Biomolecular Detection and Quantification 10 (2016) 9–14

Contents lists available at ScienceDirect

Biomolecular Detection and Quantification

journal homepage: www.elsevier.com/locate/bdq

Review

Applicability of digital PCR to the investigation of pediatric-onset genetic disorders

Matthew E.R. Butchbach [a,b,c,d,*]

[a] Center for Applied Clinical Genomics, Nemours Biomedical Research, Nemours Alfred I. duPont Hospital for Children, Wilmington, DE, USA
[b] Center for Pediatric Research, Nemours Biomedical Research, Nemours Alfred I. duPont Hospital for Children, Wilmington, DE, USA
[c] Department of Biological Sciences, University of Delaware, Newark, DE, USA
[d] Department of Pediatrics, Thomas Jefferson University, Philadelphia, PA, USA

ARTICLE INFO

Article history:
Received 20 April 2016
Received in revised form 8 June 2016
Accepted 21 June 2016
Available online 8 August 2016
Handled by Jim Huggett

Keywords:
Molecular diagnostics
Digital PCR
Droplet PCR
Copy number variation
Pediatrics
Mendelian diseases

ABSTRACT

Early-onset rare diseases have a strong impact on child healthcare even though the incidence of each of these diseases is relatively low. In order to better manage the care of these children, it is imperative to quickly diagnose the molecular bases for these disorders as well as to develop technologies with prognostic potential. Digital PCR (dPCR) is well suited for this role by providing an absolute quantification of the target DNA within a sample. This review illustrates how dPCR can be used to identify genes associated with pediatric-onset disorders, to identify copy number status of important disease-causing genes and variants and to quantify modifier genes. It is also a powerful technology to track changes in genomic biomarkers with disease progression. Based on its capability to accurately and reliably detect genomic alterations with high sensitivity and a large dynamic detection range, dPCR has the potential to become the tool of choice for the verification of pediatric disease-associated mutations identified by next generation sequencing, copy number determination and noninvasive prenatal screening.

Contents

1. Introduction

Genetic disorders account for about one fifth of pediatric hospitalizations and create an even greater burden on inpatient care [1,2]. Even though genetic disorders are uncommon when considered individually, they affect *in toto* more than 20 million people in the United States of America [3]. Additionally, it is becoming increasingly important to accurately measure the amount of mod-

* Corresponding author at: Nemours Biomedical Research, Nemours Alfred I. duPont Hospital for Children, 240 Rockland Center One, 1600 Rockland Road, Wilmington, DE 19803, USA.
E-mail address: butchbach@nemoursresearch.org

http://dx.doi.org/10.1016/j.bdq.2016.06.002

ifier genes whose copy number – like *SMN2* in spinal muscular atrophy (SMA) [4] – is related to disease severity. Thus there is a real need for a reliable, accurate and sensitive means to measure genomic variants. Most of the currently available technologies for the identification of disease-associated single nucleotide and copy number variants are not sufficiently sensitive, not quantitative without the use of external calibrator or too expensive for routine implementation.

Digital PCR (dPCR) provides a way for the absolute quantification of a target locus in a DNA sample. In dPCR, the target locus is distributed across a large number of partitions by limiting dilution so that a single DNA molecule is present in some, but not all, of the partitions [5,6]. As a result, some of the partitions may be devoid of target molecule to amplify during PCR. The abundance of the target locus can be measured by counting the number of partitions with a positive end-point reaction as well as the negative partitions. The Poisson equation ($\lambda = -\ln(1 - p)$ where λ is the average number of molecules in the sample and p is the fraction of positive reactions) corrects for the underestimation of the true number of molecules by counting negative end-point reactions [7]. Because of this end-point measurement of abundance, dPCR does not require the use of external calibration curve for calculating abundance of the target locus making this assay an absolute quantification.

Early incarnations of dPCR used limiting dilution so that each PCR reaction contained a single DNA molecule. Unfortunately, this approach was very labor intensive and not very precise. Partitioning the DNA so that a single molecule would be present in a PCR would be a more efficient approach for dPCR. dPCR currently uses one of two platforms to partition target DNA into very small volumes: nanofluidic chambers, or arrays, and nanodroplet emulsion [7]. Nanofluidic arrays divide the target DNA into numerous reaction chambers of nanoliter volumes wherein PCRs are run and the number of positive reactions is counted by fluorescence imaging [8–11]. Another means of partitioning involves the emulsification of target DNA and PCR master mixes into thousands of nanodroplet; the nanodroplets are counted after the PCR run for the number of positive reactions [12–14]. Both approaches allow for the accurate absolute quantification of the target DNA in a sample.

2. Applications of digital PCR to pediatric genetics

2.1. Identification of genetic alterations associated with disease

Chromosomal abnormalities involving multiple genes or whole chromosomes have been detected historically with fluorescence *in situ* hybridization (FISH) or array comparative genomic hybridization (aCGH) panels. These approaches are labor intensive and costly. Chromosomal polyploidies like trisomy 21, trisomy 18 and trisomy 13 can also be readily and easily measured with dPCR [15,16]. Droplet dPCR can also readily detect the presence of a mosaic supernumerary marker isochromosome 12p (iso12p) in DNA samples from patients with Pallister-Killian syndrome [17]. Chromosome 22q11 microdeletion syndrome, which is associated with neurological, cognitive and behavioral deficits, can be rapidly detected in patient DNA samples using dPCR to quantify copy number changes within the deleted region [18,19]. Furthermore, dPCR determines the endpoints of this deletion within chromosome 22q11 [19]. Deletions within the *DFNB1* locus – which include the connexin genes *GJB2* and *GJB6* – can be quantitatively measured in DNA from patients with autosomal recessive nonsyndromic sensorineural hearing loss [20]. In addition to identifying larger-scale changes within the genome, dPCR can detect deletions of single genes associated with disorders like SMA (*SMN1* (*survival motor neuron 1*)) [21,22] and South East Asian-type α(0)-thalassemia (*HBA1*/*HBA2* (*α-globin*)) [23].

dPCR can be used to screen patient samples for intragenic mutations linked to numerous monogenic disorders. Examples include verification of point mutations in *GCM2* (*glial cells missing homolog 2*; *GCM2(T370M)* and *GCM2(R367Tfs*)*) associated with hypoparathyroidism [24], in *MAP3K3* (*mitogen-activated protein kinase kinase kinase 3*; *MAP3K3(I441M)*) with verrucous venus malformation [25], in *PIK3CA* (α *catalytic subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase*; *PIK3CA(C420R)*, *PIK3CA(E542K)*, *PIK3CA(E545K)*, *PIK3CA(H1047R)* and *PIK3CA(H1047L)*) with lympathic malformation and Klippel-Trenaunay syndrome [26], in *SMN1* (*SMN1(Y272C)*) with SMA [21] and in *GNAS* (*stimulatory* α *subunit of G protein*, $G_s\alpha$; *GNAS(R201C)*) with McCune-Albright syndrome [27]. In many cases, the disease-associated intragenic mutations were initially identified using next generation sequencing [24–26]. The disease-associated *GNAS* mutation associated with McCune-Albright syndrome could not be detected in the patient until after death and only in certain tissues, suggesting somatic mosaicism [27]. With the assistance of a peptide nucleic acid (PNA) oligomer to lower the detection limit, Uchiyama et al. [28] can detect low frequency somatic mutations of *GNAQ* (*G protein* α *subunit q*, $G_q\alpha$; *GNAQ(R183Q)*) in patients with Sturge-Weber syndrome, a rare congenital neurocutaneous multisystem disorder, using droplet dPCR.

In addition to identifying early-onset genetic diseases, dPCR can detect subclonal mutations in children with various cancers. Using dPCR, subclonal *SETBP1* (*SET binding protein 1*) point mutations are detectable in a cohort of patients with juvenile myelomonocytic leukemia (JMML) [29]. Standard deep sequencing could not detect these subclonal point mutations. These somatic *SETBP1* mutations were associated with poor prognoses in these patients. dPCR shows somatic loss of one wild-type *NF1* (*neurofibromin-1*) allele within a malignant melanoma of a patient with neurofibromatosis type I [30]. Congenital hemangiomas are rare vascular tumors that develop prenatally as a result of somatic mutations; ddPCR can detect subclonal mutations in *GNAQ* (*GNAQ(E209L)* and *GNAQ(E209P)*) and *GNA11* (*G protein subunit* α *11*, $G_{11}\alpha$; *GNA11(E209L)*) in these tumors [31]. Within tumors, dPCR is a powerful tool to detect subclonal mutations affecting prognosis as well as loss of heterozygosity.

2.2. Noninvasive detection of genetic alterations in plasma

Cell-free fetal DNA (cffDNA) containing small fragments of fetal genomic DNA represent a small proportion (about 10%) of the DNA present in maternal plasma [32]. dPCR can reliably detected very small quantities of cffDNA in maternal plasma making noninvasive prenatal diagnosis feasible [12]. Noninvasive prenatal diagnosis of genetic disorders can be accomplished by comparing either the copy number of a target chromosome against that of a reference chromosome (relative chromosome dosage; RCD) or the amount of the mutant allele relative to the wild-type allele (relative mutation dosage; RMD). RCD can detect fetal trisomy 21 in maternal plasma [33]. RCD can also determine the sex of the fetus from cffDNA [34]. As a proof of concept, RMD detects single nucleotide changes or small deletions in *HBB* (*β-globin*; HbE, (*HBB(E26K)*) and CD41/42 allele (a 4 nucleotide deletion at codons 41 and 42)) associated with β-thalassemia [35]. RMD can be used to prenatally diagnose hemophilia (*F8*, *coagulation factor VIII*; *F9*, *coagulation factor IX*), sickle cell anemia (HbS, *HBB(E6V)*) and cystic fibrosis (*CFTRΔF508*; loss of phenylalanine 508 in *cystic fibrosis transmembrane conductance regulator* (*CFTR*)) [36–38]. Using massively parallel deep sequencing, cffDNA can be scanned globally for paternally inherited single nucleotide polymorphisms (SNPs) by relative haplotype dosage (RHDO) [39]. Lam and colleagues identify mutations in *HBB* that are associated with β-thalassemia via a targeted RHDO approach [40].

One disadvantage of using RCD, RMD or RHDO for fetal genetic screening is that a large number of DNA molecules need to be detected and counted in order to detect increases in dosage. The use of fetal-specific markers can enhance the sensitivity of the genetic screen. For example, epigenetic-genetic chromosome dosage (EGCD) detects trisomy 21 with a hypermethylated fetal DNA marker on chromosome 21 (within the promoter of *HLCS*; *holocarboxylase synthetase*) and reference gene on the Y chromosome (*ZFY*, *Y-linked zinc finger protein*) [41] or on the X chromosome (*ZFX*; *X-linked zinc finger protein*) [42]. The use of rs6636, a paternally-inherited, autosomal SNP within *TMED8* (*transmembrane emp24 protein transport domain containing 8*) on chromosome 14, makes the EGCD assay independent of sex chromosomes as reference markers [43]. The epigenetic-genetic dosage approach along with targeted Sanger sequencing were used to identify a point mutation in *FGFR3* (*fibroblast growth factor receptor 3*; *FGFR3(G380R)*) associated with achondroplasia [44].

Aside from screening for fetal genetic disorders, dPCR can help identify maternal genetic factors which could affect the fetus. One potential complication during pregnancy is hemolytic disease of the fetus and newborn (HDFN), which is a fetal alloimmune condition resulting in anemia to heart failure (hydrops fetalis) and death. The RhD (Rh blood group D) antigen is one factor implicated in HDFN. If a woman without functional RhD carries a fetus who inherited a functional RhD from his father (RhD^+ pregnancy), she will develop maternal antibodies against RhD. These maternal anti-RhD antibodies could cause HDFN in subsequent pregnancies by crossing the placental barrier and destroying fetal erythrocytes. Standard genetic screen can identify RhD^+ pregnancies in mothers completely lacking *RHD* but not in those mothers harboring intragenic mutations in *RHD*. dPCR can detect paternally-inherited, fetal *RHD* in maternal plasma [34,45]. dPCR can accurately detect maternal *RHD* variants like *RHD(IVS3 + 1G > A)* in the presence of cffDNA containing paternally-inherited RHD [46].

In addition to noninvasive prenatal genetic testing, dPCR can be used to identify somatic tumor mutations in the plasma of children with cancer. Neuroblastoma-associated mutations in *ALK* (*anaplastic lymphoma receptor tyrosine kinase*; *ALK(F1174L)* and *ALK(R1275Q)*) can be detected in cell-free DNA using dPCR [47]. *MYCN* (*N-Myc*) copy numbers are elevated in cell-free DNA from the sera of patients with childhood solid tumors [48].

2.3. Determination of copy number variations

Larger scale structural variations such as copy number variations (CNVs) and segmental duplications in the human genome have been linked with numerous diseases. aCGH and SNP microarrays are currently used to detect CNVs but these techniques are costly and have difficulty detecting duplications or higher order increases in copy number [49]. dPCR can precisely and reliably detect CNVs within the genome. SMA is a genetically homogeneous early-onset motor neuron disease with a large variability in phenotype severity. *SMN2*, a nearly perfect duplicate gene to *SMN1*, copy number is inversely related to disease severity in SMA (reviewed in Ref. [4]). Droplet and array dPCR can detect a wide range of *SMN2* copy numbers, i.e. 0–5 copies, more accurately than other quantitative PCR techniques at detecting high copy numbers (>4 copies) [21,22]. Higher copy numbers of both *BHLHA9* (*basic helix-loop-helix family member A9*) and *YWHAE* (*14-3-3ε*) can be detected by dPCR patients with split-hand malformations associated with double quadruplication in 17p13.3 [50]. In addition to measuring higher order CNVs, digital PCR can be used to determine mechanisms underlying higher order amplification of genomic intervals like quadruplications within chromosome 17p13.3 in split-hand malformations [50] and within the *PLP1* (*proteolipid protein 1*) locus on the X chromosome in Pelizaeus-Merzbacher disease [51].

CNVs associated with diseases having a non-Mendelian inheritance pattern, like childhood-onset obesity, can be measured with dPCR. Fachi and colleagues [52] demonstrate an association between low *AMY1* (*salivary amylase*) copy number and increased body mass index (BMI) and risk of obesity. This study used quantitative PCR to measure *AMY1* copy numbers. A subsequent study using dPCR fails to validate this inverse relationship between *AMY1* copy numbers and obesity risk in a different adult population [53]. However, other studies using dPCR have confirmed this relationship in early-onset obesity. An inverse correlation between *AMY1* copy number and risk of early-onset obesity as well as BMI is found using dPCR in affected females but not males from Finland [54]. In a cohort of children from Mexico, there is a strong relationship between high *AMY1* copy number and reduced risk of obesity [55]. Future studies will determine if this inverse relationship between *AMY1* copy number and obesity risk is unique to early-onset obesity.

2.4. Measurement of allelic imbalance

Preferential allelic imbalance (PAI) occurs when a disease-associated heritable SNP is preferentially retained relative to the wild-type allele. PAI of specific SNPs have been observed in tumor DNA from many different cancers including acute lymphoblastic leukemia (ALL), which is the most common childhood cancer. Tumor PAI can be detected by Sanger sequencing and SNP genotyping but neither technique is quantitative. The somatic mutation allelic ratio test using digital droplet PCR (SMART-ddPCR) quantifies PAI in ALL tumor DNA [56,57]. SMART-ddPCR uses a similar strategy to RMD and RHDO (Section 2.2). This approach has identified subclonal somatic CNVs using SNPs within *CDKN2A* (*cyclin-dependent kinase inhibitor 2A*) and *IKZF1* (*Ikaros*) in ALL patients [56] and found ALL-associated PAI of the *CDKN2A(A148T)* risk allele [57]. The *CDKN2A* and *IKZF1* copy numbers measured with SMART-ddPCR were in agreement with those measured by multiplex ligation-dependent probe amplification (MLPA) [56].

2.5. Detection of disease-associated epigenetic changes

In type I diabetes, the loss of pancreatic β cells is believed to occur before the clinical presentation of hyperglycemia. It would, therefore, be desirable to detect β cell death in these individuals prior to clinical onset. The promoter region of the *INS* (*preproinsulin*) gene is uniquely unmethylated in β cells [58]. Elevated levels of unmethylated *INS* promoter DNA can be detected in the sera of patients with type I diabetes [58]. Increased levels of unmethylated *INS* relative to methylated *INS* are detected by dPCR in children with recent onset type I diabetes [59]. Increased unmethylated *INS* correlates to decreased insulin secretion and also indicates pancreatic β cell death [60]. When measuring methylated and unmethylated *INS* levels independently, both forms of *INS* DNA are elevated in the serum at onset of type I diabetes [61]. The elevations in both methylated and unmethylated *INS* DNA gradually diminish as the disease progressed over time with the rate of unmethylated *INS* DNA decline being faster than that for methylated *INS* DNA [61]. These studies demonstrate the feasibility of dPCR for early detection of childhood disease with complex genetics.

3. Comparison of digital PCR to other techniques

dPCR is an end-point counting of the number of negative partitions. Since dPCR is an absolute quantification of a target locus, there is no need for an externally generated calibration curve. The independence of dPCR from an external calibration curve gives it an advantage over qPCR. Another advantage of dPCR is that it is more tolerant to the inhibitory effects of substances like heparin

and SDS (sodium dodecylsulfate) than qPCR [62]. dPCR is more precise and reproducible than qPCR [63,64]. Both array and droplet dPCR are more accurate at DNA copy number quantification than other PCR-based approaches [65,66]. When compared against each other, array and droplet dPCR are equally precise and accurate.

dPCR is more sensitive than conventional qPCR techniques for measuring CNVs [67]. For example, dPCR can accurately and reliably measure between 0 and 5 copies of *SMN2* in genomic DNA samples from SMA patients while other techniques like qPCR have a narrower range, i.e. 0–3 copies [21,22]. The increased range in CNV detection is due to the linear amplification that occurs in dPCR which allows for small fold change (i.e. less than 2-fold) differences to be measured.

In addition to being more sensitive at measuring single unit changes in copy number, dPCR can detect low abundance of a target locus within a heterogeneous mix of DNA. The increased sensitivity of dPCR at detecting low levels of target DNA within a complex mixture of DNA has been demonstrated by detecting subclonal *SETBP1* point mutations in patients with JMML, a low frequency somatic *GNAQ(R183Q)* mutation in patients with Sturge-Weber syndrome [28] and a *GNAS1(R201C)* mutation within some tissues of McCune-Albright syndrome post-mortem case [27,29]. In cffDNA, dPCR is more sensitive than standard qPCR in detecting fetal *RHD* as well as the sex of the fetus in maternal plasma [34,45]. dPCR, therefore, is better capable of analyzing complex mixtures of DNA to detect rare locus events than qPCR.

Variability in the partition volume can affect the accuracy of the target locus quantification in dPCR, usually resulting in an underestimation [10,14,65,66]. This variability tends to be a greater concern for droplet dPCR than for array dPCR since the partition size within the nanofluidic chamber is constant. There may be, however, small differences in partition size between different batches of nanofluidic chambers which can contribute to error. In a recent study, the uncertainty associated with variable partition volume was shown to be very low, between 0.7% and 2.9% depending on the platform [65]. The error resulting from this variability may be small relative to other potential sources of error that are not unique to dPCR, like sample processing and loading.

dPCR assumes that there is a random distribution of template DNA throughout the partitions. In some cases, copies of the target DNA are arranged in close proximity to each other on the template DNA; in order words, two (or more) copies of the target DNA will always be in a single partition. As a result, there will be an underestimation of the measured copy number [67,68]. This limitation can be circumvented by disrupting the template DNA biochemically with a restriction endonuclease that cleaves between the copies of the target DNA or mechanically using DNA shearing [22].

When performing dPCR, the reaction mix must first be partitioned by either nanofluidic chambers or nanodroplets. After the dPCR run is completed, the partitions need to be read fluorimetrically using a separate apparatus. At present, dPCR is, therefore, more labor intensive than qPCR and requires specialized equipment. This post-PCR handling of samples in dPCR is one disadvantage over qPCR, however, future developments in sample automation will minimize the impact of post-PCR handling on dPCR. Additionally, air bubbles can be introduced during the loading of the samples which can adversely affect the readout. New dPCR technologies such as the use of a centrifugal microfluidic disk are being developed to circumvent some of these limitations [69]. Despite these limitations, dPCR does have many advantages, highlighted in this section, over conventional qPCR.

Next generation sequencing (NGS) approaches – like whole exome (WES) and whole genome (WGS) sequencing – are increasingly being used to diagnose genetic disorders in children [3,70]. WGS is particularly useful in rapidly identifying a molecular diagnosis for a monogenic disease whose genetic basis has not yet been ascertained. This rapid testing would be particularly beneficial for neonatal onset disorders [71]. Although these next generation sequencing approach offer some advantages over standard diagnostic procedures including dPCR, WES and WGS require highly specialized equipment and detailed bioinformatic analysis of a large set of sequencing data. Deep sequencing can also miss identifying subclonal *SETBP1* point mutations in a JMML which can be detected by dPCR [29]. NGS approaches are better suited for genetic discovery but dPCR has advantages over NGS in the detection of known mutations.

Another advantage of NGS is that it can also analyze multiple target genes at the same time making it a remarkable platform for multiplexing. dPCR can some multiplexing capability in that, to date, up to five different target regions can be detected at the same time using two different fluorophores [21]. The multiplexing capacity of dPCR can be further increased in the future by increasing the number of fluorophores, designing better probes which can produce larger differential signals and using one-to-one droplet fusions containing the template DNA and different probes [13,21].

Before dPCR approaches can be regularly used for molecular diagnostics, many of these aforementioned considerations need to be adequately addressed [68,72]. The fact that the primers and probes designed for conventional qPCR-based diagnostic tests can also be easily used by dPCR should facilitate its routine use in diagnostics. The relatively easy readout used by dPCR make it more suitable for a broader group of individuals as it does not require specialized skills. The dPCR readout is also amenable to higher throughput and automated formats. These newer formats should make dPCR more cost effective and reduce the amount of time it takes to complete the diagnostic assay.

4. Conclusions

In summary, dPCR provides a way to accurately and reliably detect genomic alterations including gene deletions and point mutations, or single nucleotide polymorphisms. Because of its sensitivity, dPCR can detect a specific target locus at a very low concentration within a genomic DNA mixture, making this technique particularly amenable to track somatic subclonal mutations as well as cffDNA. dPCR has a large dynamic range of detection which permits the accurate quantification of CNVs in genes associated with pediatric diseases as well as in their modifiers. Because of these advantages, dPCR shows great prognostic – and potentially diagnostic – value in caring for and ultimately treating children with genetic diseases.

Conflicts of interest

None.

Funding source

Nemours Foundation, National Institute of General Medical Sciences (P20GM103464), National Institute of General Medical Sciences (P30GM114736).

Author contribution

The author confirms being the sole contributor of this work and approves it for publication.

Acknowledgments

I would like to thank Dr. Katia Sol-Church and the members of the Motor Neuron Diseases Research Laboratory for their input.

References

[1] S.E. McCandless, J.W. Brunger, S.B. Cassidy, The burden of genetic disease on inpatient care in a children's hospital, Am. J. Hum. Genet. 74 (2004) 121–127.
[2] L. Colvin, C. Bower, A retrospective population-based study of childhood hospital admission with record linkage to a birth defects registry, BMC Pediatr. 9 (2009) 32.
[3] S.F. Kingsmore, D.L. Dinwiddie, N.A. Niller, S.E. Soden, C.J. Saunders, The Children's Mercy Genomic Medicine Team, Adopting orphans: comprehensive genetic testing of Mendelian diseases of childhood by next-generation sequencing, Expert Rev. Mol. Diagn. 11 (2011) 855–868.
[4] M.E.R. Butchbach, Copy number variations in the *Survival Motor Neuron* genes: implications for spinal muscular atrophy and other neurodegenerative diseases, Front. Mol. Biosci. 3 (2016) 7.
[5] B. Vogelstein, K.W. Kinzler, Digital PCR, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 9236–9241.
[6] P.J. Sykes, S.H. Neoh, M.J. Brisco, E. Hughes, J. Condon, A.A. Morley, Quantitation of targets for PCR by use of limiting dilution, Biotechniques 13 (1992) 444–449.
[7] E. Day, P.H. Dear, F. McCaughan, Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine, Methods 59 (2013) 101–107.
[8] S. Dube, J. Qin, R. Ramakrishnan, Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device, PLoS One 3 (2008) e2876.
[9] T.K.F. Yung, K.C.A. Chan, T.S.K. Mok, J. Tong, K.F. To, Y.M.D. Lo, Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients, Clin. Cancer Res. 15 (2009) 2076–2084.
[10] S. Bhat, J. Herrmann, P. Armishaw, P. Corbisier, K.R. Emslie, Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number, Anal. Bioanal. Chem. 394 (2009) 457–467.
[11] A.C. Hatch, J.S. Fisher, A.R. Tovar, A.T. Hsieh, R. Lin, S.L. Pentoney, D.L. Yang, A.P. Lee, 1-Million droplet array with wide-field fluorescence imaging for digital PCR, Lab Chip 11 (2011) 3838–3845.
[12] B.J. Hindson, K.D. Ness, D.A. Masquelier, P. Belgrader, N.J. Heredia, A.J. Makarewicz, I.J. Bright, M.Y. Lucero, A.L. Hiddessen, T.C. Legler, T.K. Kitano, M.R. Hodel, J.F. Petersen, P.W. Wyatt, E.R. Steenblock, P.H. Shah, L.J. Bousse, C.B. Troup, J.C. Mellen, D.K. Wittmann, N.G. Erndt, T.H. Cauley, R.T. Koehler, A.P. So, S. Dube, K.A. Rose, L. Montesclaros, S. Wang, D.P. Stumbo, S.P. Hodges, S. Romine, F.P. Milanovich, H.E. White, J.F. Regan, C.A. Karlin-Neumann, C.M. Hindson, S. Saxonov, B.W. Colston, High-throughput droplet digital PCR system for absolute quantitation of DNA copy number, Anal. Chem. 83 (2011) 8604–8610.
[13] D. Pekin, Y. Skhiri, J.C. Baret, D. Le Corre, L. Mazutis, C. Ben Salem, F. Millot, A. El Harrak, J.B. Hutchison, J.W. Larson, D.R. Link, P. Laurent-Puig, A.D. Griffiths, V. Taly, Quantitative and sensitive detection of rare mutations using droplet-based microfluidics, Lab Chip 11 (2011) 2156.
[14] L.B. Pinheiro, V.A. Coleman, C.M. Hindson, J. Herrmann, B.J. Hindson, S. Bhat, K.R. Emslie, Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification, Anal. Chem. 84 (2011) 1003–1011.
[15] H.C. Fan, S.R. Quake, Detection of aneuploidy with digital polymerase chain reaction, Anal. Chem. 79 (2007) 7576–7579.
[16] H.C. Fan, Y.J. Blumenfeld, Y.Y. El-Sayed, J. Cheuh, S.R. Quake, Microfluidic digital PCR enables rapid prenatal diagnosis of fetal aneuploidy, Am. J. Obstet. Gynecol. 200 (2009) 543, e1-543, e7.
[17] K. Fujiki, K. Shirahige, M. Kaur, M.A. Deardorff, L.K. Conlin, I.D. Krantz, K. Izumi, Mosaic ratio quantification of isochromosome 12p in Pallister-Killian syndrome using droplet digital PCR, Mol. Genet. Genom. Med. 4 (2016) 257–261.
[18] D. Pretto, D. Maar, C.M. Yrigollen, J. Regan, F. Tassone, Screening newborn blood spots for 22q11.2 deletion syndrome using multiplex droplet digital PCR, Clin. Chem. 61 (2015) 182–190.
[19] V.J. Hwang, D. Maar, J. Regan, K. Angkustsiri, T.J. Simon, F. Tassone, Mapping the deletion endpoints in individuals with 22q11.2 deletion syndrome by droplet digital PCR, BMC Med. Genet. 15 (2014) 106.
[20] A.N. Abou Tayoun, H. Mason-Suares, A.L. Frisella, M. Bowser, E. Duffy, L. Mahanta, B. Funke, H.L. Rehm, S.S. Amr, Targeted droplet-digital PCR as a tool for novel deletion discovery at the DFNB1 locus, Hum. Mutat. 37 (2016) 119–126.
[21] Q. Zhong, S. Bhattacharya, S. Kotsopoulos, J. Olson, V. Taly, A.D. Griffiths, D.R. Link, J.W. Larson, Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR, Lab Chip 11 (2011) 2167–2174.
[22] D.L. Stabley, A.W. Harris, J. Holbrook, N.J. Chubbs, K.W. Lozo, T.O. Crawford, K.J. Swoboda, V.L. Funanage, W. Wang, W. Mackenzie, M. Scavina, K. Sol-Church, M.E.R. Butchbach, *SMN1* and *SMN2* copy numbers in cell lines derived from patients with spinal muscular atrophy as measured by array digital PCR, Mol. Genet. Genom. Med. 3 (2015) 248–257.
[23] S. Pornprasert, W. Prasing, Detection of alpha(0)-thalassemia South-East Asian-type deletion by droplet digital PCR, Eur. J. Haematol. 92 (2014) 244–248.
[24] T. Mitsui, S. Narumi, M. Inokuchi, K. Nagasaki, M. Nakazawa, G. Sasaki, T. Hasegawa, Comprehensive next-generation sequencing analyses of hypoparathyroidism: identification of novel GCM2 mutations, J. Clin. Endocrinol. Metab. 99 (2014) E2421–E2428.
[25] J.A. Couto, M.P. Vivero, H.P.W. Kozakewich, A.H. Taghinia, J.B. Mulliken, M.L. Warman, A.K. Greene, A somatic MAP3K3 mutation is associated with verrucous venous malformation, Am. J. Hum. Genet. 96 (2015) 480–486.
[26] V.L. Luks, N. Kamitaki, M.P. Vivero, W. Uller, R. Rab, J.V.M.G. Bovée, K.L. Rialon, C.J. Guevara, A.I. Alomari, A.K. Greene, S.J. Fishman, H.P.W. Kozakewich, R.A. Maclellan, J.B. Mulliken, R. Rahbar, S.A. Spencer, C.C. Trenor III, J. Upton, D. Zurakowski, J.A. Perkins, A. Kirsh, J.T. Bennett, W.B. Dobyns, K.C. Kurek, M.L. Warman, S.A. McCarroll, R. Murillo, Lymphatic and other vascular malformative/overgrowth disorders are caused by somatic mutations in PIK3CA, J. Pediatr. 166 (2015) 1048–1054.
[27] V. Vasilev, A.F. Daly, A. Thiry, P. Petrossians, F. Fina, L. Rostomyan, M. Silvy, A. Enjalbert, A. Barlier, A. Beckers, McCune-Albright syndrome: a detailed pathological and genetic analysis of disease effects in an adult patient, J. Clin. Endocrinol. Metab. 99 (2014) E2029–E2038.
[28] Y. Uchiyama, M. Nakashima, S. Watanabe, M. Miyajima, M. Taguri, S. Miyatake, N. Miyake, H. Saitsu, H. Mishima, A. Kinoshita, H. Arai, K. Yoshiura, N. Matsumoto, Ultra-sensitive droplet digital PCR for detecting a low-prevalence somatic *GNAQ* mutation in Sturge-Weber syndrome, Sci. Rep. 6 (2016) 22985.
[29] E. Stieglitz, C.B. Troup, L.C. Gelston, J. Haliburton, E.D. Chow, K.B. Yu, J. Akutagawa, A.N. Taylor-Weiner, Y.L. Liu, Y.D. Wang, K. Beckman, P.D. Emanuel, B.S. Braun, A. Abate, R.B. Gerbing, T.A. Alonzo, M.L. Loh, Subclonal mutations in *SETBP1* confer a poor progrosis in juvenile myelomonocytic leukemia, Blood 125 (2015) 516–524.
[30] A. Rübben, B. Bausch, A. Nikkels, Somatic deletion of NFI gene in neurofibromatosis type I-associated malignant melanoma demonstrated by digital PCR, Mol. Cancer 5 (2006) 36.
[31] U.M. Ayturk, J.A. Couto, S. Hann, J.B. Mulliken, K.L. Williams, A.Y. Huang, S.J. Fishman, T.K. Boyd, H.P.W. Kozakewich, J. Bischoff, A.K. Greene, M.L. Warman, Somatic activating mutations in GNAQ and GNA11 are associated with congenital hemangioma, Am. J. Hum. Genet. 98 (2016) 789–795.
[32] Y.M.D. Lo, R.W.K. Chiu, Genomic analysis of fetal nucleic acids in maternal blood, Annu. Rev. Genom. Hum. Genet. 13 (2012) 285–306.
[33] Y.M.D. Lo, F.M.F. Lun, K.C.A. Chan, N.B.Y. Tsui, K.C. Chong, T.Z. Lau, T.Y. Leung, B.C.Y. Zee, C.R. antor, R.W.K. hiu, Digital PCR for the molecular detection of fetal chromosomal aneuploidy, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 13116–13121.
[34] K.A. Sillence, L.A. Roberts, H.J. Hollands, H.P. Thompson, M. Kiernan, T.E. Madgett, C.R. Welch, N.D. Avent, Fetal sex and *RHD* genotyping with digital PCR demonstrates greater sensitivity than real-time PCR, Clin. Chem. 61 (2015) 1399–1407.
[35] F.M.F. Lun, N.B.Y. Tsui, K.C.A. Chan, T.Y. Leung, P. Charoenkway, K.C.K. Chow, W.Y.W. Lo, C. Wanapirak, T. Sanguansermsri, C.R. Cantor, R.W.K. Chiu, Y.M.D. Lo, Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma, Proc. Natl. Acad. Sci. U. S. A. 105 (2016) 19920–19925.
[36] N.B.Y. Tsui, R.A. Kadir, K.C.A. Chan, C. Chi, G. Mellars, E.G. Tuddenham, T.Y. Leung, T.K. Lau, R.W.K. Chiu, Y.M.D. Lo, Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA, Blood 117 (2011) 3684–3691.
[37] A.N. Barrett, T.C.R. McDonnell, K.C.A. Chan, L.S. Chitty, Digital PCR analysis of maternal plasma for noninvasive detection of sickle cell anemia, Clin. Chem. 58 (2012) 1026–1032.
[38] E. Debrand, A. Lykoudi, E. Bradshaw, S.K. Allen, A non-invasive droplet digital PCR (ddPCR) assay to detect paternal CFTR mutations in the cell-free fetal DNA (cffDNA) of three pregnancies at risk of cystic fibrosis via compound heterozygosity, PLoS One 10 (2015) e0142729.
[39] Y.M.D. Lo, K.C.A. Chan, H. Sun, E.Z. Chen, P. Jiang, F.M.F. Lun, Y.W. Zheng, T.Y. Leung, T.K. Lau, C.R. Cantor, R.W.K. Chiu, Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus, Sci. Transl. Med. 2 (2010) 61ra91.
[40] K.W.G. Lam, P. Jiang, G.J.W. Liao, K.C.A. Chan, T.Y. Leung, R.W.K. Chiu, Y.M.D. Lo, Noninvasive prenatal diagnosis of monogenic diseases by targeted massively parallel sequencing of maternal plasma: application to β-thalassemia, Clin. Chem. 58 (2012) 1467–1475.
[41] Y.K. Tong, S. Jin, R.W.K. Chiu, C. Ding, K.C.A. Chan, T.Y. Leung, L. Tu, T.K. Lau, Y.M.D. Lo, Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach, Clin. Chem. 56 (2010) 90–98.
[42] F.M.F. Lun, R.W.K. Chiu, K.C.A. Chan, T.Y. Leung, T.K. Lau, Y.M.D. Lo, Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma, Clin. Chem. 54 (2008) 1664–1672.
[43] Y.K. Tong, R.W.K. Chiu, R. Akolekar, T.Y. Leung, T.Z. Lau, K.H. Nicolaides, Y.M.D. Lo, Epigenetic-genetic chromosome dosage approach for fetal trisomy 21 detection with an autosomal genetic reference marker, PLoS One 5 (2010) e15244.
[44] L. Orhant, O. Anselem, M. Fradin, P.H. Becker, C. Beugnet, N. Deburgrave, G. Tafuri, F. Letourneur, F. Goffinet, L.A. El Khattabi, F. Leturcq, T. Bienvenu, V. Tsatsaris, J. Nectoux, Droplet digital PCR combined with minisequencing, a new approach to analyze fetal DNA from maternal blood: application to the non-invasive prenatal diagnosis of achondroplasia, Prenat. Diagn. 36 (2016) 397–406.
[45] I. Svobodová, E. Pazourková, A. Horínek, M. Novotná, P. Calda, M. Korabecná, Performance of droplet digital PCR in non-invasive fetal *RHD* genotyping—comparison with a routine real-time PCR based approach, PLoS One 10 (2015) e0142572.

[46] N.B.Y. Tsui, C.A. Hyland, G.J. Gardener, D. Danon, N.M. Fisk, G. Millard, R.L. Flower, Y.M.D. Lo, Noninvasive fetal *RHD* genotyping by microfluidics digital PCR using maternal plasma from two alloimmunized women with the variant *RHD*(IVS3 + 1G > A) allele, Prenat. Diagn. 33 (2013) 1214–1216.
[47] V. Combaret, I. Iacono, A. Bellini, S. Bréjon, V. Bernard, A. Marabelle, C. Coze, G. Pierron, E. Lapouble, G. Schleiermacher, J.Y. Blay, Detection of tumor ALK status in neuroblastoma patients using peripheral blood, Cancer Med. 4 (2015) 540–550.
[48] S. Kurihara, Y. Ueda, Y. Onitake, T. Sueda, E. Ohta, N. Morihara, S. Hirano, F. Irisuna, E. Hiyama, Circulating free DNA as non-invasive diagnostic biomarker for childhood solid tumors, J. Pediatr. Surg. 50 (2015) 2094–2097.
[49] C.R. Coughlin II, G.H. Scharer, T.H. Shaikh, Clinical impact of copy number variation analysis using high-resolution microarray technologies: advantages, limitations and concerns, Genome Med. 4 (2012) 80.
[50] S. Gu, J.E. Posey, B. Yuan, C.M.B. Carvalho, H.M. Luk, K. Erikson, I.F.M. Lo, G.K.C. Leung, C.R. Pickering, B.H.Y. Chung, J.R. Lupski, Mechanisms for the generation of two quadruplications associated with split-hand malformation, Hum. Mutat. 37 (2016) 160–164.
[51] C.R. Beck, C.M.B. Carvalho, L. Banser, T. Gambin, D. Stubbolo, B. Yuan, K. Sperle, C.M. McCahan, M. Henneke, P. Seeman, J.Y. Garbern, G.M. Hobson, J.R. Lupski, Complex genomic rearrangements at the PLP1 locus include triplication and quadruplication, PLoS Genet. 11 (2015) e1005050.
[52] M. Falchi, J.S. El-Sayed Moustafa, P. Takousis, F. Pesce, A. Bonnefond, J.C. Andersson-Assarsson, P.H. Sudmant, R. Dorajoo, M.N. Al-Shafai, L. Bottolo, E. Ozdemir, H.C. So, R.W. Davies, A. Patrice, R. Dent, M. Mangino, P.G. Hysi, A. Dechaume, M. Huyvaert, J. Skinner, M. Pigeyre, R. Caiazzo, V. Raverdy, E. Vaillant, S. Field, B. Balkau, M. Marre, S. Visvikis-Siest, J. Weill, O. Poulain-Godefroy, P. Jacobson, L. Sjostrom, C.J. Hammond, P. Deloukas, P.C. Sham, R. McPherson, J. Lee, E.S. Tai, R. Sladek, L.M.S. Carlsson, A. Walley, E.E. Eichler, F. Pattou, T.D. Spector, P. Froguel, Low copy number of the salivary amylase gene predisposes to obesity, Nat. Genet. 46 (2014) 492–497.
[53] C.L. Usher, R.E. Handsaker, T. Esko, M.A. Tuke, M.N. Weedon, A.R. Hastie, H. Cao, J.E. Moon, S. Kashin, C. Fuchsberger, A. Metspalu, C.N. Pato, M.T. Pato, M.I. McCarthy, M. Boehnke, D.M. Altshuler, T.M. Frayling, J.N. Hirschhorn, S.A. McCarroll, Structural forms of the human amylase locus and their relationships to SNPs, haplotypes and obesity, Nat. Genet. 47 (2015) 921–925.
[54] H. Viljakainen, J.C. Andersson-Assarsson, M. Armenio, M. Pekkinen, M. Pettersson, H. Valta, M. Lipsanen-Nyman, O. Mäkitie, A. Lindstrand, Low copy number of the *AMY1* locus is associated with early-onset female obesity in Finland, PLoS One 10 (2015) e0131883.
[55] M.A. Mejía-Benítez, A. Bonnefond, L. Yengo, M. Huyvaert, A. Dechaume, J. Peralta-Romero, M. Klünder-Klünder, J.G. Mena, J.S. El-Sayed, M. Moustafa, M. Falchi, P. Cruz, Froguel, Beneficial effect of high number of copies of salivary amylase *AMY1* gene on obesity risk in Mexican children, Diabetologia 58 (2015) 290–294.
[56] A.J. de Smith, K.M. Walsh, H.M. Hansen, A.A. Endicott, J.K. Wiencke, C. Metayer, J.L. Wiemels, Somatic mutation allelic ratio test using ddPCR (SMART-ddPCR): an accurate method for assessment of preferential allelic imbalance in tumor DNA, PLoS One 10 (2015) e0143343.
[57] K.M. Walsh, A.J. de Smith, H.M. Hansen, I.V. Smirnov, S. Gonseth, A.A. Endicott, J. Xiao, T. Rice, C.H. Fu, L.S. McCoy, D.H. Lachance, J.E. Eckel-Passow, J.K. Wiencke, R.B. Jenkins, M.R. Wrensch, X. Ma, C. Metayer, J.L. Wiemels, A heritable missense polymorphism in *CDKN2A* confers strong risk of childhood acute lymphoblastic leukemia and is preferentially selected during clonal evolution, Cancer Res. 75 (2015) 4884–4894.
[58] M.I. Husseiny, A. Kaye, E. Zebadua, F. Kandeel, K. Ferreri, Tissue-specific methylation of human insulin gene and PCR assay for monitoring beta cell death, PLoS One 9 (2014) e94591.
[59] S. Usmani-Brown, J. Lebastchi, A.K. Steck, C. Beam, K.C. Herold, M. Ledizet, Analysis of β-cell death in type 1 diabetes by droplet digital PCR, Endocrinology 155 (2014) 3694–3698.
[60] K.C. Herold, S. Usmani-Brown, T. Ghazi, J. Lebastchi, C.A. Beam, M.D. Bellin, M. Ledizet, J.M. Sosenko, J.P. Krischer, J.P. Palmer, Type 1 diabetes TrialNet Study Group, β Cell death and dysfunction during type 1 diabetes development in at-risk individuals, J. Clin. Invest. 125 (2015) 1163–1173.
[61] M.M. Fisher, R.A. Watkins, J. Blum, C. Evans-Molina, N. Chalasani, L.A. DiMeglio, K.J. Mather, S.A. Tersey, R.G. Mirmira, Elevations in circulating methylated and unmethylated preproinsulin DNA in new-onset type 1 diabetes, Diabetes 64 (2015) 3867–3872.
[62] T.C. Dingle, R.H. Sedlak, L. Cook, K.R. Jerome, Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances, Clin. Chem. 59 (2013) 1670–1672.
[63] C.M. Hindson, J.R. Chevillet, H.A. Briggs, E.N. Gallichotte, I.K. Ruf, B.J. Hindson, R.L. Vessella, M. Tewari, Absolute quantification by droplet digital PCR versus analog real-time PCR, Nat. Methods 10 (2013) 1003–1005.
[64] R. Sanders, J.F. Huggett, C.A. Bushell, S. Cowen, D.J. Scott, C.A. Foy, Evaluation of digital PCR for absolute DNA quantification, Anal. Chem. 83 (2011) 6474–6484.
[65] L. Dong, Y. Meng, Z. Sui, J. Wang, L. Wu, B. Fu, Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material, Sci. Rep. 5 (2015) 13174.
[66] P. Corbisier, L. Pinheiro, S. Mazoua, A.M. Kortekaas, P.Y.J. Chung, T. Gerganova, G. Roebben, H. Emons, K. Emslie, DNA copy number concentration measured by digital and droplet digital quantitative PCR using certified reference materials, Anal. Bioanal. Chem. 407 (2015) 1831–1840.
[67] A.S. Whale, J.F. Huggett, S. Cowen, V. Speirs, J. Shaw, S. Ellison, C.A. Foy, D.J. Scott, Comparison of microfluidic digital PCR and conventational quantitative PCR for measuring copy number variation, Nucleic Acids Res. 40 (2014) e82.
[68] J.F. Huggett, S. Cowen, C.A. Foy, Considerations for digital PCR as an accurate molecular diagnostic tool, Clin. Chem. 61 (2015) 79–88.
[69] F. Schuler, M. Trotter, M. Geltman, F. Schwemmer, S. Wadle, E. Dominguez-Garrido, M. López, C. Cervera-Acedo, P. Santibáñez, F. von Stetten, R. Zengerle, N. Paust, Digital doplet PCR on disk, Lab Chip 16 (2016) 208–216.
[70] J.E. Petrikin, L.K. Willig, L.D. Smith, S.F. Kingsmore, Rapid whole genome sequencing and precision neonatology, Semin. Perinatol. 39 (2015) 623–631.
[71] C.J. Saunders, N.A. Miller, S.E. Soden, D.L. Dinwiddie, A. Noll, N.A. Alnadi, N. Andraws, M.L. Patterson, L.A. Krivohlavek, J. Fellis, S. Humphray, P. Saffrey, Z. Kingsbury, J.C. Weir, J. Betley, R.J. Grocock, E.H. Marguiles, E.G. Farrow, M. Artman, N.P. Safina, J.E. Petrikin, K.P. Hall, S.F. Kingsmore, Rapid whole-genome sequencing for genetic disease diagnosis in neonatal intensive care units, Sci. Transl. Med. 4 (2012) 154ra135.
[72] J.F. Huggett, C.A. Foy, V. Benes, K. Emslie, J.A. Garson, R. Haynes, J. Hellemans, M. Kubista, R.D. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, S.A. Bustin, The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments, Clin. Chem. 59 (2013) 892–902.