แนวทางใหม่ในการหาปริมาณดีเอ็นเอเป้าหมายอย่างสมบูรณ์โดยใช้ปฏิกิริยาลูกโซ่พอลิเมอเรสแบบดรอปเลทดิจิตอล

Droplet Digital Polymerase Chain Reaction: A Novel Method for Absolute Quantification of the Target DNA

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บทคัดย่อ

ปฏิกิริยาลูกโซ่พอลิเมอเรสหรือพีซีอาร์เป็นเทคนิคสำหรับเพิ่มปริมาณชิ้นส่วนของสารพันธุกรรมเป้าหมายภายใต้สภาวะควบคุม เทคนิคปฏิกิริยาลูกโซ่พอลิเมอเรสแบบดิจิตอลถูกพัฒนาเพื่อให้สามารถหาปริมาณดีเอ็นเอเป้าหมายสุทธิได้โดยตรงจากการตรวจวัดสัญญาณการเรืองแสงฟลูออเรสเซนต์หลังการสิ้นสุดของปฏิกิริยาโดยไม่ต้องใช้ดีเอ็นเอมาตรฐานภายใต้สถิติการแจกแจงความน่าจะเป็นของปัวส์ซอง ซึ่งสามารถอธิบายโอกาสการเกิดหรือไม่เกิดของดีเอ็นเอเป้าหมายที่ถูกกระจายออกเป็นส่วนย่อยขนาดเล็ก ปัจจุบันหนึ่งในเทคนิคปฏิกิริยาลูกโซ่พอลิเมอเรสแบบดิจิตอลซึ่งได้รับการพัฒนาขึ้นใหม่ ได้แก่ การทำให้เกิดดีเอ็นเอโมเลกุลเดี่ยวแบบดรอปเลท หลักการทำงานของเทคนิคดังกล่าวเป็นการเพิ่มปริมาณของดีเอ็นเอเป้าหมายในหยดอีมัลชั่นภายในเครื่องเพิ่มปริมาณสารพันธุกรรมทั่วไป ก่อนการนำไปตรวจวัดการเรืองแสงฟลูออเรสเซนต์ของโพรบแทคแมน ณ จุดสิ้นสุดของปฏิกิริยา ปฏิกิริยาลูกโซ่พอลิเมอเรสแบบดรอปเลทดิจิตอลนั้นสามารถประยุกต์ใช้ในงานทางชีววิทยาโมเลกุลต่างๆ ตั้งแต่ การตรวจหาการแปรผันของจำนวนชุดดีเอ็นเอ การตรวจวิเคราะห์ตัวบ่งชี้ทางชีวภาพของดีเอ็นเอในตัวอย่างพลาสม่าซึ่งปราศจากเซลล์ จนถึงการตรวจหาดีเอ็นเอของยีนก่อกลายพันธ์ต่างๆ

คำสำคัญ: ดีเอ็นเอเป้าหมาย แทคแมน ปฏิกิริยาลูกโซ่พอลิเมอเรส ปฏิกิริยาลูกโซ่พอลิเมอเรสแบบดรอปเลทดิจิตอล

Abstract

The polymerase chain reaction (PCR) is a method for amplifying specific segments of the target DNA under controlled conditions. Under the concept of no use of the standard, digital PCR (dPCR) was developed for absolute quantification of the target DNA by using endpoint measurement. On the basis of the Poisson distribution statistic of dPCR, the DNA target was randomly distributed into an individual partition, it then employs the signal detection of the positive or negative partitions to calculate the amount of the target DNA. The droplet digital PCR (ddPCR) is a novel type of dPCR, which bases on generating the DNA into water-in-oil droplets. The ddPCR requires Taqman assay as the detection method similarly to the real time PCR, to provide a fluorescence signal for determination. The working process of ddPCR runs from generating the emulsion droplets, thermal cycling droplets in a normal thermal cycler till the endpoint, and analyzing the data by using a droplet reader assembled with fluorescent detection machine. The applications of ddPCR nowadays run into 3 areas including analysis of copy number of variations (CNVs), absolute quantification of biomarker DNA in a cell-free plasma sample, and detection of rare allele with small fold of target differences such as mutation gene in a wild type DNA.

Keywords: droplet digital PCR, PCR, polymerase chain reaction, target DNA, Taqman

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Introduction

The polymerase chain reaction (PCR) is a method for amplifying specific segments of target DNA by generating multiple copies using DNA polymerase enzymes under controlled conditions and detecting the PCR product by visualization techniques using dyes, or DNA-based fluorescence resonance energy transfer probes.[1-2] Although conventional PCR analyzes minimal starting quantities of nucleic acid, it requires post-PCR method such as gel electrophoresis leading to possibility of laboratories contamination.[3] Additionally, detecting the PCR product using ethidium bromide-contained gel is classified only a semi-quantitative analysis.[4] The next generation of PCR is real time PCR, which requires comparison of cycle threshold (Ct) values of an unknown to its standard to obtain quantitative information.[3] Digital PCR (dPCR) was developed since 1990s[5] for quantification of target nucleic acid in a sample that was discrete in partition and detected by using endpoint measurement.[6] The recently commercialized dPCR format is droplet digital PCR (ddPCR) that can be applied for determination of copy number variations, rare alleles, and cell-free DNA in plasma.[7]

In this review, a principle and workflow of ddPCR will be described including its application. Advantage and disadvantage among conventional PCR, real time PCR, and ddPCR will also be discussed.

Principle of PCR

PCR is a molecular biology technique that allows quick replication of the target DNA by amplifying a specific sequence lying between known positions on double strand DNA. PCR contains three-step process that requires a target DNA, a DNA polymerase, a supply of the 4 nucleotide bases, and primers, referred as a cycle. One PCR cycle consists of the following steps; Denaturation, Annealing, and Extension steps.[8-11]

Along the PCR program, it can be divided into four major phases including baseline region, early exponential phase, linear phase, and plateau phase.[12] At the baseline, there are no fluorescence signals detected. During the exponential phase, the theoretical doubling of the product at every cycle creates exponential signal growth and the reaction is very specific and precise. This phase is employed as detection region of DNA for real-time PCR method. The next phase is the linear phase that contains high variability due to the reaction components are starting to become limiting and the reaction efficiency is falling so the signal no longer grows exponentially. And the final phase is plateau phase which reaction components have been exhausted and the reaction generates no more fluorescence. This phase usually uses as an end-point of gel detection for conventional PCR method.

Limitation of conventional PCR and discovery of real time PCR

Although PCR has to be a powerful tool for quantify nucleic acid, RNA, and DNA. The conventional PCR method has several disadvantages.[13] These include the fact that there is no or less relationship between end point of PCR and initial target sequence. Moreover, the post-PCR method, such as gel electrophoresis, Southern blotting, and ELISA-like systems[13] is required to detect the DNA target that adds more time of analysis and may lead to laboratory contamination. Another reason is limitation on sensitivity, specificity, and reproducibility of the assay and limitation of screening large numbers of sample that make the conventional PCR not suitable for the routine used.[3, 14-15] Because of limitation of the conventional technique, real time PCR was developed for quantification. This technique bases on measurement of DNA amplification from the fluorescent signal at each PCR cycle[12] with no need for post-PCR handling[3] and it focuses on the exponential phase of the reaction that provides precise and accurate data for quantification of initial DNA copy number.[3, 16] However, a series of standards is needed for comparing the Ct values of samples of unknown concentration with the standards to determine the amount of target DNA.[12, 15]

One of the common detection methods of real time PCR is Taqman hydrolysis probes (or known as 5'-nuclease assay[17]), which base on Förster (Fluorescence) Resonance Energy Transfer (FRET).[2] The probes consist of oligonucleotides that are labeled with the fluorescent dyes. When the probe bound to the target

sequence, quenched dye will be degraded by Taq DNA polymerase during the extension step of the PCR, which separates the reporter from the quencher dye and results in increasing of fluorescence emission proportional to the amount of product formed.[17-18]

Digital PCR

Digital PCR based an end point measurement that works by random distribution of the sample into discrete partitions.[6] This concept was started in 1992 by Sykes et al. to quantify the total number of initial targets present in a sample using limiting dilution of PCR, and Poisson distribution statistics. The PCR has been optimized to provide an all-or-none end point at very low DNA target numbers that means some portions of these reactions contain the target molecule (positive) while others contain no template (negative), and these partitions are thermally cycled to end point, and then lead to determine the fraction of positive partitions to calculate copy number without standards.[6] Currently there are three approaches employed by commercially available digital PCR systems. The first approach is microfluidic dPCR[19] based on standard 5'-nuclease probe (TaqMan) chemistry and primer–probe design rules by using single used microfluidic chip to split the sample into the nanoliter individual cDNA partitions, then detection using a PCR end-point scan after thermocycled.[20] The second approach, called BEAMing dPCR based on 4 principle including beads, emulsion, amplification, and magnetic.[21-22] Recently there is another new approach that uses water-in-oil droplet, called droplet digital PCR (ddPCR) system in 96-well plate workflow.

Droplet digital PCR and process of working

Droplet digital PCR (ddPCR) based on forming the all or none reaction of nanometer water-in-oil emulsion droplet before performing the PCR and detecting the DNA products at the end point. The ddPCR is required a sample preparation as the real-time PCR assay by using specific primers and Taqman probes.[6-7] The first step of ddPCR is forming the emulsion droplets of the relevant forward and reverse primers, the TagMan probe, the DNA target, and other PCR components (*i.e.*, DNA polymerases, dNTPs, $MgCl_2$) in droplet generator, which required the droplet generating oil and 8 channels cartridge for this process. After the droplets are generated, they are transferred to a conventional 96-well PCR plate and amplified to end point (35-45 cycles) by using a conventional thermal cycler. Then, the plate is transferred to an auto-sampler droplet reader. In this process, the droplets from each well are sipped and lined for detection toward the two color fluorescence detector on both FAM and VIC fluorescence channels.[6-7]

Detection of droplet digital PCR

Because of specific cleavage of TaqMan probes form the detection method of ddPCR, each droplet has an intrinsic fluorescence signal resulting from the fluorogenic probes (FAM/VIC) that provide specific duplexed detection of target and reference genes. From the fluorescence amplitude, the droplets that contain the target template will generate a strong fluorescence signal. Therefore, the simple threshold can assign each droplet as positive or negative. As the droplet volume is known, the absolute concentration of target sequences is calculated by using fraction of positive droplets.

To estimate DNA target concentration, the concept of calculation is based on Poisson distribution statistic that the target copies of starting sample are portioned into positive and negative droplets by random distribution of independent events. Therefore, from this statistic, fraction of positive partition is used to determine absolute copy number in the form of unit of average of DNA copy number per droplet by using the followed equation: $\lambda = -\ln(1-\rho)$; if, λ as average number of target DNA molecules per partition (Copy number/droplet); as the fraction of positive partition which is the number of partitions containing amplified product divided by the number of partition analyzed.[7]

Comparison of conventional PCR, real time PCR, and droplet digital PCR

The comparisons of the three PCR methods are performed in the aspects of detection, quantification, and cost (Table1).

Application of droplet digital PCR

A. Determination of copy number variation (CNV)

Copy number variations (CNVs) are structural variation relative with deletions and amplifications of genome segments ranging from 1,000 to 100,000 nucleotide bases. This genetic variation found that 1,447 CNV regions cover approximately 12% of the human genome.[23] CNVs are shown to influence gene expression, phenotypic variability, and some may associate with causing disease.[24] The previous studies showed that copy number of *CCL3L1* was associated with susceptibility to human immunodeficiency virus-1 (HIV-1) infection[25] and low copy number of *FCGR3B* was associated with susceptibility to glomerulonephritis in the autoimmune disease systemic lupus erythematosus.[26] Common ways for detecting CNVs is using microarray-based methods[27] or next-generation sequencing technologies.[28] Recently, it is enabled using ddPCR.[7]

CNV analysis in HapMap samples for MRGPRX1, chromosome X, and CYP2D6 - Haplotype map of the human genome, called HapMap is a resource that describes the common pattern of human DNA sequence variation that is expected to be a key to find genes affecting health, disease, and responses to drugs and environmental factors. Mas-related G-protein-coupled receptors, member X1 (*MRGPRX1*) is a gene that probably involved in the function of nociceptive sensory neurons and may regulate the sensation or modulation of pain. Although MRGP superfamily consists of over 50 members in mouse genome, several pseudogenes are identified.[29] Among cytochrome P450 enzymes that have roles for the first phase in the metabolism and elimination of many substances, CYP2D6, an enzyme that metabolizes approximately 25% of drugs currently used including antidepressants, antipsychotics, antiarrhythmics, antiemetics, beta-blockers and opioids, has been reported to consist of variation in the encoding *CYP2D6* gene that affects enzymatic activity.[30-31] Multiplication of *CYP2D6*1* and *CYP2D6** alleles showed to increase CYP2D6 enzyme activity that results in rapid metabolism of CYP2D6 substrates.[32] The HapMap samples were screened to determine of CNV states for 3 target genes including *MRGPRX1*, *chromosome X*, and *CYP2D6*. The results showed the detection of CNV of the 3 genes from the lower CNV states (Fig. 1).[7]

B. Quantification of DNA in cell-free plasma

A clinical sample, circulating cell-free DNA (cfDNA) in plasma/serum has been suggested as a biomarker. Many studies use the alterations of cfDNA as diagnostic, prognostic, and monitoring markers in cancer patients.[33] Additionally, a noninvasive prenatal diagnosis of fetal gender using cfDNA of fetus in maternal plasma has been developed and useful for pregnancies that have risk of sex-linked genetic conditions.[34-35] The challenges of developing tools for quantification are natures of cfDNA in plasma that are highly fragmented and present at low levels.[36] In the application study of ddPCR, the maternal cell-free plasma was used for quantifying fetal DNA and total DNA for fetal sex determination. The male and female fetal DNAs were quantified by using fetal DNA markers including *SRY* (Sex-determining region Y) gene from *Chromosome Y* along with hypermethylated *RASSF1* gene. (Fig.2)[7] The level of fetal DNA from both *SRY* and hypermethylated *RASSF1* and total DNA were measured for both male and female fetuses from 19 maternal plasma samples taken between 10 and 20 weeks gestational age. Detection of a male fetus was based on the amplification of *SRY* in Y-chromosome sequences which described as the ratios of *SRY* to total DNA concentration and the detection of *RASSF1* which describes as the ratio of *RASSF1* to total DNA concentration. The results of male fetuses showed a correlation of 97.3% between *SRY* and hypermethylated *RASSF* that means female fetuses can be inferred from negative result of *SRY*. This analysis was successfully confirmed by ultrasound technique within 6 weeks after sample collection.[7]

C. Detection of rare mutation alleles

BRAF or v-raf murine sarcoma viral oncogene homolog B1 is a gene that encoding a protein belonging to the raf/mil family of serine/threonine protein kinases. Mutations of this gene are associated with various cancers such as malignant melanoma, non-Hodgkin lym-

phoma, colorectal cancer, thyroid carcinoma, and lung cancers.[37-38] The ddPCR was applied for detecting the *BRAF*V600E rare mutant allele. Serial dilutions of the mutant cell line DNA were prepared in a background of homologous wild type DNA. The results showed that the ddPCR could detect 0.001% mutant fraction, 1000 times lower than real-time PCR.[7]

Conclusion

PCR is a powerful tool for amplifying specific sequences of nucleic acid using an enzymatic reaction that involves 3 step processes, denaturation, annealing, and extension, in a thermal cycler. Several PCR techniques have been continually developed. Currently, PCR can be categorized into 2 generations including conventional PCR and real-time PCR. Another approach which newly leads to be the third one called digital PCR. First generation conventional PCR uses a post-PCR analysis such as gel electrophoresis to determine the accumulated DNA product. In real time PCR, DNA concentration of an unknown is measured by comparing Ct value with the standard in the exponential phase, in which the obtained data is not absolute quantitative data. The DNA product in real time PCR can be measured by several techniques. The famous and common one is Taqman assay, which bases on FRET, and works by increasing fluorescent emission proportional to amount of the DNA product after specific cleavage of the Taqman probes. The digital PCR approach bases on a Poisson distribution statistic, which randomly distributes the DNA target into a small partition. One of these approaches is the ddPCR that generates DNA sample into water-in-oil droplet and cyclers to endpoint. The ddPCR uses a common detection method similarly to real time PCR, which a specific detection of DNA target by Taqman probes provide a fluorescence signal to determine positive and negative droplets that make the absolute concentration. The application of ddPCR is summarized into 3 areas; *i.e.,* analysis of CNVs, absolute quantification of DNA in a clinical sample cell-free plasma, and detection of rare mutation allele in wild type DNA.

Consideration to the PCR applications, to obtained quantitative results, both real time PCR and ddPCR techniques are utilized. However these two techniques consume higher cost than conventional one. The real time PCR is quite suited for investigation of gene expression whereas, for measurement of the low levels of a specific DNA such as rare alleles, CNVs, or a mutant gene, the ddPCR is more suitable. Therefore, the key to select the appropriate PCR method is finally depended on proposes of the study.

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Table 1. Comparisons of conventional PCR, real time PCR, and droplet digital PCR

	Conventional PCR	**Real time PCR**	**Droplet digital PCR**
Phase of detection	Plateau phase	Exponential phase	Plateau phase
Detection method	Require post PCR method	Fluorescence-specific method	Fluorescence-specific method
Quantification	Semi-quantification	Comparing the Ct values of samples with a series of standards	Calculation absolute quantitative data by Poisson statistic
Facility	Be easy to access by a general thermal cycler machine	Need a real time PCR machine	Additionally need a droplets generator and a reader device
Cost/Sample	60 baht/sample	140 baht/sample	120-900 baht/sample

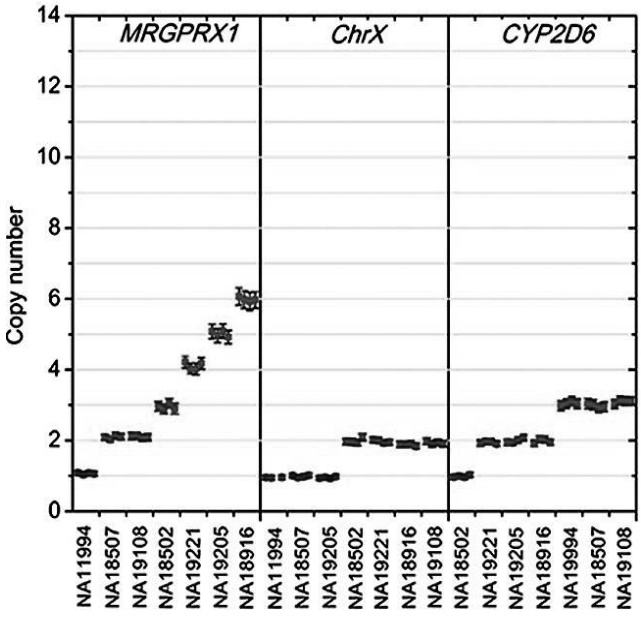

Figure 1 Determination copy number of *MRGPRX1, Chromosome X, and CYP2D6* in HapMap samples[7]

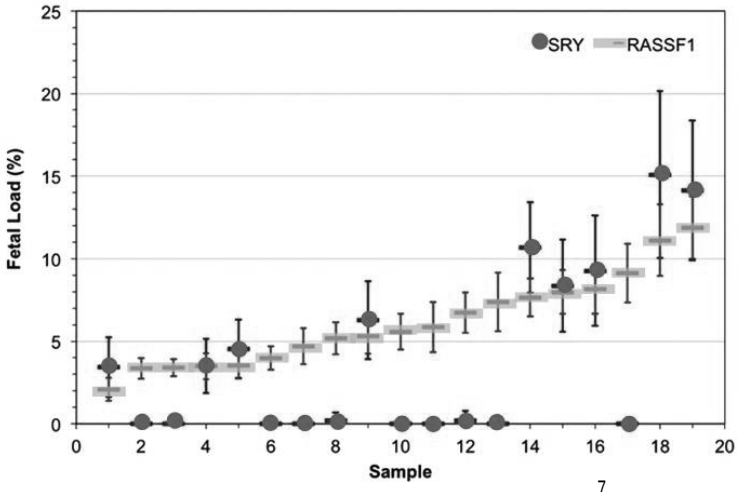

Figure 2 Fetal load of *SRY* and *RASSF1*[7]