing 9,216 wells. From their website: www.fluidigm.com.

Results

If a 20% fetal DNA enhancement can be achieved, then 2,609 counts would be sufficient to achieve a 99% DR for a 1% FPR and potentially feasible with readily available plates. At 10%, the newer 9,000 plates are required. However, if only a 2% increase is seen, then 220,816 counts will be necessary, and over 110,000 would be needed just to achieve 95% for a 5% FPR – both far beyond current commercially available technology (table 1).

Discussion

Over the past two decades, there have been a number of methods that have been tried or have been proposed as methods for noninvasive prenatal diagnosis (NIPD) (table 2). Fetal cells were the first target with two decades, and millions of USD were spent from NIH studies and companies trying to isolate these cells for which there are no known fetal-specific antigens [1, 2, 13]. Since the end of the NICHD funded NIFTY trial in 2002 [2], several companies have tried to continue the work mostly with frustration [13]. Newer methods of cell identification and

Table 1. Power analysis for digital PCR

FPR	DR	20%	10%	5%	2%
0.01	0.99	2,609	9,536	36,379	220,816
0.01	0.95	1,902	6,948	26,504	160,868
0.05	0.99	1,899	6,946	26,501	160,865
0.05	0.95	1,304	4,767	18,187	110,392

Number of wells required to achieve stated FPR and DR.

Table 2. Evolving methods under consideration for NIPD

Fetal cells	Decades of promise. Isolation and identification method to find 'needle in haystack'
Digital PCR	Depends upon enrichment levels and number of wells available
ff RNA	Much too labile for routine use. Data integrity and stock manipulation stain
Next Gen sequencing	Works but expensive. Evolving methods to plex to reduce costs
DNA methylation	Fetal DNA sequences shorter, potentially significantly cheaper than sequencing

automated scanning have brought this approach back as a possibility [1].

The sentinel discovery by Lo et al. [14] in 1997 that ff DNA was relatively abundant in maternal plasma suggested an entirely new frame for the approach to identification of fetal material. The first approach of quantitative DNA differences among non-pregnant, normal pregnant, and Down syndrome-carrying pregnant women proved too technically challenging as the maximum difference in total chromosome 21 DNA amounts were perhaps only 2–3%. Even using digital PCR as it existed even only a few years ago meant that proper differentiation was essentially impossible (table 1). Using 48 × 48 (2,304) or 96 × 96 (9,216) well plates simply did not give enough wells to count to effect a statistically reliable differentiation among the groups.

Attention then turned to RNA – i.e. searching for a placental-specific RNA (e.g. PLAC 4) [6, 7]. This was scientifically very appealing, but the lability of RNA (hours – not centuries as for DNA) and the 'stain' from the scientific 'mishandling' of data coupled with criminal charges and one guilty plea, the hype to rush to market, and not enough actual science effectively killed that approach [8].

Next-generation sequencing clearly 'works,' but the challenge is in making it practical. Considerable advances have been documented in the past couple of years [4, 5, 12]. Related approaches such as differential analysis of fetal DNA methylation are likewise promising but will require a lot more study before being considered to be practical possibilities [11].

A second generation for digital PCR methodologies is potentially likely to be successful, but only if substantial, increased fetal DNA counts than currently speculated can be achieved or if the number of wells investigated can be dramatically increased. Preliminary analyses by differing groups have suggested anywhere from 10 to 20% enrichment down to only 1–2% [1]. With substantial enrichment of the specimen with ff DNA, then existing methods would likely be sufficient to achieve acceptable segregation and useful diagnosis. However, if only 1–2% is possible, the digital PCR technique will not be powerful enough to do the job. The currently marketed generation of 10,000-well plates is still not sufficient to reliably distinguish Down syndrome. A new generation of 20,000 wells might be sufficient even if anticipated increases in DNA levels prove correct. If not, then 200,000 will likely be needed, and much will depend upon the costs of such analyses. The pendulum, of which methods of NIPD could be marketable, will likely swing back and forth yet again to fetal cells, per se, or any one of the DNA approaches.

References

1 Evans MI, Kilpatrick M: Non-invasive prenatal diagnosis & screening; in Odibo A, Krantz DA (eds): Prenatal Screening and Diagnosis – An Issue of Clinics in Laboratory Medicine. Philadelphia, Saunders, 2010, pp 655–664.

2 Bianchi DW, Simpson JL, Jackson LG, Elias S, Holzgreve W, Evans MI, Dukes KA, Sullivan LM, Klinger KW, Bischoff FZ, Hahn S, Johnson KL, Lewis D, Wapner RJ, de la Cruz F: Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I Data. Prenat Diagn 2002;22:609–615.

3 Chiu RW, Akolekar R, Zheng YWL, Leung TY, Sun H, Chan KCA, Lun FMF, et al: Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. BMJ 2011; 342:c7401.

4 Ehrlich M, Deciu C, Zwiefelhofer T, Tynan JA, Cagasan L, Tim R, et al: Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting. Am J Obstet Gynecol 2011;204:205.e1–e11.
5 Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, et al: DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med, DOI: 10.1097/GIM.0b013e3182368a0e.
6 Lo YMD: Noninvasive prenatal detection of fetal chromosomal aneuploidies by maternal plasma nucleic acid analysis: a review of the current state of the art. Br J Obstet Gynaecol 2009;116:152–157.
7 Lo YMD, Tsui NB, Chiu RWK, Lau TK, Leung TN, Heung MM, et al: Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nat Med 2007;13:218–223.
8 Pollack A: Biotech company fires chief and others over handling of data. New York Times, Sept 29, 2009. http://www.nytimes.com/2009/09/29/business/29drug.html?sq=Elizabeth&adxnnl=1&adxnnlx=1329174249-+xa79JGOFIMENYHBPrFWNg.
9 Chiu RW, Chan A, Gao Y, Lau VYM, Zheng W, Leung TY, et al: Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci 2008;105:20458–20463.
10 Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR: Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci 2008;105:16266–16271.
11 Papageorgiou EA, Karagrigoriou A, Tsaliki E, Velissariou V, Carter NP, Patsalis PC: Fetal specific DNA methylation ratio permits non-invasive prenatal diagnosis of trisomy 21. Nat Med 2010;17:510–513.
12 Ashoor G, Syngelaki A, Wagner M, Birdir C, Nicolaides KH: Chromosome selective sequencing of maternal plasma cell-free DNA for first trimester detection of trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012, in press.
13 Seppo A, Frisova V, Ichetovkin I, Kim Y, Evans MI, Antsaklis A, Nicolaides KH, Tafas T, Tsipouras P, Kilpatrick MW: Detection of circulating fetal cells utilizing automated microscopy: potential for non-invasive prenatal diagnosis of chromosomal aneuploidies. Prenat Diag 2008;28:815–821.
14 Lo YMD, Corbetta N, Chamberlain PF, et al: Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–487.