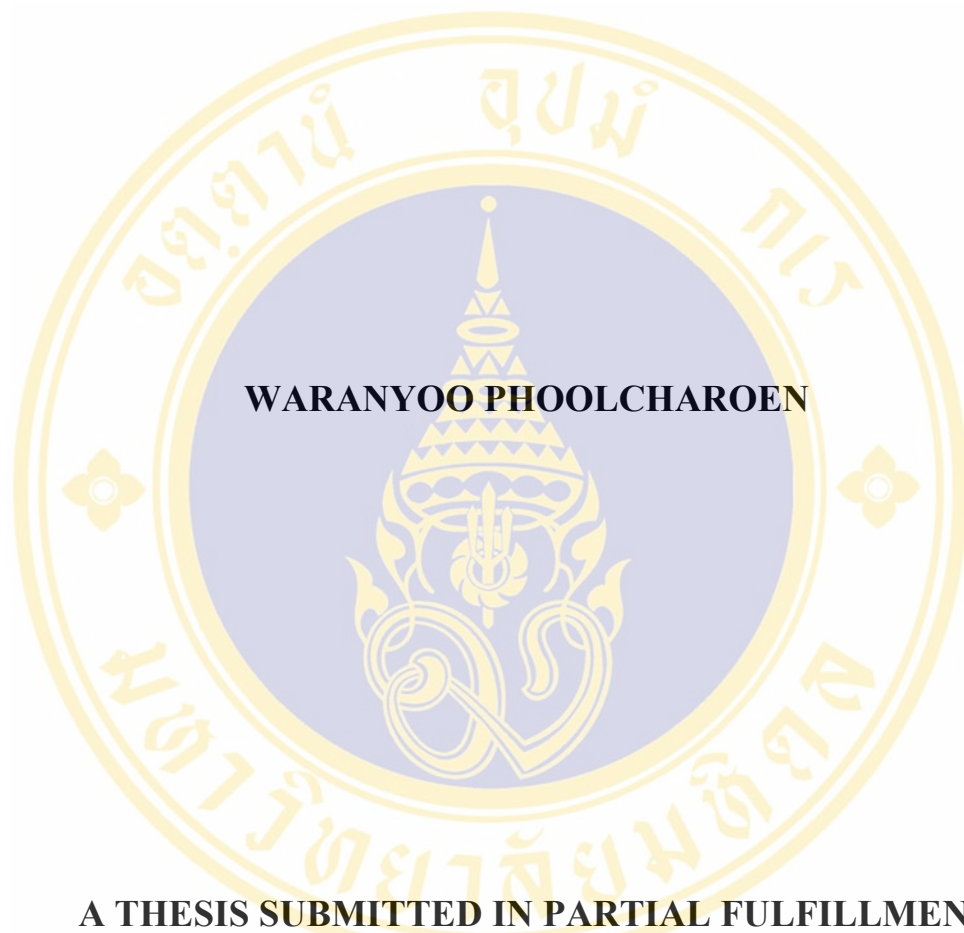


**THE RELATIONSHIP BETWEEN THE HUMAN HEPATOMA
(HepG2) CELL CYCLE AND DENGUE VIRUS INFECTION**



**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
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GENETICS AND GENETIC ENGINEERING)
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Thesis
Entitled

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(HepG2) CELL CYCLE AND DENGUE VIRUS INFECTION**



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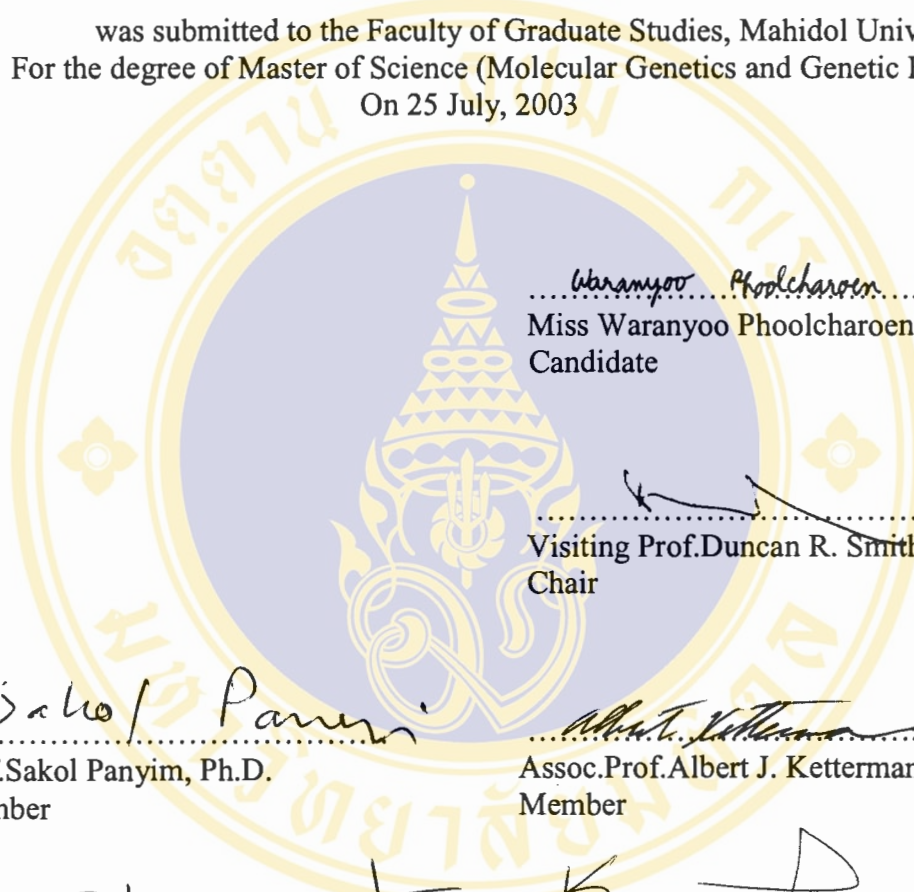
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THE RELATIONSHIP BETWEEN THE HUMAN HEPATOMA (HepG2) CELL CYCLE AND DENGUE VIRUS INFECTION

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ABSTRACT

The dengue virus, a mosquito-borne member of the family *flaviviridae*, causes dengue fever and the more severe forms, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). At present dengue fever is distributed throughout the world, especially in Southeast Asia and has become one of the most important international health problems because of the absence of a dengue vaccine or a specific treatment. The pathogenesis of DHF and DSS is not well understood. As such, knowledge about the receptors for dengue is necessary because the initial binding of a virus to a target cell is a critical determinant of cell and tissue specific tropism and thus of pathogenesis. The liver is suspected as a target organ for dengue infection. Previous studies have shown that some viral receptors are expressed in a cell cycle dependent manner, such as the Epstein-Barr virus (EBV) receptor and the Adenovirus receptor. The aim of this thesis is to determine whether the receptors for dengue are expressed depending upon the cell cycle stage of the target cells, which are HepG2 cells and Vero cells. HepG2 and Vero cells were synchronized by several methods such as the thymidine double block method to synchronize cells into S phase; paclitaxel to synchronize cells into M phase and serum starvation method to synchronize HepG2 cells into G₂ phase and to synchronize Vero cells into the G₀ phase. After the cells were synchronized, the differential susceptibility of the synchronized cells to infection with the dengue virus, as compared to unsynchronized cells, was determined. The results showed that dengue serotype 2 and 3 production from HepG2 and Vero cells in each phase of the cell cycle is different. Next the percent infectivity of dengue serotype 2 and 3 in HepG2 and Vero cells in each phase of the cell cycle was determined. The percent infectivity of dengue serotype 2 and 3 in HepG2 cells is different among each phase of the cell cycle whereas in Vero cells is not different. These results imply that the receptors of dengue serotype 2 and 3 in HepG2 cells may be expressed differently in each phase of the cell cycle. In contrast, the receptors for Vero cells may be expressed at a constant level in each phase of the cell cycle or alternatively the dengue virus may use a different method of entry into Vero cells (a non-target tissue) such as direct membrane fusion and not receptor-mediated endocytosis.

KEY WORDS: DENGUE VIRUS / RECEPTOR / CELL CYCLE

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ความสัมพันธ์ระหว่างวัฏจักรของเซลล์ตับ (เซลล์ HepG2) และการติดเชื้อไวรัสไข้เลือดออก
(THE RELATIONSHIP BETWEEN THE HUMAN HEPATOMA (HepG2) CELL
CYCLE AND DENGUE VIRUS INFECTION)

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บทคัดย่อ

โรคไข้เลือดออกเกิดจากการติดเชื้อไวรัสไข้เลือดออก ซึ่งสามารถติดต่อผ่านทางยุงลาย โรคนี้เป็นปัญหาสุขภาพที่สำคัญของโลก เนื่องจากยังไม่มีวัคซีนป้องกันและการรักษาที่จำเพาะ ปัจจุบันความรู้ทางพยาธิวิทยาของโรคยังไม่เป็นที่ทราบแน่ชัด แต่มีการพบว่าขั้นตอนแรกของการที่ไวรัสเข้าสู่เซลล์เป้าหมายและเพิ่มจำนวนได้คือ การจับกับตัวตอบรับบนผิวเซลล์ เป็นขั้นตอนที่สำคัญ โดยมีตัวเป็นอวัยวะเป้าหมายอย่างหนึ่ง จากการศึกษาเบื้องต้นพบว่า ตัวตอบรับของไวรัสบางชนิด เช่น Epstein-Barr Virus และ Adenovirus มีการแสดงออกบนผิวเซลล์ที่ติดเชื้อไวรัสแตกต่างกันในแต่ละช่วงของวัฏจักรของเซลล์ งานวิจัยนี้จัดทำขึ้นเพื่อศึกษาการแสดงออกของตัวตอบรับของไวรัสไข้เลือดออกในแต่ละช่วงของวัฏจักรของเซลล์ โดยได้ทำการศึกษาในเซลล์เป้าหมายของไวรัส คือ เซลล์ HepG2 และเซลล์ที่ไม่ใช่เซลล์เป้าหมาย คือ เซลล์ Vero ขั้นตอนแรกของการวิจัยคือการแยกเซลล์ให้อยู่ในแต่ละช่วงของวัฏจักรของเซลล์ เช่น ใช้ thymidine มายับยั้งการสร้าง DNA ซึ่งจะช่วยให้เซลล์อยู่ในระยะ S, ใช้ paclitaxel ทำให้เซลล์อยู่ในระยะ M และเพาะเลี้ยงเซลล์ในอาหารเลี้ยงเซลล์ที่ปราศจากซีรัมเพื่อทำให้เซลล์ HepG2 อยู่ในระยะ G₂ และทำให้เซลล์ Vero อยู่ในระยะ G₀ จากนั้นจึงนำเซลล์ในแต่ละช่วงมาติดเชื้อไวรัสไข้เลือดออกซีโรไทป์ 2 และ 3 พบว่า ไวรัสไข้เลือดออกทั้งสองซีโรไทป์ ออกจากเซลล์ HepG2 และ เซลล์ Vero ในแต่ละช่วงแตกต่างกัน แต่เมื่อทำการวิเคราะห์เปอร์เซ็นต์การติดเชื้อของเซลล์ในแต่ละช่วงของวัฏจักรของเซลล์ พบว่า เปอร์เซ็นต์การติดเชื้อของเซลล์ HepG2 ในแต่ละช่วงมีความแตกต่างกัน ในขณะที่เปอร์เซ็นต์การติดเชื้อของเซลล์ Vero ในแต่ละช่วงนั้นไม่ต่างกัน จากผลการศึกษาดังกล่าว ตัวตอบรับของไวรัสไข้เลือดออกซีโรไทป์ 2 และ 3 บนผิวเซลล์ HepG2 แสดงออกในแต่ละช่วงของวัฏจักรของเซลล์แตกต่างกัน แต่ในเซลล์ Vero นั้น การแสดงออกของตัวตอบรับของไวรัสไข้เลือดออกทั้งสองซีโรไทป์ไม่แตกต่างกันในแต่ละช่วงของวัฏจักรของเซลล์ หรืออาจเป็นไปได้ว่า ไวรัสไข้เลือดออกทั้งสองซีโรไทป์ เข้าไปในเซลล์ Vero โดยวิธี Membrane fusion ซึ่งไม่จำเป็นต้องอาศัยตัวตอบรับในกระบวนการเข้าเซลล์

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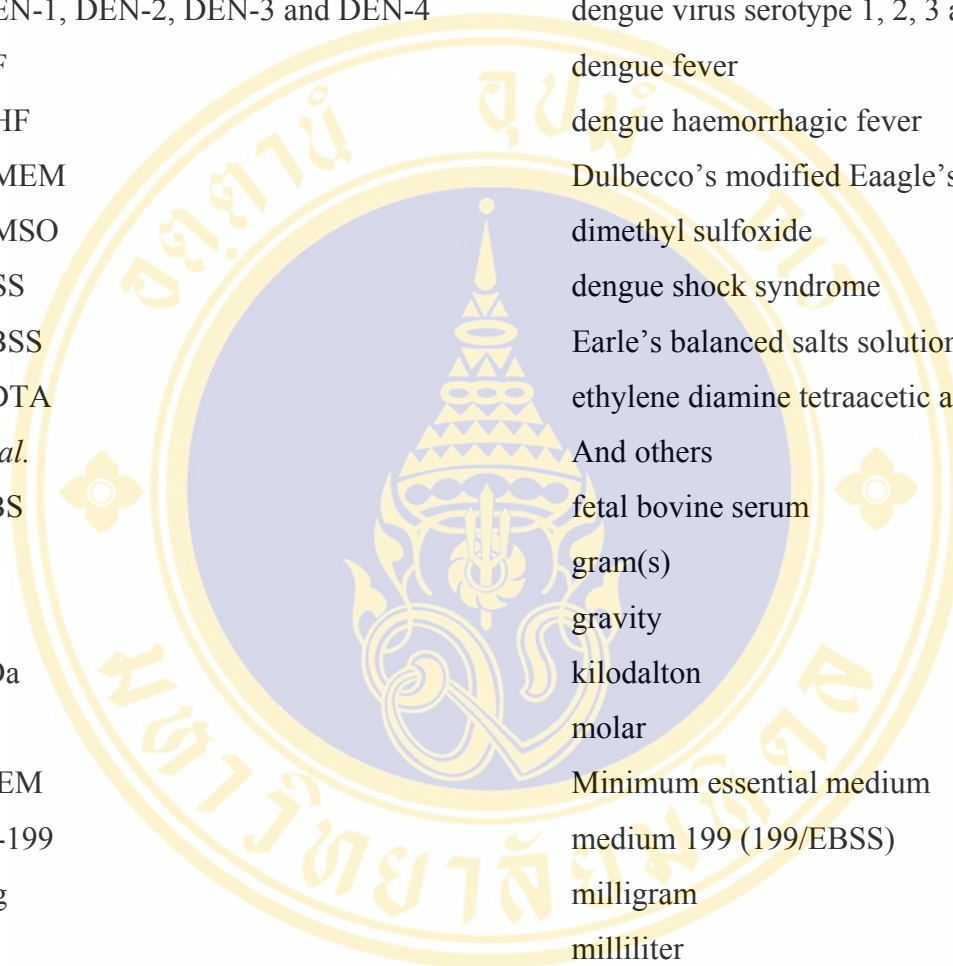
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LIST OF ABBREVIATIONS



DEN-1, DEN-2, DEN-3 and DEN-4	dengue virus serotype 1, 2, 3 and 4
DF	dengue fever
DHF	dengue haemorrhagic fever
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DSS	dengue shock syndrome
EBSS	Earle's balanced salts solution
EDTA	ethylene diamine tetraacetic acid
<i>et al.</i>	And others
FBS	fetal bovine serum
g	gram(s)
g	gravity
kDa	kilodalton
M	molar
MEM	Minimum essential medium
M-199	medium 199 (199/EBSS)
mg	milligram
ml	milliliter
mM	millimolar
moi	multiplicity of infection
NaCl	sodium chloride
°C	degree Celsius
pfu	plaque forming unit
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

LIST OF ABBREVIATIONS (CONT.)

TEMED	N, N, N',N' tetramethylenediamine
Tris-HCl	Tris-(hydroxymethyl)-aminomethane Hydrochloride
μg	microgram
μl	microliter
μM	micromolar



CHAPTER I

INTRODUCTION

1. Epidemiology

Dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) caused by the dengue virus have been globally prevalent over the past 40 years. In 1996, the World Health Organization (WHO) estimated that more than 2.5 billion people now live in areas where dengue is endemic. Up to 20 million people are afflicted annually by the disease, resulting in more than 24,000 deaths. Additionally, there are around 500,000 pediatric cases of DHF which demand intensive cares in hospital each year (1).

Dengue fever was first documented at the beginning of the nineteenth century. However the global epidemiology and transmission dynamic of the dengue virus were changed dramatically in Southeast Asia during the Second World War. Because of the war, hundreds of thousand of Japanese and allied soldiers, most of them susceptible to dengue virus infection, were moving in and out of this area. This gave ideal condition for movement of viruses between cities, countries and regions, as well as providing susceptible individuals for epidemic transmission. Moreover, the disruption and changes in the ecology expanded the geographical distribution and increased densities of the principal mosquitoes vector, which is *Aedes aegypti*, making many countries in this region highly permissive for epidemic transmission. In the years after the war, most countries in Southeast Asia were hyperendemic and a few years later DHF occurred in this region. The first epidemic occurred in Manila, the Philippines in 1953-1954. After that an epidemic occurred in Bangkok, Thailand in 1958. Later on, other Southeast Asia countries as well as some Pacific Islands had evidenced dengue epidemics (2-4).

In Thailand, dengue has long been among the top five of the most common communicable diseases in the country, which are: diarrheal diseases, conjunctivitis, pneumonia, DHF, and malaria(5). In the beginning, about 50 to 100 cases were

diagnosed annually until the first large outbreak of the disease in 1958 when 2,158 cases and 300 deaths were reported. Since then, the reported number of cases has been gradually increasing. In the first few outbreaks, the disease was mainly found in Bangkok and its surrounding areas and the majority of cases were children aged between 2 and 6 years. Since 1965, the disease has been reported in all regions of the country. There are many factors that facilitate the spread of DHF in Thailand. Rapid demographic and societal changes have taken place in the past 20 years. The movement of population from rural areas to Bangkok and other big cities has resulted in uncontrolled urbanization with poor sewage management systems, which facilitated the breeding of mosquitoes. The essential spread of used automobile tyres and plastic materials further intensified the already uncontrolled DHF vector multiplication. Moreover, shortage of piped water supply in both urban and rural areas has created the practice of conserving water in household containers that are potential breeding places of *Aedes* mosquito, the main vector of DHF in Thailand. The massive population movement in recent years has further facilitated the spread of the dengue virus from high endemic areas to the rest of the country. All these factors coupled with ineffective DHF control measures in the past have resulted in high morbidity caused by DHF and the dramatically increasing trend of the disease in Thailand (6).

2. Clinical features and pathogenesis

Dengue virus infection causes a spectrum of illness ranging from inapparent or mild febrile illness to severe and fatal haemorrhagic disease. Infection with any one of four serotypes causes a similar clinical presentation that may vary in severity. The incubation period varies from 3 to 14 days. Dengue fever (DF) is a non-fatal febrile disease associated with sudden onset, extreme malaise, and pain of the muscles, back, limbs and eyes; rash is common as other non-specific constitutional symptoms such as nausea, vomiting and headache. The severe forms of the disease are dengue haemorrhagic fever (DHF) and DHF with plasma leakage that may lead to hypovolaemic shock; dengue shock syndrome (DSS) (figure 1)

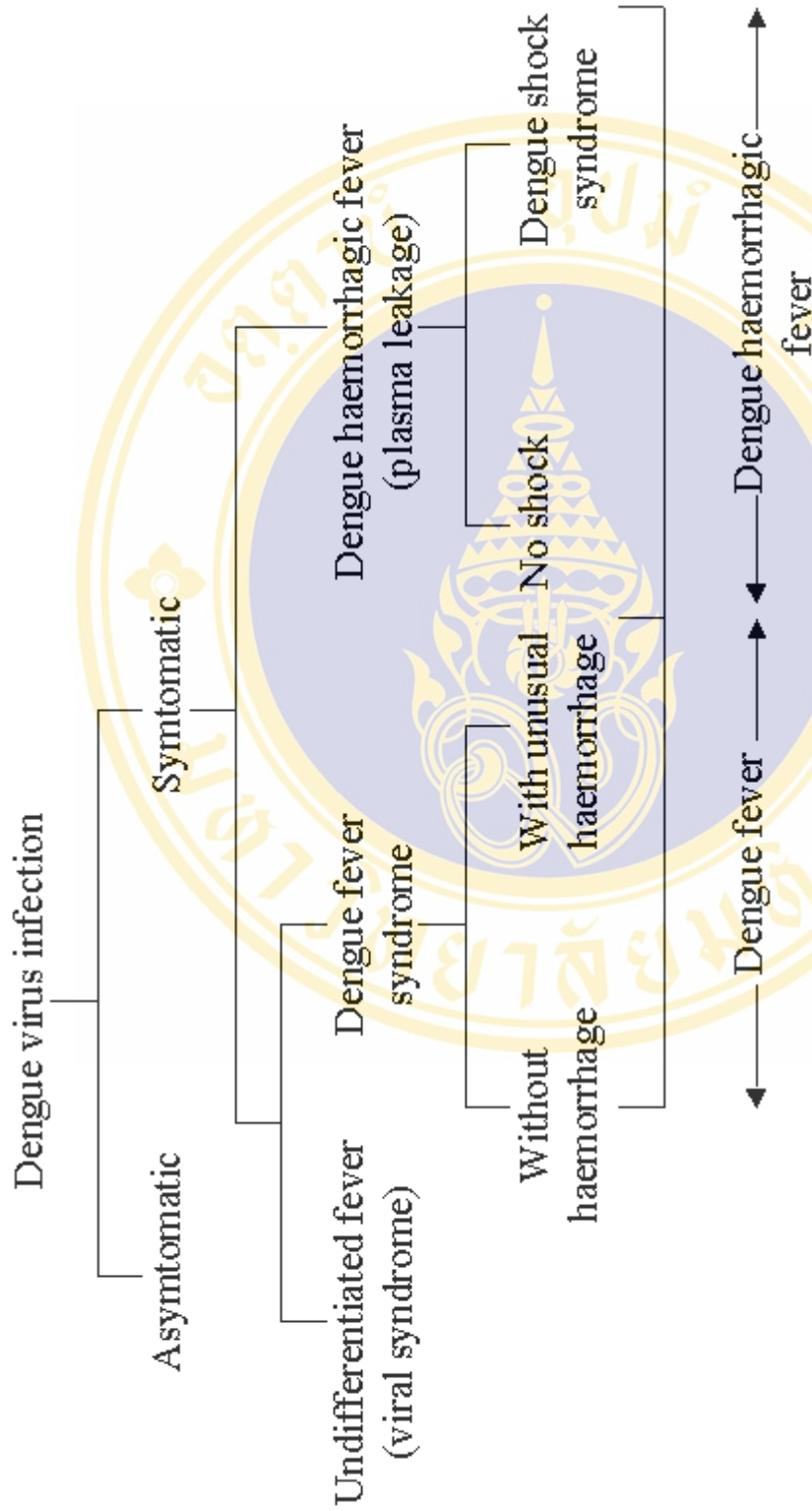


Figure 1. Manifestation of dengue virus infection (1)

The severity of DHF is further graded according to clinical criteria. Grade I, the fever accompanies with nonspecific constitutional symptoms. The only haemorrhagic manifestation is a positive tourniquet test. In grade II, there is spontaneous bleeding, usually from the skin, nose or gums, in addition to the manifestation of grade I. In grade III the circulatory failure manifests by a rapid, weak pulse with narrowing of pulse pressure (<20mm Hg) or hypotension. In grade IV, moribund patients have undetectable blood pressure and pulse. Grade III and IV are called DSS. DHF encompasses all four grades (7).

Two main pathophysiological changes occur in DHF/DSS. One is an increased vascular permeability that gives rise to loss of plasma from the vascular compartment. This results in haemoconcentration, low pulse pressure and other signs of shock, if plasma loss becomes critical. The second change is a disorder in haemostasis involving vascular changes, thrombocytopenia and coagulopathy.

A constant finding in DHF/DSS is the activation of the complement system, with profound depression of C3 and C5 levels. The mediators that increase vascular permeability and the precise mechanism(s) of the bleeding phenomena seen in dengue infection have not been identified yet. Platelet defects may be both qualitative and quantitative. For example, some circulating platelets during the acute period of DHF may be incapable of normal function. Therefore, even a patient with a platelet count greater than 100000 per mm³ may still have a prolonged bleeding time.

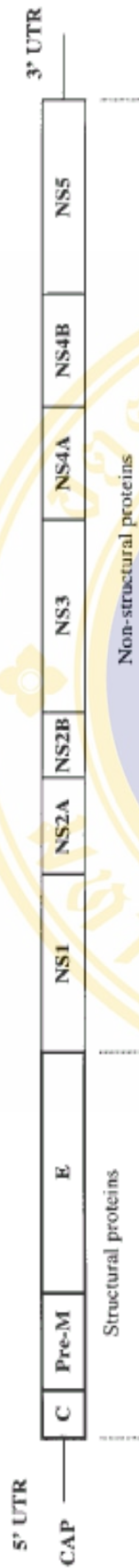
A mechanism that may contribute to the development of DHF/DSS is enhancement of virus replication in macrophages by heterotypic antibodies. In secondary infection with a virus of a different serotype from that causing the primary infection, cross-reactive antibodies that fail to neutralize the virus may increase the number of infected monocytes as dengue virus-antibody complexes are taken into these cells. This may result in the activation of cross-reactive CD4⁺ and CD8⁺ cytotoxic lymphocytes. The rapid release of cytokines caused by the activation of T cells and by the lysis of infected monocytes mediated by cytotoxic lymphocytes may result in the plasma leakage and haemorrhage that occur in DHF (1).

3. Dengue Virus

Dengue virus is a member of the genus *Flavivirus* within the family *Flaviviridae*. There are four serotypes, called DEN-1, DEN-2, DEN-3 and DEN-4, which are antigenically related. Dengue has spherical shape of about 40-50 nm in diameter, with a lipid envelope enclosing an isomeric nucleocapsid core of 30 nm in diameter. The genome of dengue is a positively single-stranded sense RNA of length 10188, 10173, 10170 and 10158 nucleotides for DEN 1-4 respectively and consists of a single open reading frame that encodes a polyprotein precursor of 3396, 3391, 3390 and 3386 amino acids for DEN 1-4 respectively. After the polyprotein precursor is processed by host and viral protease, it generates 10 mature proteins including 3 structural proteins, which are capsid (C), membrane (M) and envelope (E) protein, and 7 non-structural proteins, which are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (2;8;9).(figure 2)

3.1 Cellular targets of virus

Dengue virus has been detected in cells of the monocyte-macrophage lineage in the lymphoid organ, lung, spleen, bone marrow and liver of patients with dengue infection and there are reports of isolation of dengue virus from peripheral blood mononuclear cells during the viraemic period (10-12). Even though monocytes and macrophages are shown to be the major targets for dengue infection, liver involvement in clinical pathogenesis has been corroborated by demonstration of dengue virus RNA by reverse transcription (RT)-PCR in liver tissue samples obtained from children with fatal dengue fever (13;14). Moreover dengue virus antigen has been detected in hepatocytes, and virus particle was recovered from the liver biopsy specimens of DHF patients (11). In addition, the levels of aspartate transaminase (AST) and alanine transaminase (ALT), which are synthesized by liver, in serum were significantly higher (15-17). The cited reports indicate malfunction of the liver, which may be caused by either direct dengue virus infection of liver cells or dengue virus- mediated host immune responses and suggest that the liver is one of the target organ of dengue infection.



Figur e2. Polyprotein of dengue virus

This figure shows polypeptide precursor of dengue virus containing viral structural and non-structural proteins. The dengue virus consists of 4 serotypes with different nucleotide and amino acid length;

DEN-1 : 3396 amino acids; 10188 nucleotides

DEN-2 : 3391 amino acids; 10173 nucleotides

DEN-3 : 3390 amino acids; 10170 nucleotides

DEN-4 : 3386 amino acids; 10158 nucleotides

3.2 Mechanism of dengue entry into cells

Binding of virus particles to receptors brings them in intimate physical contact with the cell surface and sets the stage for crossing the membrane barriers. Viruses then enter the cells either by direct fusion with the plasma membrane or by adsorptive or receptor-mediated endocytosis. Hase *et al.* (18) studied the entry modes of dengue virus into C6/36 mosquito cells and of DEN-2 virus into human peripheral blood monocytes *in vitro*. They found that inoculation of dengue virus into C6/36 cells and human peripheral blood monocytes resulted in direct penetration of the virions into the cytoplasm at the cell surface. In adsorptive endocytosis, the receptor-virus complex is internalized in clathrin-coated pits and delivered to endosomes. The acidic interior of the endosome triggers the viral fusion protein conformational change. Dengue enter into host cells by utilizing one of the two pathways preferentially or by both mechanisms (19).

3.3 Intracellular viral replication

After dengue virus binds to cell surface, it is uptaken by endocytosis or direct fusion of viral cellular membrane. (figure 3) Once in the endocytic vesicle and following lowering of the pH in the endosomal milieu the virus envelope proteins undergoes an irreversible conformational change, from a dimer to a trimer (18). This change facilitates the subsequent fusion of the virus envelope and host cell endosomal membrane, and the nucleocapsid is released into the cytoplasm. This is followed by the immediate translation of the uncoated viral genome, a single stranded positive-sense RNA. After that the 5' untranslated region (5'UTR) directs the RNA to the ribosomes, where the translation of the single open reading frame into a precursor polyprotein occurs. The viral polyprotein is processed co- and posttranslationally into individual and functional viral proteins. Cellular and viral proteases carry out this process. The RNA-dependent RNA polymerase associated with cofactors, produces minus-strand single-stranded RNA, which in turn serves as a template for the production of new plus-strand single-stranded RNA. After replication, the viral genome is encapsidated in the nucleocapsid proteins and directed to the endoplasmic

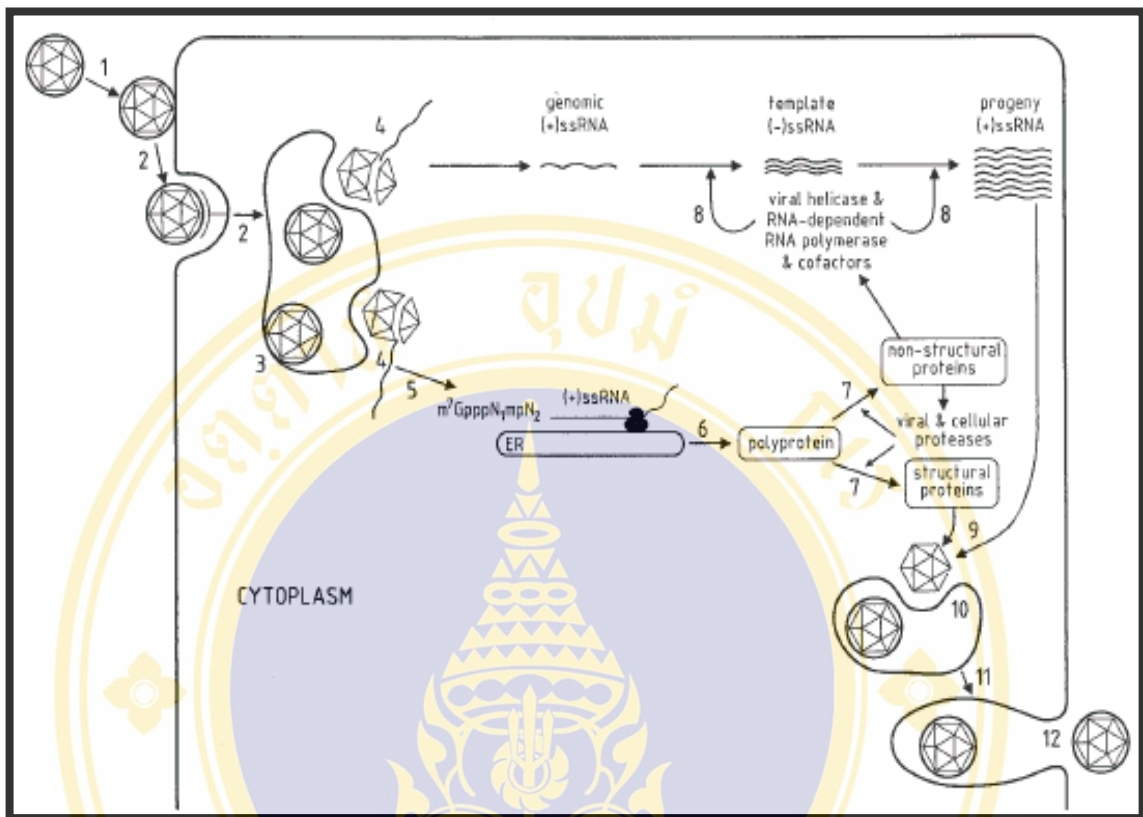


Figure 3. The replicative cycle of members of the Flavivirus. (2)

reticulum or other membranous structures induced by viral infection, where the immature virus, surrounded by a lipid envelope containing viral proteins, buds off into the lumen. Finally, the enveloped proteins become glycosylated and mature viruses are released into the extracellular space (2).

3.4 Putative receptor(s) for dengue virus

The identification of dengue virus receptors on target cells is still not definitive, although the involvement of the virus envelope protein in the process has not been debated. Infection of some cells may involve anti-dengue antibody-mediated immune adherence by means of the Fc domain of antibody that bound to virus, mediating binding to cells. Daughaday *et al.* (20) described that dengue virus could infect cultured monocytes in two ways: through a trypsin sensitive receptor or through trypsin insensitive Fc receptor (FcR), while Chen *et al.* (21) reported that a recombinant dengue virus envelope-Fc fusion protein were unable to detect binding to human monocyte other than via the FcR. Later, another cell that can use FcR to mediate dengue infection is human platelet (22).

In addition, there are many trypsin sensitive proteins, which have been reported to be receptors for dengue infection. Moreno-Altamirano *et al.* (23) studied about the putative receptors on monocyte-macrophages and they found that possible receptors for DEN-2 on monocyte-macrophages are membrane protein with apparent molecular masses of 27,45,67 and 87 kDa. Moreover Salas-Benito *et al.* (24) reported that dengue virus binding molecules on the surface of C6/36 cells are glycoproteins of 40 and 45 kDa. Other cells that use trypsin sensitive proteins as the putative receptors are human hepatoma HepG2 cells and simian kidney Vero cells (25).

Additionally, Glycoaminoglycans such as heparan sulphate (HS) can be used for initial binding to the target cells. HS is a repeating highly negatively charged linear copolymer of variably sulfated uronic acid and glucosamine carbohydrate residues. For example, there are studies that found that HS is the important host components for dengue virus infection in human liver cell line-such as HuH-7, HA22T, Hep3B, PLC, and Chang liver (26;27), Vero, CHO cells (28)and baby hamster kidney cells (29).

Another putative receptor that has been studied is CD14 associated molecule. Chen *et al.* (30) studied about dengue infection in monocyte/ macrophages. They found that bacterial lipopolysaccharide blocked dengue virus entry into human

monocyte/macrophages via its receptor CD14 and that a CD14-associated cell surface structure may be essential for the initiation of dengue virus infection.

4. Receptors and cell cycle

There are many receptors that are cell cycle dependent receptors.

4.1 Cell cycle

A cell reproduces by carrying out an orderly sequence of events in which it duplicates its contents and then divides in two. This cycle of duplication and division, known as the cell cycle, is the essential mechanism by which all living things reproduce. In unicellular organisms, such as bacteria or yeast, each cell division produce a complete new organism, while in multicellular organisms, many rounds of cell division are required to make a new individual from the single-celled egg.

The duration of the cell cycle varies greatly from one cell type to another. Single-celled yeast can divide every 90-120 minutes in ideal conditions, while a mammalian liver cell divides on average less than once a year (table1) (31). A typical eukaryotic cell cycle is illustrated by human cell in culture, which divide approximately every 24 hours.

Table 1 Some eukaryotic cell-cycle frequency (31)

Cell Type	Cell-Cycle Frequency
Early frog embryo cells	30 minutes
Yeast cells	1.5-3 hours
Intestinal epithelial cells	~12 hours
Mammalian fibroblasts in culture	~20 hours
Human liver cells	~1 year

As viewed under the microscope, the cell cycle is divided into two parts: mitosis and interphase. Mitosis (nuclear division) is the most dramatic stage of the cell cycle, corresponding to the separation of daughter chromosomes and usually ending with cell division (cytokinesis). These two processes together constitute the M phase of the cell cycle. However the M phase last only about an hour, so approximately 95% of the cell cycle is spent in interphase. During interphase, the chromosomes are decondensed and distributed throughout the nucleus, so the nucleus appears morphologically uniform. At the molecular level, however, interphase is the time during which both cell growth and DNA replication occur in an orderly manner in preparation for cell division. Interphase is divided into the remaining three phases of the cell cycle. During S phase (S = synthesis), the cell replicates its nuclear DNA, an essential prerequisite for cell division. S phase is flanked by two phases where the cell continues to grow. The G₁ phase (G = gap) is the interval between the completion of M phase and the beginning of S phase (DNA synthesis). The G₂ phase is the interval between the end of S phase and the beginning of M phase. In addition, entrance into and exit out of the cell cycle occurs as a cell passes between active proliferation and a quiescent or G₀ state, in which the fundamental metabolism of the cell is depressed, including many of its usually active functions such as transcription and protein synthesis. Deprivation of growth factors can cause a cell to exit into G₀, whereas stimulation with growth factors can signal a cell to re-enter the active cycle (32).(figure 4)

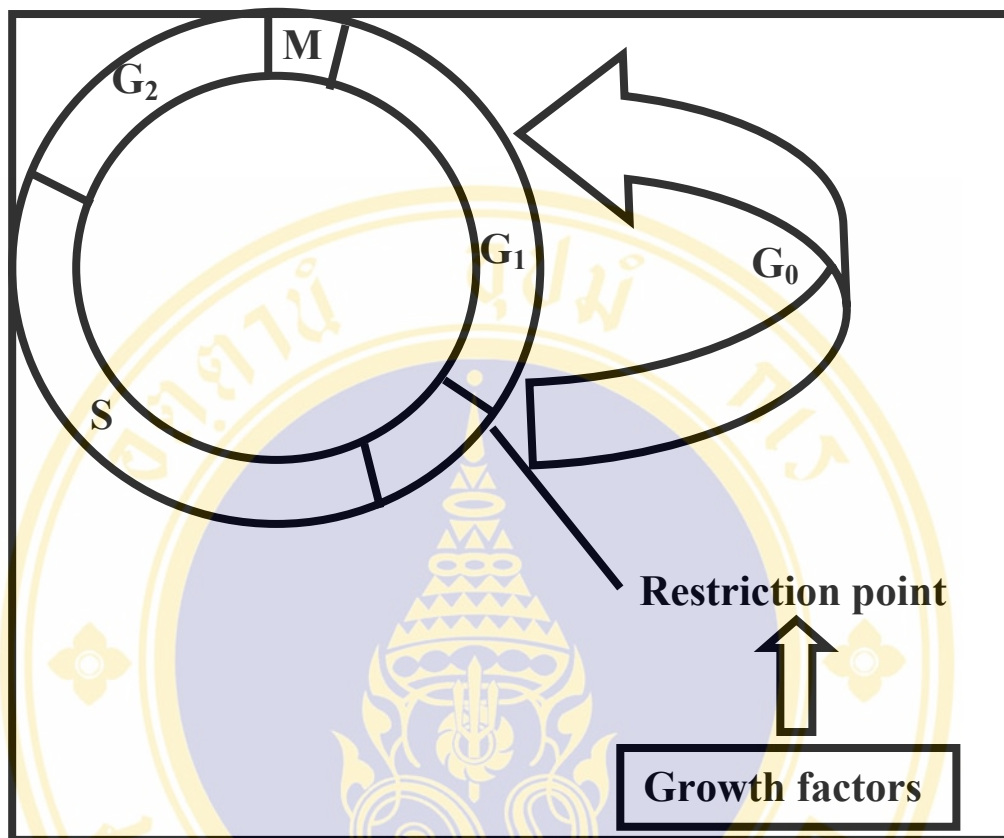


Figure 4. Regulation of animal cell cycles by growth factors

The availability of growth factors controls the animal cell cycle at a point in late G₁ called the restriction point. If the growth factors are not available during G₁, the cells enter quiescent stage of the cycle called G₀ (32).

The DNA content can distinguish cells at different stages.(figure 5) For example, animal cells in G_1 are diploid (containing two copies of each chromosome). So their DNA content is referred to as $2n$ (n designates the haploid DNA content of the genome). During S phase, replication increases the DNA content of the cell from $2n$ to $4n$ so cells in S have DNA contents ranging from $2n$ to $4n$. DNA content then remains at $4n$ for cells in G_2 and M, decreasing to $2n$ after cytokinesis. Experimentally, cellular DNA content can be determined by incubation of cells with a fluorescent dye that bind to DNA, followed by analysis of the fluorescence intensity of individual cells in a flow cytometer or fluorescence-activated cell sorter, thereby distinguishing cell in the G_0/G_1 , S, and G_2/M phases of the cell cycle(32).

4.2 Receptors and cell cycle

There are many receptors that express differently in each phase of the cell cycle. For example, Cassanelli *et al.* (33) used immunofluorescence techniques to label progesterone receptor (PR) on the MCF-7 human breast cancer cell line and used image cytometry to analyze the PR expression during G_0 , G_1 , S and G_2/M cell-cycle phases. They found that PR synthesis occurs preferentially during the G_0-G_1 transition and that PR levels are constant during the G_1-G_2 transition. Not only PR, but vitamin D receptor (VDR) expression also relates to the cell cycle. Segaert *et al.* (34) studied the expression of VDR on cultured human keratinocytes. They observed a concomitant loss of VDR expression when keratinocytes were forced into quiescence by depriving growth factors. Mitogenic stimulation of these G_0 cells however quickly unregulated VDR levels several hours ahead the G_1-S transition point. Growth arrest at the G_1-S border by mimosine treatment or at the metaphase by nocodazole also down regulated VDR levels but a restoration of VDR expression was again quickly achieved after reentering the cell cycle. These finding indicated that VDR expression in keratinocytes is restricted to actively cycling cells, but not limited to one particular phase of the cell cycle. In addition, interferon- α/β receptor (IFNAR) also express depend on the cell cycle. Takane *et al.* (35) found that the expression of IFNAR mRNA significantly increased when the proportion of murine B16 melanoma cells in DNA

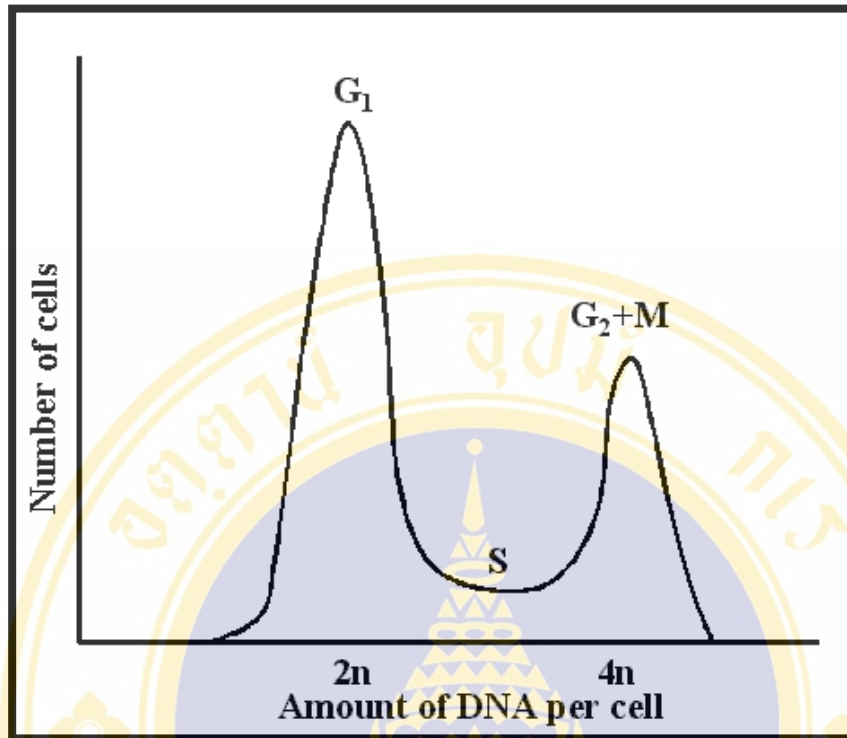


Figure 5. Determination of cellular DNA content

A population of cells is labeled with a fluorescent dye that binds DNA. The cells are then passed through a flow cytometer, which measures the fluorescence intensity of individual cells. The data are plotted as cell number versus fluorescence intensity, which is proportional to DNA content. The distribution shows two peaks, corresponding to cells with DNA contents of $2n$ and $4n$; these cells are in G_1 and G_2 / M phases of the cell cycle, respectively. Cells in S phase have DNA contents between $2n$ to $4n$ and are distributed between these two peaks (32).

synthesis (S) phase increased in vitro. Moreover, Majoul *et al.* (36) studied the receptor for cholera and shiga toxin in Vero cells. Cholera and shiga toxins bind to the cell surface via glycolipid receptors GM1 and Gb3, respectively. Their results show that Cholera toxin binds preferentially in G₀/G₁, with little binding through S-phase to telophase, whereas Shiga toxin binds maximally through G₂ to telophase but does not bind during G₀/G₁ and S phase. Furthermore, there is the receptor for *Toxoplasma gondii*, an obligate intracellular parasite that has the same manner like those previous receptors. Grimwood *et al.* (37) studied the receptor of *Toxoplasma gondii* on Chinese hamster ovary cells and bovine kidney cells. Their results suggest that *Toxoplasma gondii* bind specifically to a host cell receptor, which is upregulated in the mid-S phase of the cell cycle.

However, there are some viral receptors that express depending on the cell cycle. Wells *et al.* (38) interested in Epstein-Barr virus (EBV) receptor. EBV receptor positive cells absorbed FITC-conjugated virions and were subsequently stained for DNA content with propidium iodide. These cells were analyzed by flow cytometer to determine the relationship between cell cycle and EBV receptor expression on single cells. From their results, EBV receptor was present throughout the entire cell cycle. The level of expression, however, varied; increasing in G₁ and G₂/M while remaining approximately constantly in S phase.

Another viral receptor that also expresses differently in the cell cycle is Adenovirus (Ad) receptor. Seidman *et al.* (39) evaluated Ad association with cells in both unsynchronized and pharmacologically synchronized cell populations. In unsynchronized cell populations, elevated Ad association with cells correlated with expression of cyclin B1, a marker of entry into the M phase. The same analysis conducted on cell populations that were synchronized at M phase or at S phase confirmed that M phase cells bound three- to sixfold more than unsynchronized cells, which are primarily in the G₁ and G₂ phases. Moreover, assessment of cell surface expression of Ad receptors demonstrated that both the high-affinity coxakie-adenovirus receptor for Ad fiber protein and the low affinity α_v integrin receptor for Ad penton base protein showed increased cell surface expression at M phase. These results demonstrate that Ad infection of a homogenous population of cells can vary

depending on the cell cycle stage, with enhanced Ad binding and expression correlating with the enhanced expression of Ad receptors during M phase.

Besides, the cell cycle may effect viral infection by changing the environment in the cells. For example, the cell cycle of host cells may effect the infection of retrovirus such as Rous sarcoma virus (RSV), Murine leukemia virus (MLV) and human foamy virus (HFV) (40;41). Following entry into the cell cytoplasm, the retroviral genome must gain access to host cell chromosomes within the nucleus to achieve stable integration. During this process the retroviral DNA is present on a large complex with a subset of retroviral proteins known as the preintegration complex (PIC) (42). The physical size of the PIC is thought to exceed the upper limit for passive diffusion through nuclear pores. In nondividing cells, viral entry, uncoating, DNA synthesis, and formation of the MLV PIC occur at the same rate as in dividing cells, but integration fails to occur (43;44). During mitosis, however, the nuclear membrane disassembles, rendering the chromosome accessible to the MLV PIC.(44) These finding could explain the conclusion that infection by retroviruses such as MLV, RSV and HFV require cell division (40;41;43-46). However, the requirement for mitosis during infection is not common to all retrovirus. Indeed, lentiviruses are able to infect certain types of nondividing cells (43;47). This property is thought to be due to the ability of lentiviral PIC to be actively transported across the membrane (48). Although the precise mechanism by which nuclear entry is achieved remains elusive, nuclear localization signals (NLS) have been identified on both Vpr and matrix proteins of human immunodeficiency virus type 1 (HIV-1) and were initially thought to direct nuclear localization of the PIC (49).

CHAPTER II

OBJECTIVE

Dengue is one of the most important international health problems in tropical and subtropical region around the world. Dengue haemorrhagic fever and dengue shock syndrome are a leading cause of hospitalization in many countries in Southeast Asia. As of today, there is no drug to treat or vaccine to prevent this disease. As such dengue will continue to be a significant problem in many parts of the world.

The pathogenesis of DHF and DS is not well understood. As such knowledge about the receptors for dengue is necessary because the initial binding of a virus to a target cell is a critical determinant of cell and tissue specific tropism and thus of pathogenesis. There are many evidences suggest that liver is one of the target organs of dengue infection but the knowledge about the receptor for dengue in liver cells is not clear.

The aim of this thesis is to determine whether the receptors for dengue are expressed in a cell cycle dependent manner in both a target cell line (HepG2) and a non-target cell line (Vero).

CHAPTER III

MATERIALS AND METHODS

1. source of materials

1.1 Chemical

Bovine serum albumin fraction V (BSA)	Sigma, Missouri, USA
Crystal violet	Bio Basic Inc, Ontario, Canada
Dimethyl sulfoxide (DMSO)	Amresco®, Ohio, USA
EDTA	Bio Basic Inc, Ontario, Canada
Gentamycin	TP. Drug Laboratory, Bangkok, Thailand
Lactabumin hydrolysate	Sigma, Missouri, USA
Paclitaxel	Sigma, Missouri, USA
Penicillin/Streptomycin	Hyclone, Utah, USA
Thymidine	Sigma, Missouri, USA

1.2 Culture media and reagents

Bovine Calf serum	GIBCO BRL, Maryland, USA
Dulbecco's Modified Eagle's medium (DME / High Glucose powder)	GIBCO BRL, Maryland, USA
Dulbecco's Modified Eagle's medium (DME / Low Glucose powder)	GIBCO BRL, Maryland, USA
Fetal bovine serum	GIBCO BRL, Maryland, USA
Medium 199 (199/EBSS)	HyClone, Utah, USA
MEM	GIBCO BRL, Maryland, USA
Trypsin (1:250)	GIBCO BRL, Maryland, USA

1.3 Miscellaneous materials

Rabbit anti-GRP78 (GRP78(H-129))	Santa Cruz Biotechnology, California, USA
ImmunoPure® Goat Anti-Rabbit IgG, (H+L) peroxidase conjugated	Santa Cruz Biotechnology, California, USA

Cycle test plus DNA reagent kit Becton Dickinson, California, USA

-Solution A

contains trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic disaggregation of the solid tissue fragments and digestion of cell membranes and cytoskeletons.

-Solution B

contains trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA.

-Solution C

contains propidium iodide (PI) and spermine tetrahydrochloride in citrate stabilizing buffer.

ECL Plus detection kit Amersham Biosciences, Little Chalfont
Buckinghamshire, England

Nitrocellulose Transfer Membrane PROTRAN®, New Hampshire, USA

Prestained protein marker Fermentas, Maryland, USA

Seakem L.E. Agarose BMA, Maine, USA

Standard protein marker Bio-Rad Laboratories Ltd., California, USA

1.4 Cell lines

1.4.1 HepG2 : hepatoma cell line from *Homo sapiens* (human)

1.4.2 Vero : kidney cell line from *cercopithecus aethiops* (African green monkey)

1.5 Dengue virus

DEN-1 virus strain 16007

DEN-2 virus strain 16681

DEN-3 virus strain 16562

DEN-4 virus strain 1036

1.6 Culture medium

HepG2 : Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin

Vero : DMEM, 5% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin

2. Counting cells

The number of cells was determined by using a hemocytometer. The cells in ten squares of the 0.1 mm³ chamber formed by the 1x1 mm square and 0.1 mm height of the coverslip were counted under the microscope. Before a drop of the cell suspension was dropped to a side of the hemocytometer, the cells were trypsinized into single cells. The number of cells was calculated to an accurate cell number per milliliter of cell suspension (cells/ml).

3. Culturing of HepG2 and Vero cells

The human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37 °C / 10% CO₂, 100 units/ml penicillin and 100 µg/ml streptomycin. The African green monkey kidney cell line Vero was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat inactivated fetal bovine serum (FBS) at 37 °C / 5% CO₂, 100 units/ml penicillin and 100 µg/ml streptomycin.

4. Viral stocks

Subconfluent Vero cells were infected with dengue virus at moi 0.1. Infected cells were incubated in DMEM supplemented with 5% FBS at 37°C / 5%CO₂. The medium containing the virus was clarified by centrifugation at 1,200 rpm at room temperature for 5 minutes on the day which had the highest viral yield. The supernatant was supplemented with 20% (v/v) FBS and kept at -80 °C.

5. Viral production

1.17x10⁶ Vero cells in 10 cm diameter plate were infected with 2 ml dengue virus serotype 1-4 at moi 1 at 37°C / 5% CO₂ for 1.5 hours. After that the infected cells were washed once with PBS, treated with 1 ml acid glycine pH3 for 1 minute and washed with PBS again. DMEM supplemented with 5% FBS 10 ml was added and the infected cells were incubated at 37°C / 5% CO₂. The growth medium containing dengue virus was collected every hour for 24 hours and titered by plaque assay.

6. Synchronization of HepG2 cells into G₀ phase by serum starvation method, which is modified from the method in Yeh et al. (50)

Method 1

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ for 36 hours.

Method 2

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ for 48 hours.

Method 3

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ for 3 days.

Method 4

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM without FBS at 37°C / 10% CO₂ for 36 hours.

Method 5

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM supplemented without FBS at 37°C / 10% CO₂ for 48 hours.

Method 6

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM low glucose (GIBCO BRL) without FBS at 37°C / 10% CO₂ for 36 hours.

Method 7

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5 ml DMEM low glucose (GIBCO BRL) without FBS at 37°C / 10% CO_2 for 48 hours

7. Synchronization of HepG2 cells into G₂ phase

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5 ml MEM (GIBCO BRL) without FBS at 37°C / 10% CO_2 for 36 hours.

8. Synchronization of HepG2 cells into M phase by using paclitaxel (39;51)

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5 ml $0.1 \mu\text{M}$ paclitaxel in DMEM supplemented with 10% FBS at 37°C / 10% CO_2 for 24 hours.

9. Synchronization of HepG2 cells into G₁ phase (52)**Method 1**

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5 ml 0.1% DMSO in DMEM supplemented with 10% FBS at 37°C / 10% CO_2 for 96 hours.

Method 2

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5 ml 0.2% DMSO in DMEM supplemented with 10% FBS at 37°C / 10% CO_2 for 96 hours.

10. Synchronization of HepG2 cells into S phase

Method 1 (39)

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5ml DMEM supplemented with 10 % FBS which contains 5 $\mu\text{g/ml}$ of aphidicolin at 37°C / 10% CO_2 for 24 hours.

Method 2 (39)

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5ml DMEM supplemented with 10 % FBS which contains 5 $\mu\text{g/ml}$ of aphidicolin at 37°C / 10% CO_2 for 48 hours.

Method 3 (53)

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 2mM thymidine in 5 ml DMEM supplemented with 0.1% FBS at 37°C / 10% CO_2 for 12 hours. After that the cells were grown in 5 ml DMEM supplemented with 10% FBS at 37°C / 10% CO_2 for 10 hours. Finally the cells were incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 10% CO_2 for 12 hours.

Method 4 (54)

Approximately 1×10^6 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. The cells were then incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 10% CO_2 for 12 hours. After that the cells were grown in 5 ml DMEM supplemented with 10% FBS at 37°C / 10% CO_2 for 16 hours. Finally the cells were incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 10% CO_2 for 12 hours.

11. Synchronization of Vero cells into G₀ phase by serum starvation method, which is modified from the method in Yeh et al. (50)

Method 1

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM supplemented with 0.1% FBS at 37°C / 5% CO₂ for 36 hours.

Method 2

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM without FBS at 37°C / 5% CO₂ for 36 hours.

Method 3

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM without FBS at 37°C / 5% CO₂ for 48 hours.

Method 4

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO₂ for 1 day. The cells were then incubated in 5 ml DMEM low glucose (GIBCO BRL) without FBS at 37°C / 5% CO₂ for 36 hours.

Method 5

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO₂ for 1 day. The cells were then incubated 5 ml in DMEM low glucose (GIBCO BRL) without FBS at 37°C / 5% CO₂ for 48 hours.

Method 6

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO₂ for 1 day. The cells were then incubated in MEM (GIBCO BRL) without FBS 5 ml at 37°C / 5% CO₂ for 36 hours.

12. Synchronization of Vero cells into M phase by using paclitaxel (39;51)

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in 5-cm diameter plate at 37°C / 5% CO_2 for 1 day. The cells were then incubated in $0.1 \mu\text{M}$ paclitaxel in 5 ml DMEM supplemented with 5% FBS at 37°C / 5% CO_2 for 24 hours.

13. Synchronization of Vero cells into G_1 phase (52)

Method 1

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO_2 for 1 day. The cells were then incubated in 5 ml 0.1% DMSO in DMEM supplemented with 5% FBS at 37°C / 5% CO_2 for 96 hours.

Method 2

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO_2 for 1 day. The cells were then incubated in 5 ml 0.2% DMSO in DMEM supplemented with 5% FBS at 37°C / 5% CO_2 for 96 hours.

14. Synchronization of HepG2 cells into S phase

Method 1 (39)

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO_2 for 1 day. The cells were then incubated in 5ml DMEM supplemented with 5 % FBS which contains $5 \mu\text{g/ml}$ of aphidicolin at 37°C / 5% CO_2 for 24 hours.

Method 2 (39)

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO_2 for 1 day. The cells were then incubated in 5ml DMEM supplemented with 5 % FBS which contains $5 \mu\text{g/ml}$ of aphidicolin at 37°C / 5% CO_2 for 48 hours.

Method 3 (53)

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at $37^\circ\text{C} / 5\% \text{CO}_2$ for 1 day. The cells were then incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at $37^\circ\text{C} / 5\% \text{CO}_2$ for 12 hours. After that the cells were grown in 5 ml DMEM supplemented with 5% FBS at $37^\circ\text{C} / 5\% \text{CO}_2$ for 10 hours. Finally the cells were incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at $37^\circ\text{C} / 5\% \text{CO}_2$ for 12 hours.

Method 4 (54)

Approximately 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at $37^\circ\text{C} / 5\% \text{CO}_2$ for 1 day. The cells were then incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at $37^\circ\text{C} / 5\% \text{CO}_2$ for 12 hours. After that the cells were grown in 5 ml DMEM supplemented with 5% FBS at $37^\circ\text{C} / 5\% \text{CO}_2$ for 16 hours. Finally the cells were incubated in 5 ml 2mM thymidine in DMEM supplemented with 0.1% FBS at $37^\circ\text{C} / 5\% \text{CO}_2$ for 12 hours.

15. Flow cytometry analysis of HepG2 cells and Vero cells

Approximately 1×10^6 HepG2 cells and Vero cells, which were synchronized into each phase of the cell cycle by each method as described above, were detached from the plate by trypsinization, washed twice with PBS. The cells were resuspended in 100 μl PBS and transferred to a polystyrene tube. After that 125 μl solution A in Cycle test plus DNA reagent kit (Becton Dickinson), which contains trypsin, was added and mixed gently by tapping the tube by hand for 10 minutes at room temperature. Then 100 μl solution B, which contains trypsin inhibitor and ribonuclease A, was added and mixed gently like solution A for 10 minutes at room temperature. Finally, 100 μl solution C, which contains propidium iodide, was added and gently mixed as above for 10 minutes in the dark on ice. The sample was analyzed on the flow cytometer within 3 hours after addition of solution C.

16. Viral infection of synchronized HepG2 cells

Firstly, 7×10^5 HepG2 cells were seeded and grown in 5 ml DMEM supplemented with 10% FBS in a 5-cm diameter plate at 37°C / 10% CO_2 for 1 day. After HepG2 cells were synchronized into each phase of the cell cycle by each method as mention above, the medium was discarded. The cells were incubated with dengue virus serotype 2 or 3 at moi 1 in 1 ml BA-1 diluent buffer at 37°C / 10% CO_2 for 1 hour. The infected cells were then washed once with PBS, treated with 1 ml acid glycine pH3 for 1 minute and washed with PBS again. 5 ml DMEM supplemented with 10% FBS was added and the infected cells were incubated at 37°C / 10% CO_2 . Finally the medium containing infectious viruses was collected at 19 hours to 22 hours and was titered by plaque assay. The result comes from duplicate experiments and duplicate count by plaque assay.

17. Viral infection of synchronized Vero cells

Firstly, 7×10^5 Vero cells were seeded and grown in 5 ml DMEM supplemented with 5% FBS in a 5-cm diameter plate at 37°C / 5% CO_2 for 1 day. After Vero cells were synchronized into each phase of the cell cycle by each method as mention above, the medium was discarded. The cells were incubated with dengue virus serotype 2 or 3 at moi 1 in 1 ml BA-1 diluent buffer at 37°C / 5% CO_2 for 1 hour. The infected cells were then washed once with PBS, treated with 1 ml acid glycine pH3 for 1 minute and washed with PBS again. 5 ml DMEM supplemented with 5% FBS was added and the infected cells were incubated at 37°C / 5% CO_2 . Finally the medium containing infectious viruses was collected at 16 hours to 20 hours and was titered by plaque assay. The result comes from duplicate experiments and duplicate count by plaque assay.

18. Plaque assay

Virus samples were diluted ten fold serially in BA-1 diluent buffer. Then, confluent Vero cells in 6 well plates were infected with serially diluted virus at 37°C / 5% CO_2 for 1.5 hours. During the adsorption period, the first overlay solution was prepared. The 2X nutrient solution was incubated at 42°C until used. Autoclaved 1.6%

(w/v) Seakem LE agarose (FMC) in water was melted. After 1.5 hours, the first overlay mixture solution was gently added on the infected cells and allowed to stand for 30 minutes. The plates were incubated upside down at 37°C / 5%CO₂. On the eighth day, 3.7% formaldehyde in PBS was added to fix the cells for at least 1 hour. After that, the fixed cells were stained with crystal violet solution. The number of plaques were counted and calculated in plaque forming unit per virus sample 1 ml (pfu/ml).

Titer (pfu/ml) = number of plaques x sDF x iDF

sDF = serial dilution factor (e.g. 10⁴) of the well

iDF = injection dilution factor e.g. if the injection volume is 0.25 ml; the iDF = 1 ml / 0.25 ml = 4

19. Percent infectivity of synchronized HepG2 cells

The unsynchronized and synchronized HepG2 cells, which were synchronized into each phase of the cell cycle by each method as mention above, were incubated with dengue virus serotype 2 or 3 in 1ml BA-1 diluent buffer at 37°C / 10%CO₂ for 1 hour. The infected cells were then washed once with PBS, treated with 1 ml acid glycine pH3 for 1 minute and washed with PBS again. 10 ml DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized, counted and centrifuged at 1,200 rpm to spin the cells down. Then the infected cells were resuspended in DMEM supplemented with 10% FBS and ten fold serially diluted. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method.

$$\% \text{ Infectivity} = \frac{\text{number of plaques} \times 100}{\text{number of cells in that well}}$$

20. Percent infectivity of synchronized Vero cells

The unsynchronized and synchronized Vero cells, which were synchronized into each phase of the cell cycle by each method as mention above, were incubated with dengue virus serotype 2 or 3 in 1ml BA-1 diluent buffer at 37°C / 5%CO₂ for 1 hour.

The infected cells were then washed once with PBS, treated with 1 ml acid glycine pH3 for 1 minute and washed with PBS again. 10 ml DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized, counted and centrifuged at 1,200 rpm to spin the cells down. Then the infected cells were resuspended in DMEM supplemented with 5% FBS and ten fold serially diluted. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The way to calculate the percent infectivity is given in section 19.

21. Electrophoresis of membrane proteins of HepG2 cells

21.1 Membrane protein extraction

HepG2 cells were scraped from the plate into TBS buffer and centrifuged at 310g at room temperature for 5 minutes. After this step, the cells were always kept on ice. The supernatant was discarded and the pellet was resuspended in lysis buffer (100 mM NaCl, 20 mM Tris (pH8), 2 mM Mg Cl₂, 1 mM EDTA, 0.2% TritonX-100) by vortexing. Then the solution was centrifuged at 610g at room temperature for 5 minutes and the supernatant was taken to centrifuge at 6,000g at room temperature for 5 minutes. After that the supernatant was taken to centrifuged at 20,800g at room temperature for 5 minutes. The pellet, which is the membrane protein, was resuspended in lysis buffer and kept at -80°C.

21.2 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of proteins was performed according to the protocols of Sambrook (55). SDS polycrylamide gels consist of a resolving or separating gel and a stacking gel (Table 2). The sample was electrophoresed in Tris-glycine buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS) at constant voltage of 100 V at room temperature. After electrophoresis, the protein bands on the gel were visualized by soaking in staining solution containing 50% ethanol, 7% glacial acetic acid and 0.1% Coomassie Brilliant Blue R-250 in water for 2 hours. Background was removed by soaking the gel in destaining solution (25% ethanol and 12.5% glacial acetic acid) overnight.

Table 2. Solutions for preparing SDS-polyacrylamide gel electrophoresis (55)

Component	8% Resolving gel	5% Stacking gel
	20 ml	5 ml
30% acrylamide mix (29% (w/v) acrylamide, 1% (w/v) N, N' - methylene - bis - acrylamide)	5.3	1
1.5M Tris-HCl (pH 8.8)	5	-
1.0M Tris-HCl (pH 6.8)	-	0.75
10% SDS	0.2	0.060
H ₂ O	9.3	4.1
10% ammonium persulfate	0.2	0.060
TEMED	0.012	0.010

21.3 Electrotransfer of membrane proteins from SDS-PAGE to nitrocellulose membrane

After protein samples were separated, the SDS-PAGE gel was soaked in transfer buffer (15.6 mM Tris Base, 120 mM glycine) for 15 minutes. The nitrocellulose membrane and six pieces of filter paper were cut into same size as that of the gel and soaked in transfer buffer for 15 minutes. Wet-blot Electrophoresis Transfer cell (Bio-Rad) was used to electrophoretically transfer the protein samples from SDS gels to nitrocellulose membranes. A pre-soaked fiber pad was placed on to the side of the cassettes, followed by three pieces of filter papers, SDS gel, nitrocellulose membrane, three additional pieces of filter papers and finally a second fiber pad. This entire cassette was added into the electrophoretic tank. Precooled transfer buffer was added. The electroblotting was carried out at constant voltage of 30 V at 4°C overnight.

21.4 Detection of GRP78

After the electrotransfer process was completed, the membrane was soaked in blocking solution (5% skim milk in TBS (20 mM Tris-HCl (pH7.5), 140 mM NaCl)), which enough to cover it, for 1 hour at room temperature. The membrane was incubated for 2 hours at room temperature in the blocking solution containing a 1/500 dilution of rabbit serum anti-GRP78 antibody (Santa Cruz Biotechnology, Inc.). The excess primary antibody was removed by washing the membranes 3 times with TBS buffer at room temperature. A 1/2000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.) was added and incubated at room temperature for 1 hour. After washing 3 times with TBS buffer, the membrane was developed by ECL Plus detection kit (Amersham). The amount of antibody and ECL Plus detection kit used depended upon the size of the membrane.

22. Data analysis

All of the dengue virus infection data were analyzed by using the GraphPad Prism Program (GraphPad Software Inc, San Diego, USA). For the percent infectivity result, the data sets were compared for significantly different in order to determine that whether the percent infectivity is different among each phase of the cell cycle. The

student t-test was used to compare between the two data sets, For more than two data sets, the ANOVA test was used. The significant difference was judged at the $P < 0.05$.



CHAPTER IV

RESULTS

1. Viral production of dengue virus in Vero cells

Vero cells were infected with dengue virus at multiplicity of infection (moi) of 1 pfu/ml and incubated for 1.5 hours. The medium containing virus was collected every hour for 24 hours. Titer of infectious virus was determined by plaque assay on Vero cells. Figure 6 shows the first time that virus produced from Vero cells. DEN-1, DEN-2, DEN-3 and DEN-4 are produced from Vero cells at 12 hours, 14 hours, 13 hours and 9 hours respectively. The result of viral production on HepG2 cells was also shown in figure 7 (Thepparit C., Personal Communication). DEN-1, DEN-2, DEN-3 and DEN-4 produced from HepG2 cells first time at 18 hours, 17 hours, 17 hours and 12 hours respectively.

2. Unsynchronized HepG2 and Vero cells

HepG2 and Vero cells were detached from the plate by trypsinization, washed twice in PBS. The cells were resuspended in 100 µl PBS and transferred to the polystyrene tube. After that the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. Figure 8 and figure 9 show the result from flow cytometry of the unsynchronized HepG2 and Vero cells respectively.

3. Synchronization of HepG2 cells

3.1 Synchronization of HepG2 cells into G₀ phase

To synchronize HepG2 cells into the G₀ phase, initially, a serum starvation method (50) was utilized. HepG2 cells were incubated for 36 hours in DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂. The result (figure 10) shows that the cells were not synchronized into G₀ phase. Then the time that used to incubate HepG2 cells in DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ was extended to 48

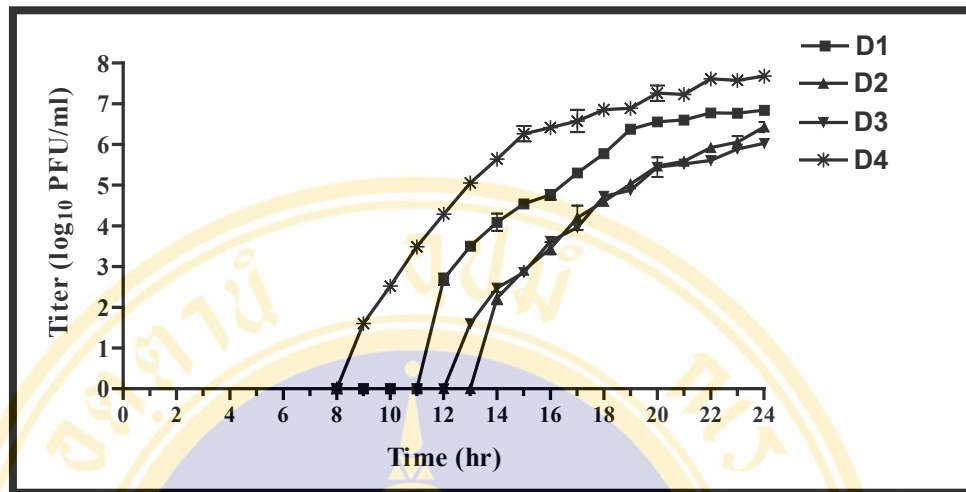


Figure 6. Dengue viral production on Vero cells

This figure shows dengue virus production on Vero cells. Vero cells were infected with dengue virus all four serotypes at moi of 1 for 1.5 hours. After treated with acid glycine infected cells were incubated in DMEM medium supplemented with 5% FBS. Samples were collected and titered by plaque assay. Each point represents the average of duplicate experiments and duplicate count.

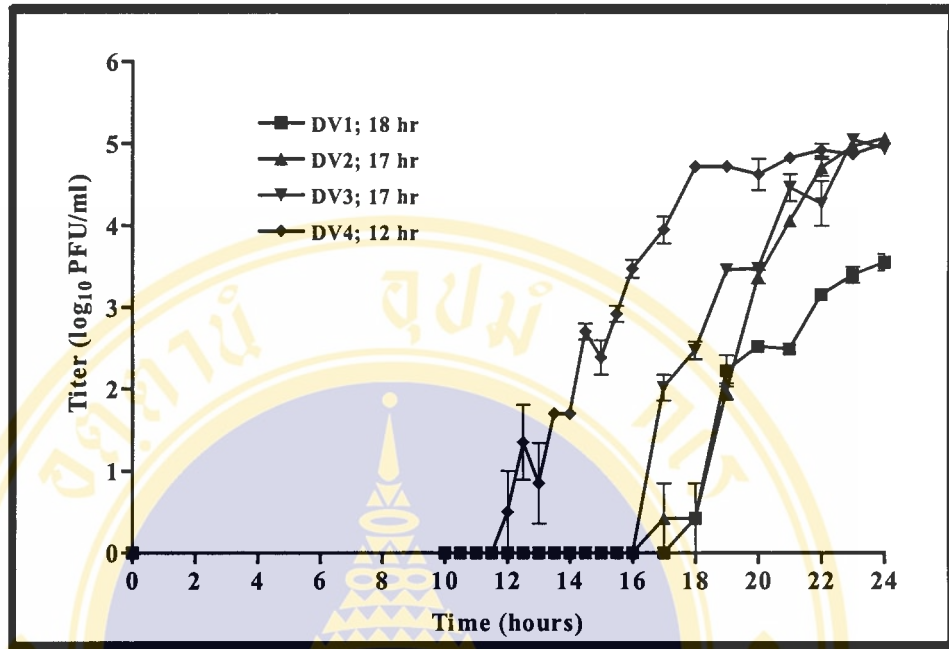


Figure 7. Dengue production on HepG2 cells (Thepparit C., Personal Communication)

This figure shows dengue virus production on HepG2 cells. HepG2 cells were infected with dengue virus all four serotypes at moi of 1. After treated with acid glycine infected cells were incubated in DMEM medium supplemented with 10% FBS. Samples were collected and titered by plaque assay. Each point represents the average of duplicate experiments and duplicate count.

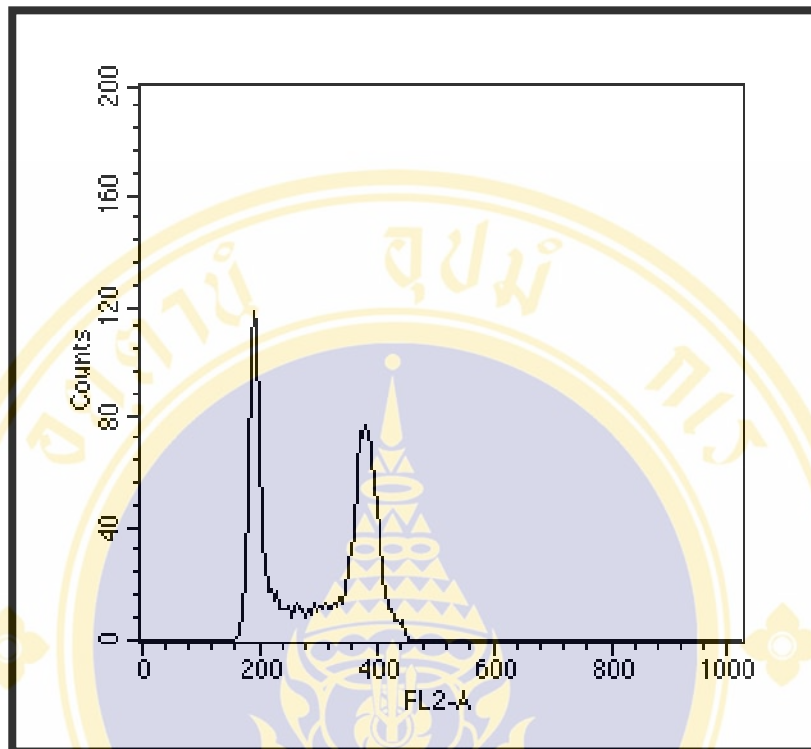


Figure 8. Flow cytometry of unsynchronized HepG2 cells

HepG2 cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 38.52%, 14.58% and 47.34% respectively.

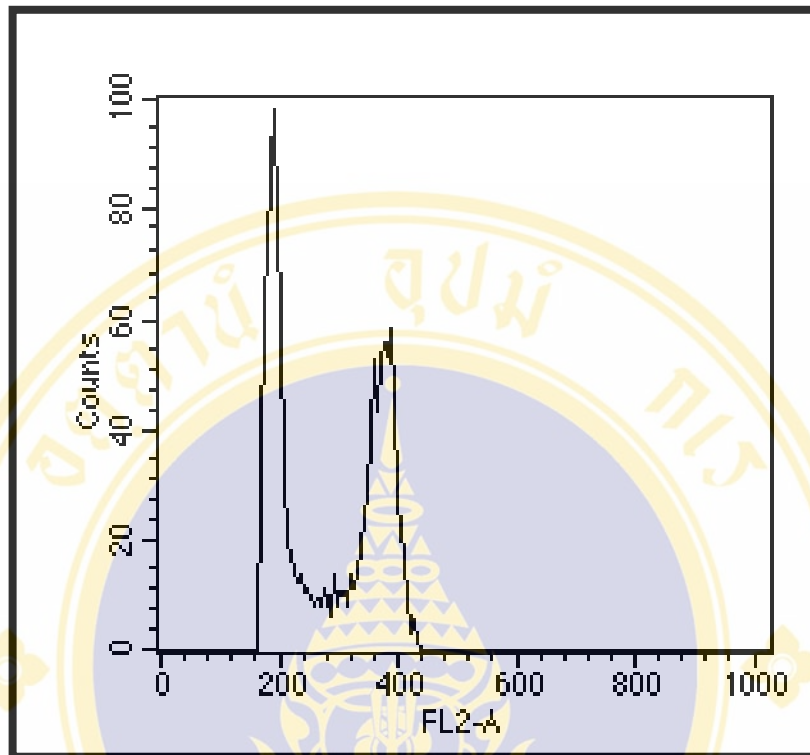


Figure 9. Flow cytometry of unsynchronized Vero cell

Vero cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G₀/G₁ phase, S phase and G₂/M phase is about 46.34%, 12.02% and 42.02% respectively.

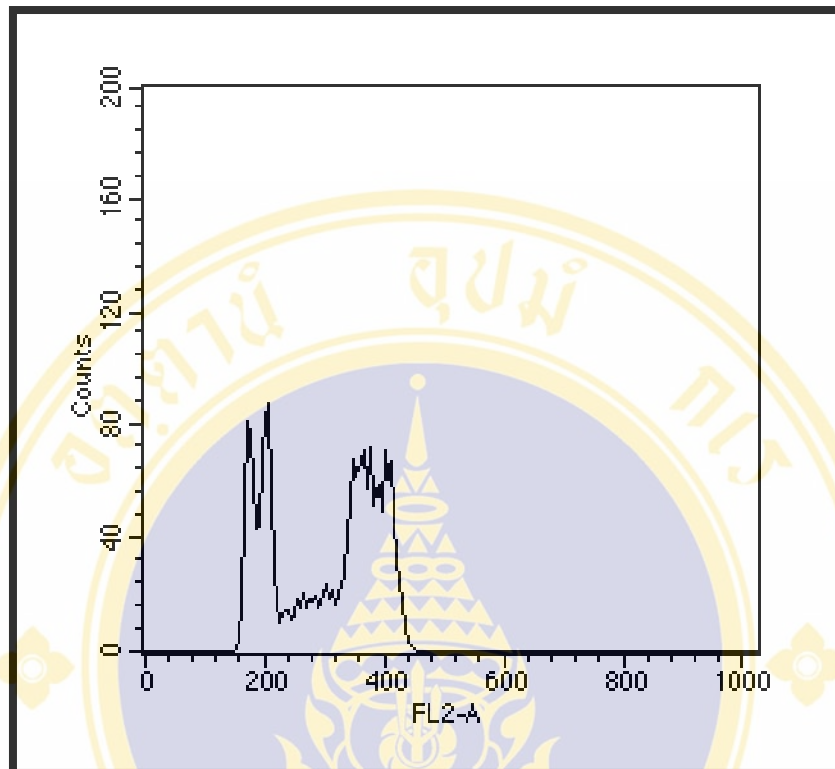


Figure 10. Flow cytometry of HepG2 cells, which were synchronized with DMEM supplemented with 0.1 % FBS for 36 hours

After HepG2 cells were incubated in DMEM supplemented with 0.1% FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 28.52%, 12.46% and 49.02% respectively.

hours. However the result (figure 11) shows that the cells were not synchronized into G₀ phase. The time that used to incubate HepG2 cells in DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ was increase to 3 days. But the result (figure 12) demonstrates that HepG2 cells were not synchronized into G₀ phase. After that HepG2 cells were changed to incubated in DMEM without FBS at 37°C / 10% CO₂ for 36 hours. The result (figure 13) shows that HepG2 cells were not synchronized into G₀ phase. Then the time that used to incubate HepG2 cells in DMEM without FBS at 37°C / 10% CO₂ was extended to 48 hours. But the result (figure 14) shows that HepG2 cells were not synchronized into G₀ phase. Moreover HepG2 cells were incubated in DMEM low glucose without FBS at 37°C / 10% CO₂ for 36 hours. The result (figure 15) indicates that HepG2 cells were not synchronized into G₀ phase. Then the time that used to incubated HepG2 cells in DMEM low glucose without FBS at 37°C / 10% CO₂ was increased to 48 hours. However the result (figure 16) demonstrates that HepG2 cells were not synchronized into G₀ phase.

3.2 Synchronization of HepG2 cells into G₂ phase

HepG2 cells were incubated in MEM without FBS at 37°C / 10% CO₂ for 36 hours. The result (figure 17) shows that HepG2 cells were synchronized into G₂ phase.

3.3 Synchronization of HepG2 cells into M phase

HepG2 cells were incubated in 0.1 μM paclitaxel in DMEM supplemented with 10% FBS 5 ml at 37°C / 10% CO₂ for 24 hours. The result (figure 18) indicates that HepG2 cells were synchronized into M phase.

3.4 The characteristic of HepG2 cells in G₂ phase and M phase

After HepG2 cells were synchronized into G₂ phase and M phase, the cells were taken photograph under the microscope. Figure 19 shows the difference of characteristic of HepG2 cells in G₂ phase and M phase.

3.5 Synchronization of HepG2 cells into G₁ phase

HepG2 cells were incubated in 0.1% DMSO in DMEM supplemented with 10% FBS 5 ml at 37°C / 10% CO₂ for 96 hours. The result (figure 20) demonstrates that HepG2 cells were not synchronized into G₁ phase. Then the amount of DMSO was increase to 0.2% DMSO in DMEM supplemented with 10 % FBS but the result (figure 21) also shows that HepG2 cells were not synchronized into G₁ phase by this method.

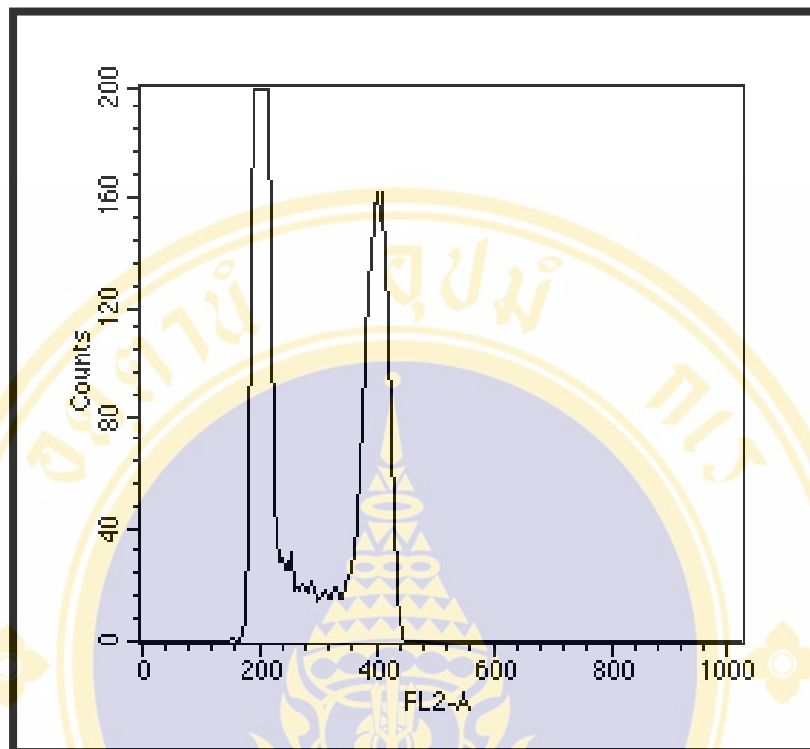


Figure 11. Flow cytometry of HepG2 cells, which were synchronized with DMEM supplemented with 0.1 % FBS for 48 hours

After HepG2 cells were incubated in DMEM supplemented with 0.1% FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G₀/G₁ phase, S phase and G₂/M phase is about 50.29%, 11.42% and 38.61% respectively.

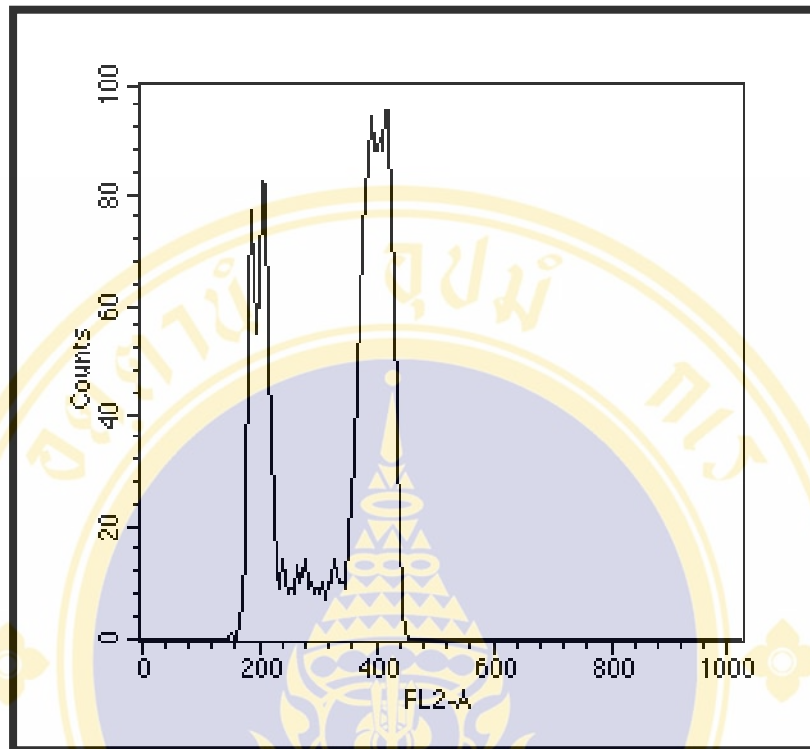


Figure 12. Flow cytometry of HepG2 cells, which were synchronized with DMEM supplemented with 0.1 % FBS for 3 days

After HepG2 cells were incubated in DMEM supplemented with 0.1% FBS for 3 days, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 28.26%, 9.76% and 62.20% respectively.

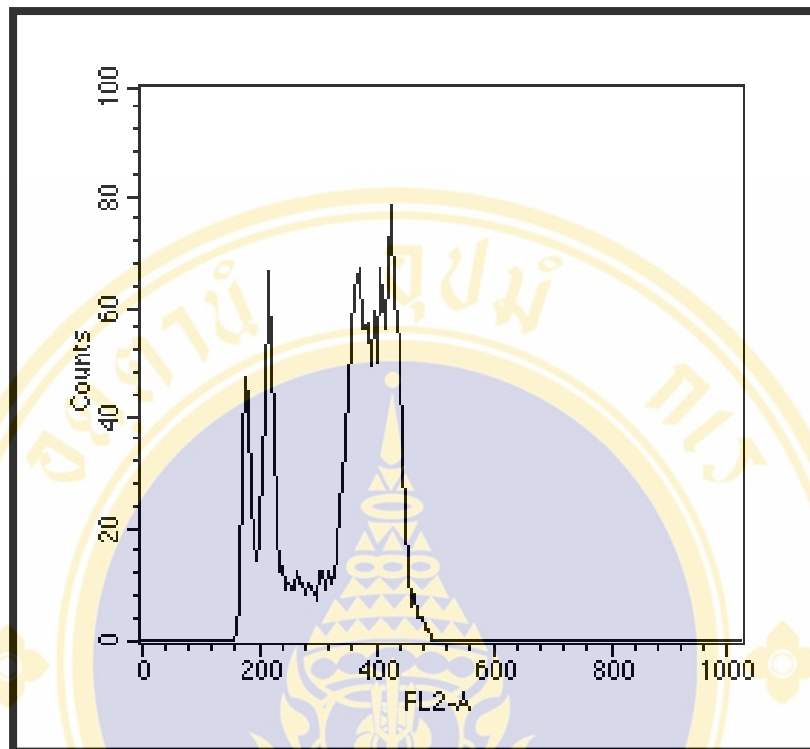


Figure 13. Flow cytometry of HepG2 cells, which were synchronized with DMEM without FBS for 36 hours.

After HepG2 cells were incubated in DMEM without FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 22.53%, 8.18% and 69.40% respectively.

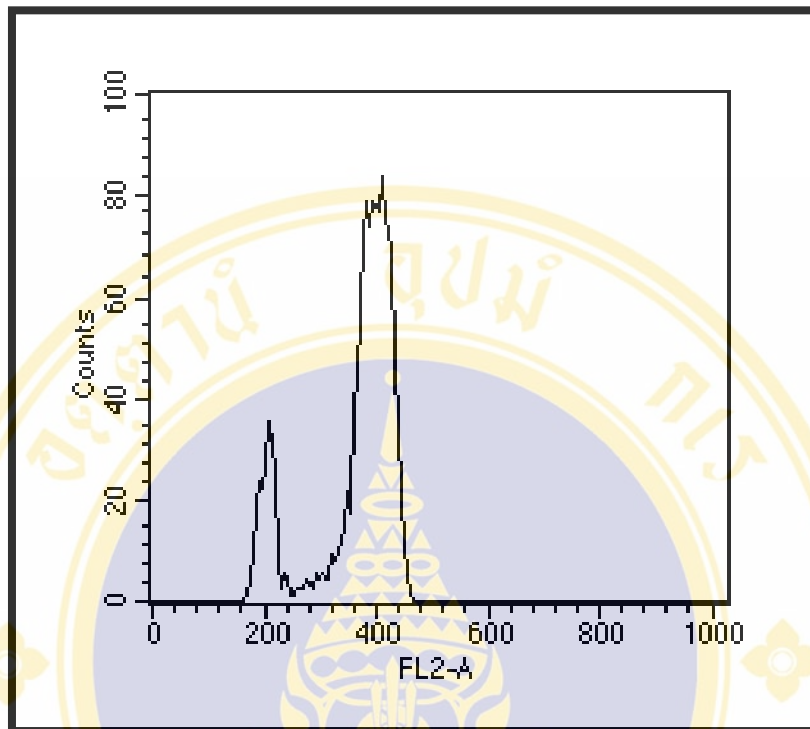


Figure 14. Flow cytometry of HepG2 cells, which were synchronized with DMEM without FBS for 48 hours.

After HepG2 cells were incubated in DMEM without FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 13.88%, 3.91% and 81.47% respectively.

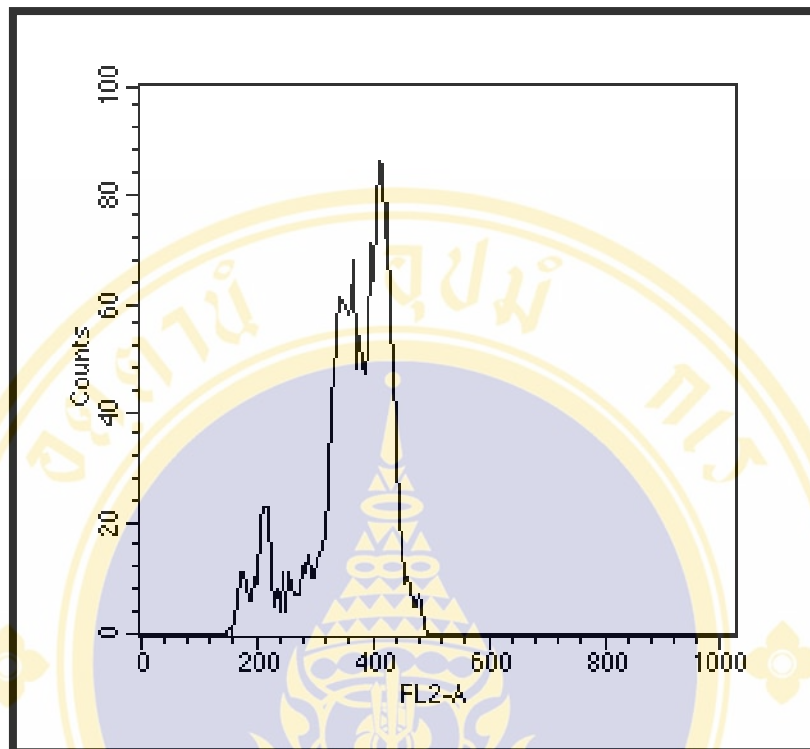


Figure 15. Flow cytometry of HepG2 cells, which were synchronized with DMEM low glucose without FBS for 36 hours

After HepG2 cells were incubated in DMEM low glucose without FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 9.17%, 7.56% and 82.52% respectively.

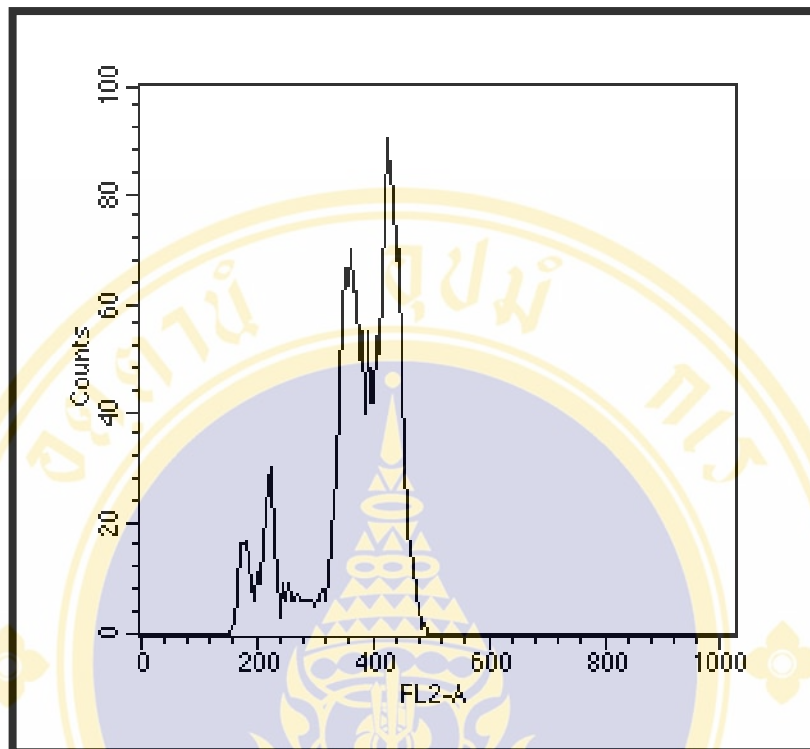


Figure 16. Flow cytometry of HepG2 cells, which were synchronized with DMEM low glucose without FBS for 48 hours

After HepG2 cells were incubated in DMEM low glucose without FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 11.34%, 4.70% and 83.34% respectively.

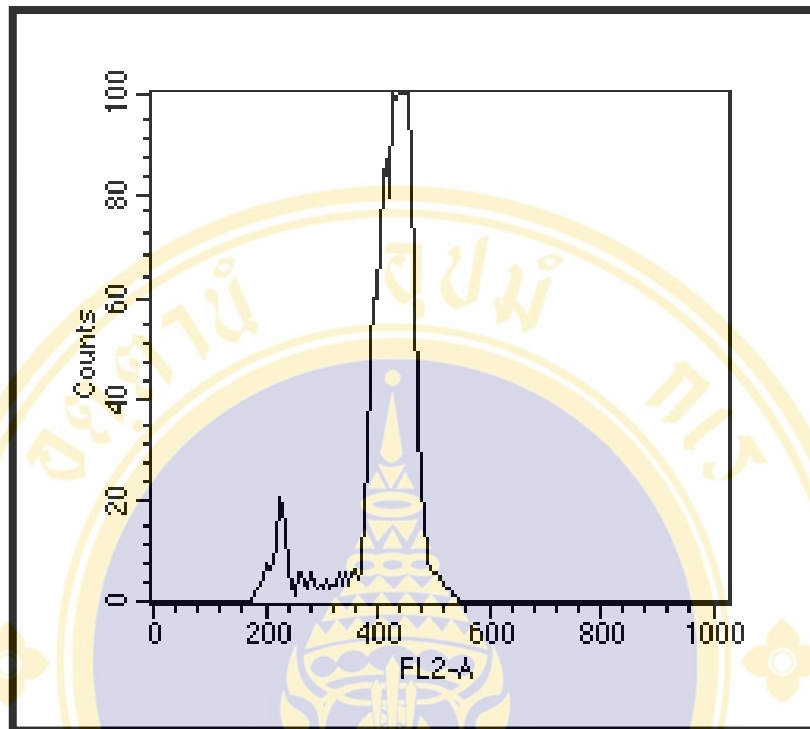


Figure 17. Flow cytometry of HepG2 cells, which were synchronized with MEM with no FBS for 36 hours

After HepG2 cells were incubated in MEM without FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 7.27%, 4.42% and 88.29% respectively.

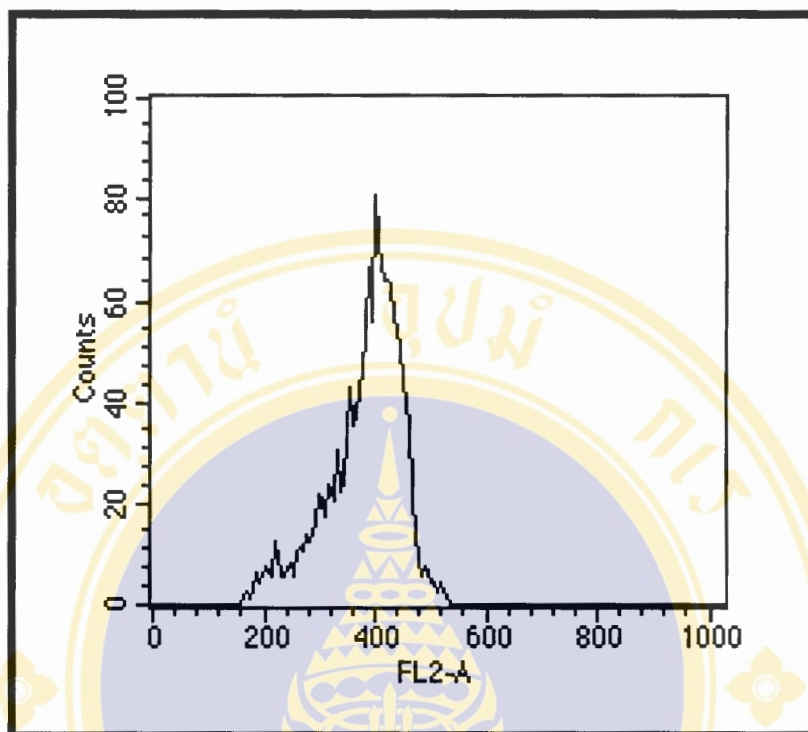


Figure 18. Flow cytometry of HepG2 cells, which were synchronized with 0.1 μ M paclitaxel in DMEM supplemented with 10% FBS for 24 hours.

After HepG2 cells were incubated in with 0.1 μ M paclitaxel in DMEM supplemented with 10% FBS for 24 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G₀/G₁ phase, S phase and G₂/M phase is about 3.20%, 20.24% and 77.01% respectively.

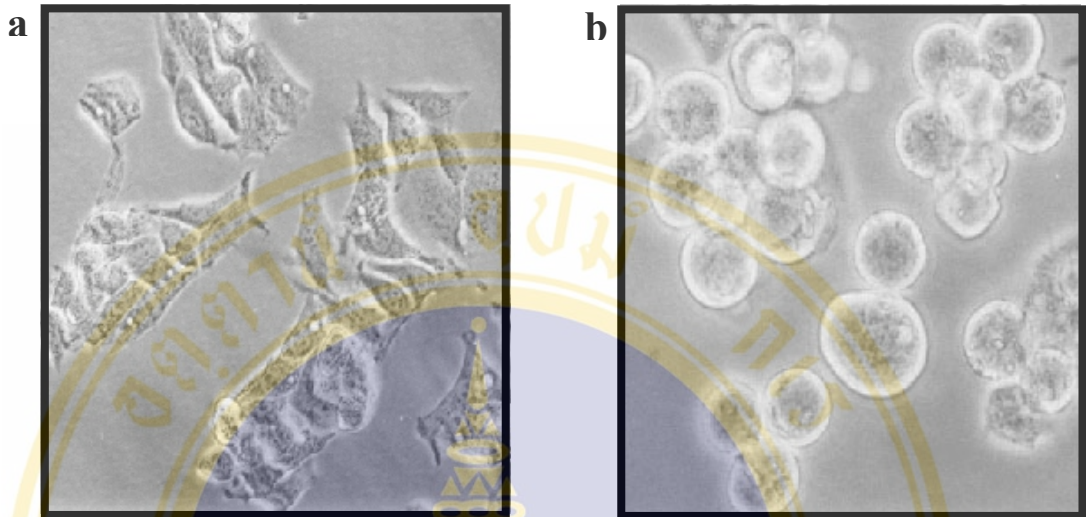


Figure 19. The characteristic of HepG2 cells in G₂ phase (a) and M phase (b)

The shape of HepG2 cells in M phase is different from G₂ phase. HepG2 cells in M phase are round whereas HepG2 cells in G₂ phase are not. The magnification is 400x.

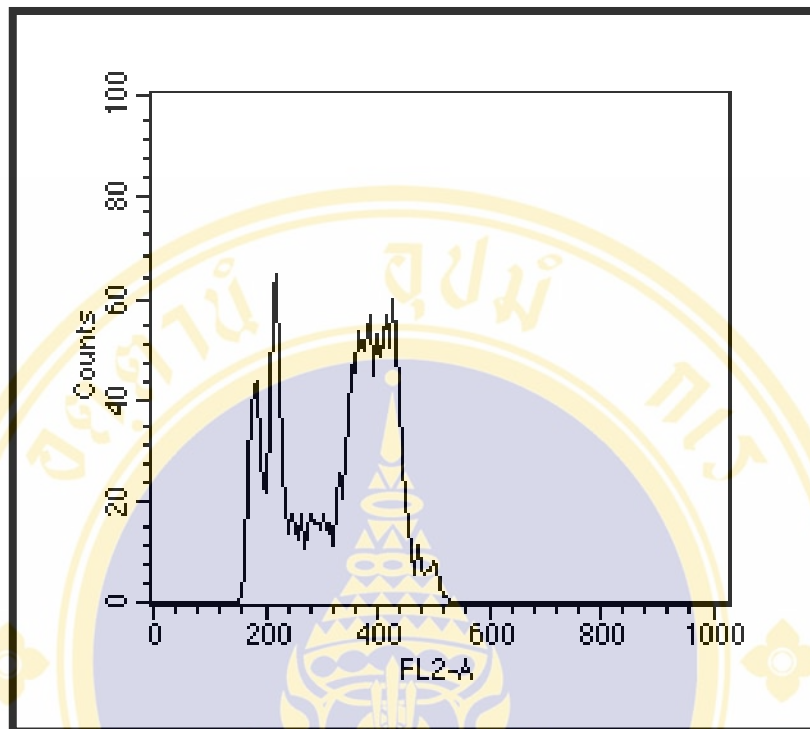


Figure 20. Flow cytometry of HepG2 cells, which were synchronized with 0.1% DMSO in DMEM supplemented with 10% FBS for 96 hours

After HepG2 cells were incubated in 0.1% DMSO in DMEM supplemented with 10% FBS for 96 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G₀/G₁ phase, S phase and G₂/M phase is about 26.66%, 12.36% and 61.27% respectively.

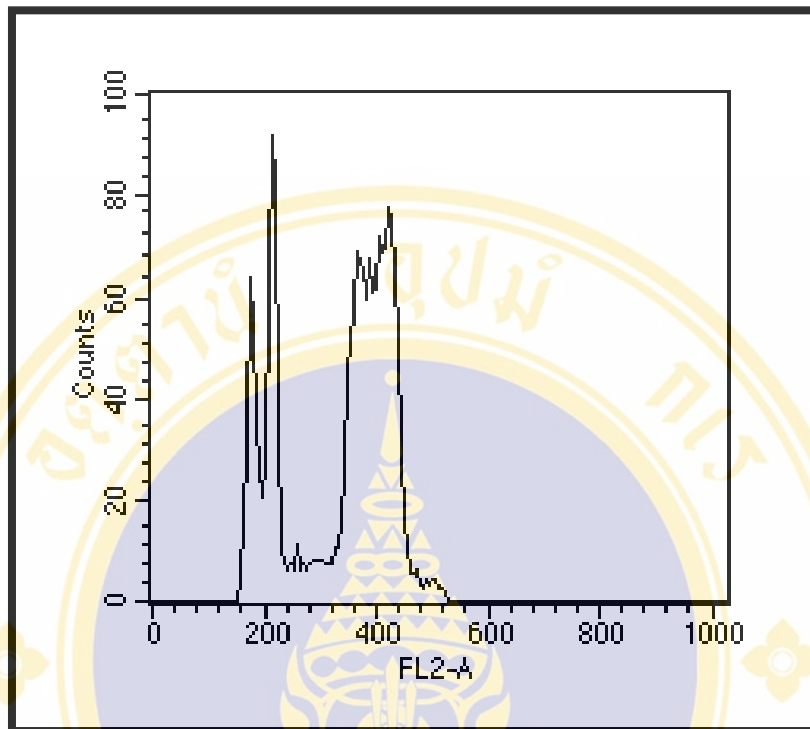


Figure 21. Flow cytometry of HepG2 cells, which were synchronized with 0.2% DMSO in DMEM supplemented with 10% FBS for 96 hours

After HepG2 cells were incubated in 0.2% DMSO in DMEM supplemented with 10% FBS for 96 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G₀/G₁ phase, S phase and G₂/M phase is about 28.53%, 5.22% and 66.37% respectively.

3.6 Synchronization of HepG2 cells into S phase

HepG2 cells were incubated in DMEM supplemented with 10% FBS, which contained 5 µg/ml of aphidicolin at 37°C / 10% CO₂ for 24 hours. The result (figure 22) shows that HepG2 cells were not synchronized into S phase. Then the time that HepG2 cells used to incubate in DMEM supplemented with 10% FBS, which contains 5 µg/ml of aphidicolin at 37°C / 10% CO₂ was extended to 48 hours. However the result (figure 23) indicates that HepG2 cells were not synchronized into S phase by this method. Then thymidine double block method (53) was utilized. HepG2 cells were incubated in 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ for 12 hours. After that the cells were changed to incubate in DMEM supplemented with 10% FBS at 37°C / 10% CO₂ for 10 hours. Finally, the cells were incubated in 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ for 12 hours. The result (figure 24) indicates that HepG2 cells were not synchronized into S phase. Then the time that HepG2 cells used to incubate in DMEM supplemented with 10% FBS at 37°C / 10% CO₂ was changed from 10 hours to 16 hours. But the time that HepG2 cells used to incubate in 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 10% CO₂ was not changed. The result (figure 25) shows that HepG2 cells were synchronized into S phase by this method.

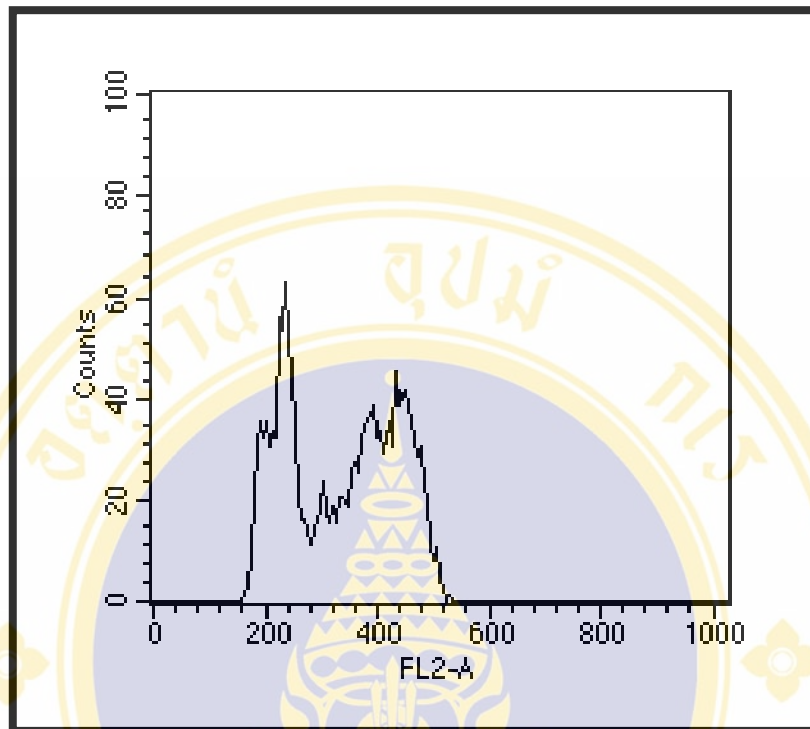


Figure 22. Flow cytometry of HepG2 cells, which were synchronized with 5 $\mu\text{g}/\text{ml}$ of aphidicolin in DMEM supplemented with 10% FBS for 24 hours

After HepG2 cells were incubated in 5 $\mu\text{g}/\text{ml}$ of aphidicolin in DMEM supplemented with 10% FBS for 24 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 35.48%, 13.95% and 50.74% respectively.

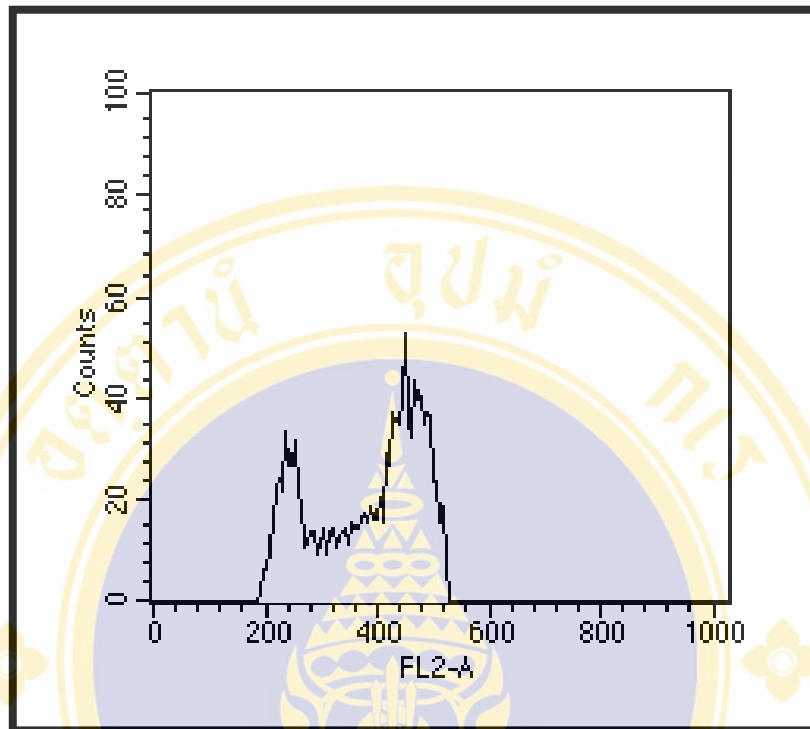


Figure 23. Flow cytometry of HepG2 cells, which were synchronized with 5 $\mu\text{g/ml}$ of aphidicolin in DMEM supplemented with 10% FBS for 48 hours

After HepG2 cells were incubated in 5 $\mu\text{g/ml}$ of aphidicolin in DMEM supplemented with 10% FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 28.23%, 20.79% and 51.18% respectively.

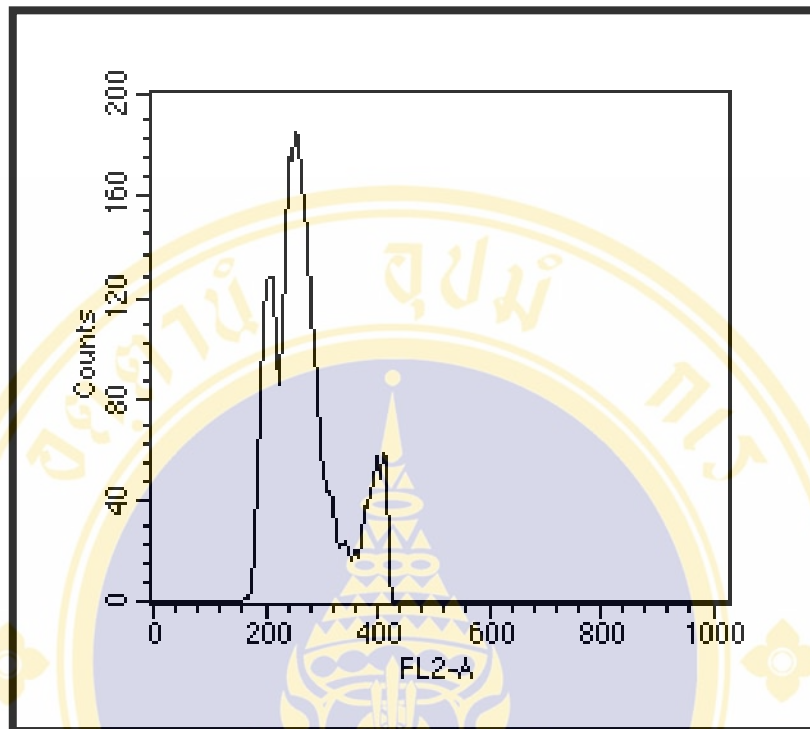


Figure 24. Flow cytometry of HepG2 cells, which were synchronized with thymidine double block method.

HepG2 cells were incubated in 2 mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours. Then the cells were grown in DMEM supplemented with 10%FBS for 10 hours and incubated in 2mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours again. After that the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The flow cytometry result shows that the population of HepG2 cells in G_0/G_1 phase, S phase and G_2/M phase is about 21.36%, 62.21% and 16.99% respectively.

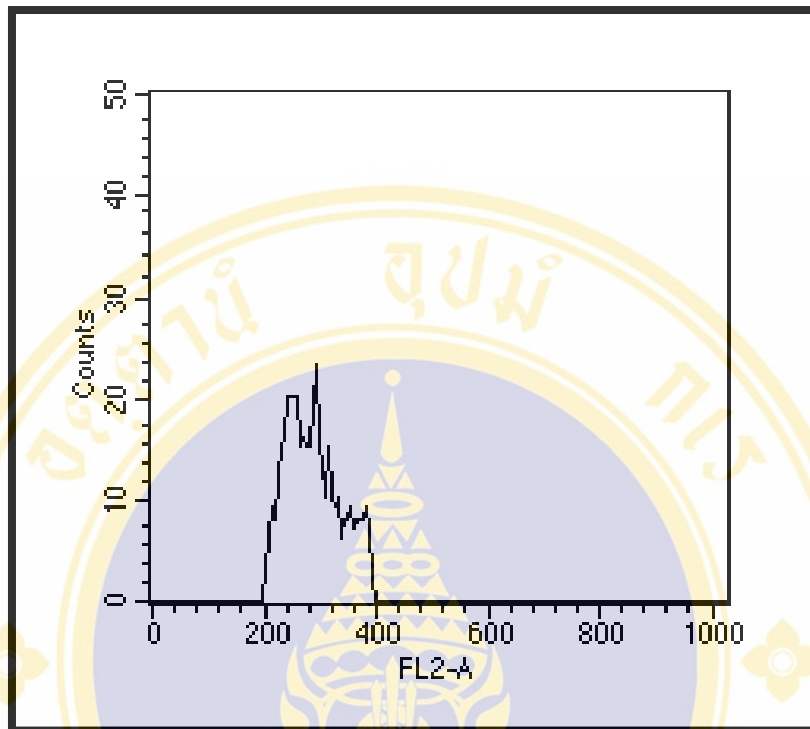


Figure 25. Flow cytometry of HepG2 cells, which were synchronized with thymidine double block method

HepG2 cells were incubated in 2 mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours. Then the cells were grown in DMEM supplemented with 10%FBS for 16 hours and incubated in 2mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours again. After that the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The flow cytometry result shows that the population of HepG2 cells in G₀/G₁ phase, S phase and G₂/M phase is about 0.98%, 71.41% and 27.67% respectively.

4. Synchronization of Vero cells

4.1 Synchronization of Vero cells into G₀ phase by serum starvation method

To synchronize Vero cells into G₀ phase, initially, a serum starvation method (50) was utilized. Vero cells were incubated for 36 hours in DMEM supplemented with 0.1% FBS at 37°C / 5% CO₂. The result (figure 26) shows that the cells were not synchronized into G₀ phase. After that Vero cells were changed to incubated in DMEM without FBS at 37°C / 5% CO₂ for 36 hours. The result (figure 27) shows that Vero cells were not synchronized into G₀ phase. Then the time that used to incubate Vero cells in DMEM without FBS at 37°C / 5% CO₂ was extended to 48 hours. But the result (figure 28) shows that Vero cells were not synchronized into G₀ phase. Moreover Vero cells were incubated in DMEM low glucose without FBS at 37°C / 5% CO₂ for 36 hours. The result (figure 29) indicates that Vero cells were not synchronized into G₀ phase. Then the time that used to incubate Vero cells in DMEM low glucose without FBS at 37°C / 5% CO₂ was increased to 48 hours. However the result (figure 30) demonstrates that Vero cells were not synchronized into G₀ phase. Finally, Vero cells were changed to incubate in MEM without FBS at 37°C / 5% CO₂ for 36 hours. The result (figure 31) shows that Vero cells were synchronized into G₀ phase.

4.2 Synchronization of Vero cells into M phase

Vero cells were incubated in 0.1 μM paclitaxel in DMEM supplemented with 5% FBS 5 ml at 37°C / 5% CO₂ for 24 hours. The result (figure 32) indicates that Vero cells were synchronized into M phase.

4.3 Synchronization of Vero cells into G₁ phase

Vero cells were incubated in 0.1% DMSO in DMEM supplemented with 5% FBS 5 ml at 37°C / 5% CO₂ for 96 hours. The result (figure 33) demonstrates that Vero cells were not synchronized into G₁ phase. Then the amount of DMSO was increase to 0.2% DMSO in DMEM supplemented with 5 % FBS. But the result (figure 34) also shows that Vero cells were not synchronized into G₁ phase by this method.

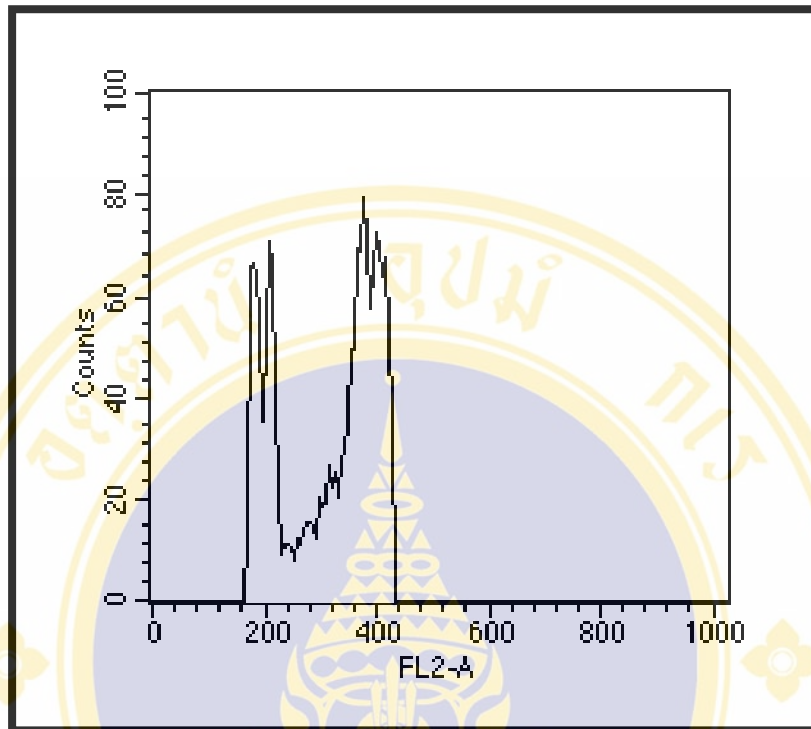


Figure 26. Flow cytometry of Vero cells, which were synchronized with DMEM supplemented with 0.1 % FBS for 36 hours.

After Vero cells were incubated in DMEM supplemented with 0.1% FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G₀/G₁ phase, S phase and G₂/M phase is about 28.48%, 13.37% and 58.43% respectively.

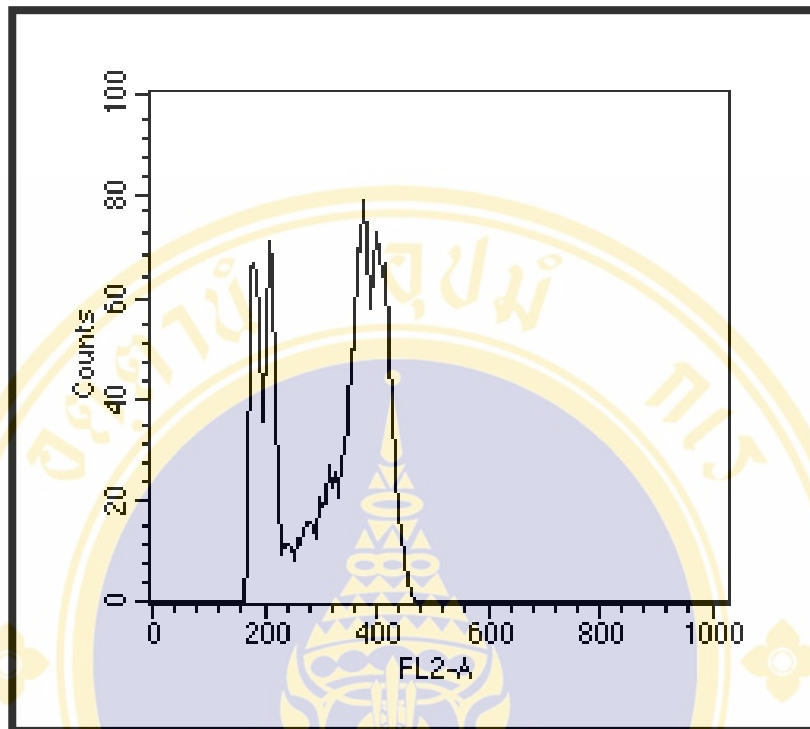


Figure 27. Flow cytometry of Vero cells, which were synchronized with DMEM without FBS for 36 hours.

After Vero cells were incubated in DMEM without FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 21.12%, 10.63% and 68.52% respectively.

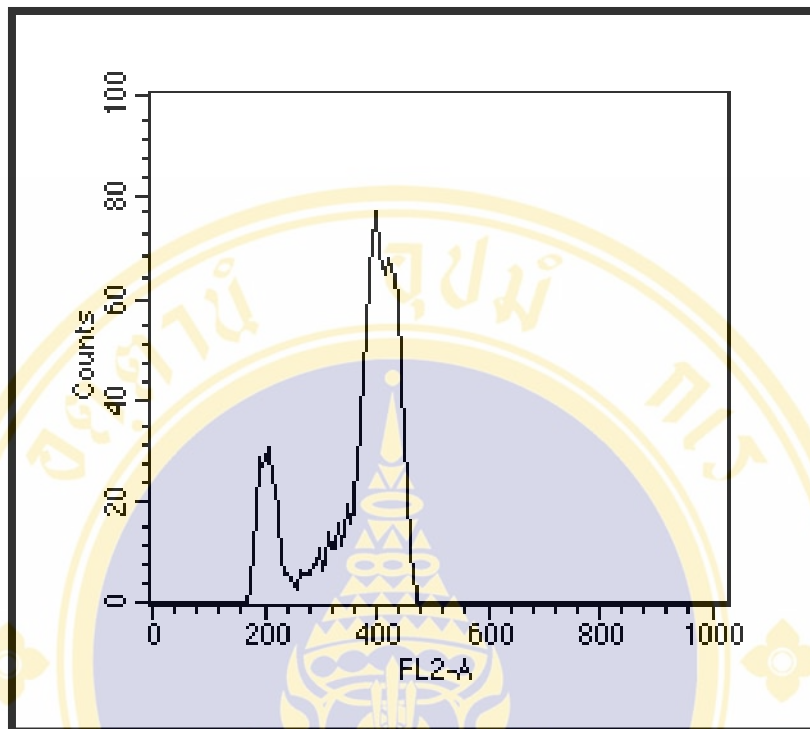


Figure 28. Flow cytometry of Vero cells, which were synchronized with DMEM without FBS for 48 hours.

After Vero cells were incubated in DMEM without FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 15.77%, 7.72% and 75.84% respectively.

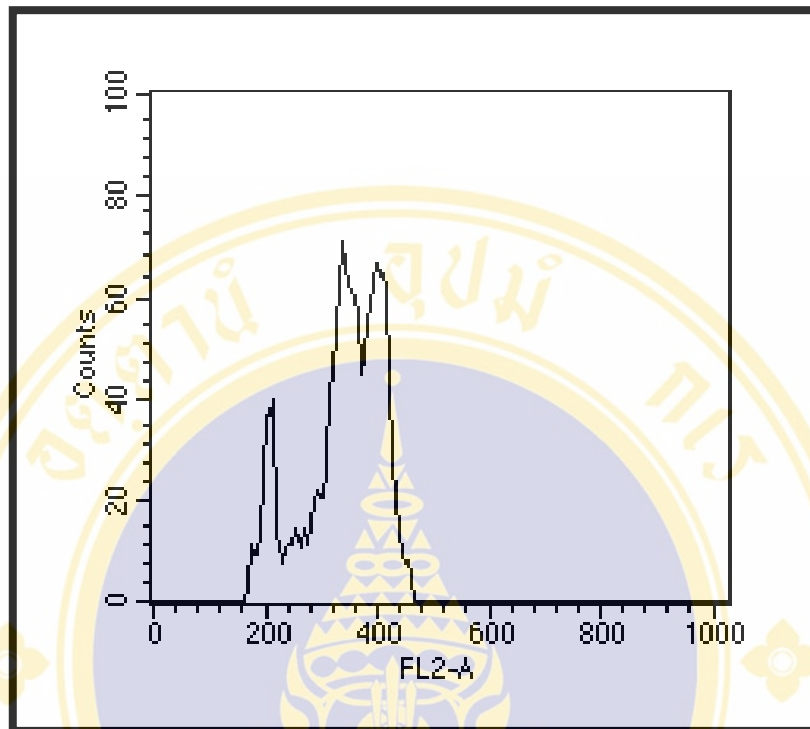


Figure 29. Flow cytometry of Vero cells, which were synchronized with DMEM low glucose without FBS for 36 hours.

After Vero cells were incubated in DMEM low glucose without FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 13.63%, 10.05% and 76.75% respectively.

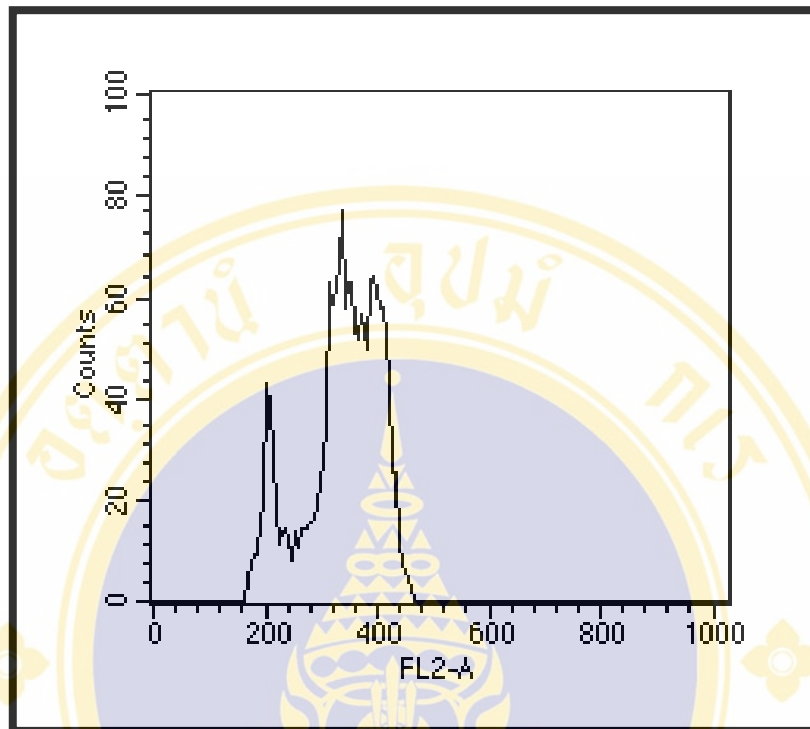


Figure 30. Flow cytometry of Vero cells, which were synchronized with DMEM low glucose without FBS for 48 hours.

After Vero cells were incubated in DMEM low glucose without FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 13.03%, 9.11% and 76.26% respectively.

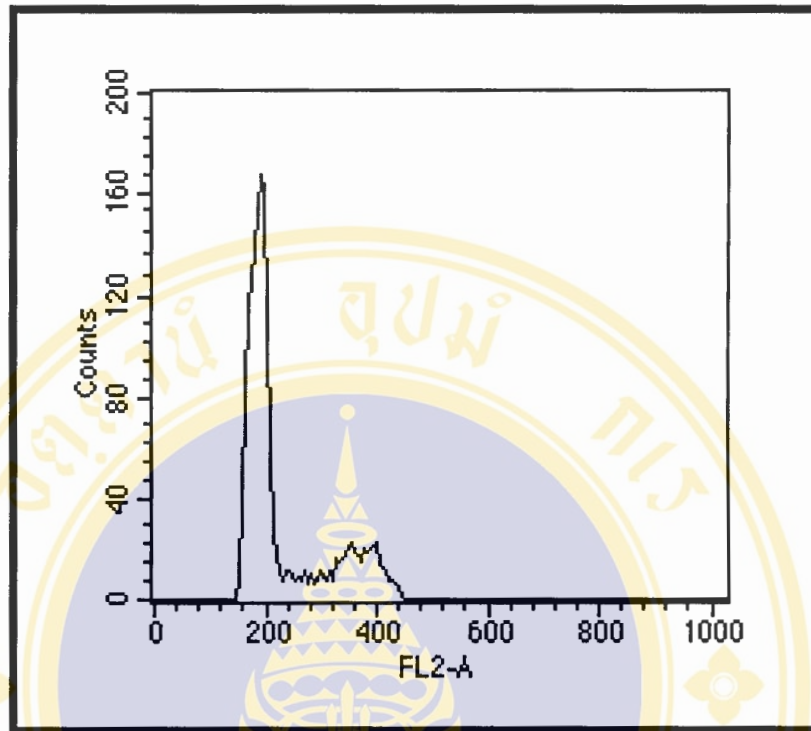


Figure 31. Flow cytometry of Vero cells, which were synchronized with MEM with no FBS for 36 hours.

After Vero cells were incubated in MEM without FBS for 36 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 83.98%, 2.24% and 13.28% respectively.

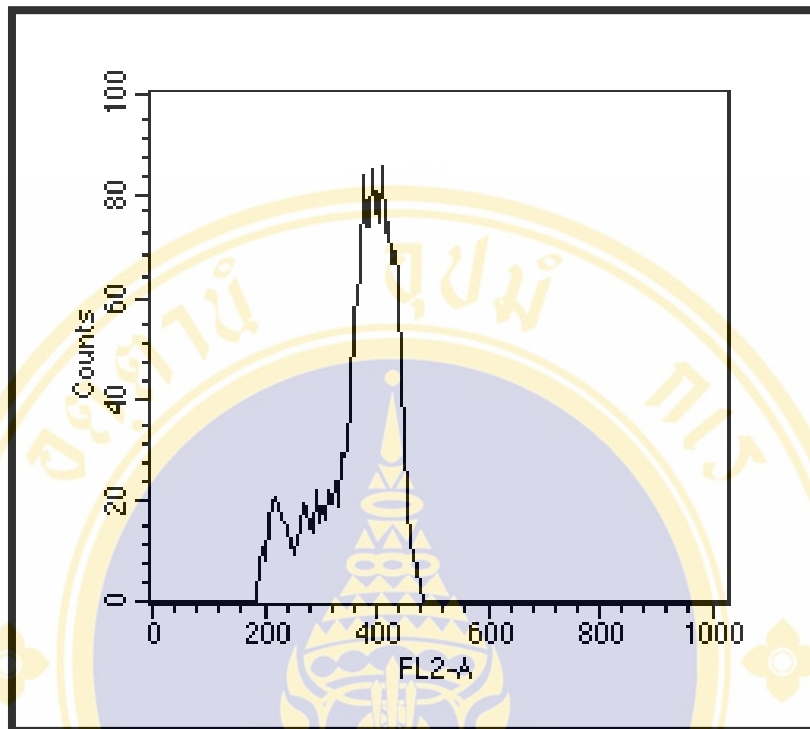


Figure 32. Flow cytometry of Vero cells, which were synchronized with $0.1\mu\text{M}$ of paclitaxel in DMEM supplemented with 5% FBS for 24 hours.

After Vero cells were incubated in $0.1\mu\text{M}$ of paclitaxel in DMEM supplemented with 5% FBS for 24 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 21.12%, 10.63% and 68.52% respectively.

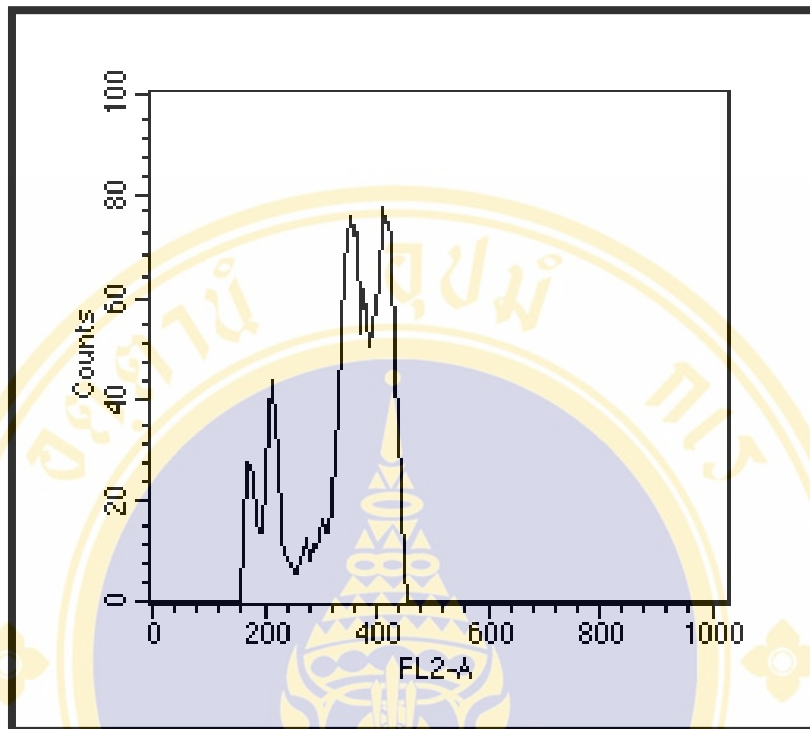


Figure 33. Flow cytometry of Vero cells, which were synchronized with 0.1%DMSO in DMEM supplemented 5% FBS for 96 hours.

After Vero cells were incubated in 0.1% DMSO in DMEM supplemented with 5% FBS for 96 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G₀/G₁ phase, S phase and G₂/M phase is about 17.07%, 7.14% and 76.07% respectively.

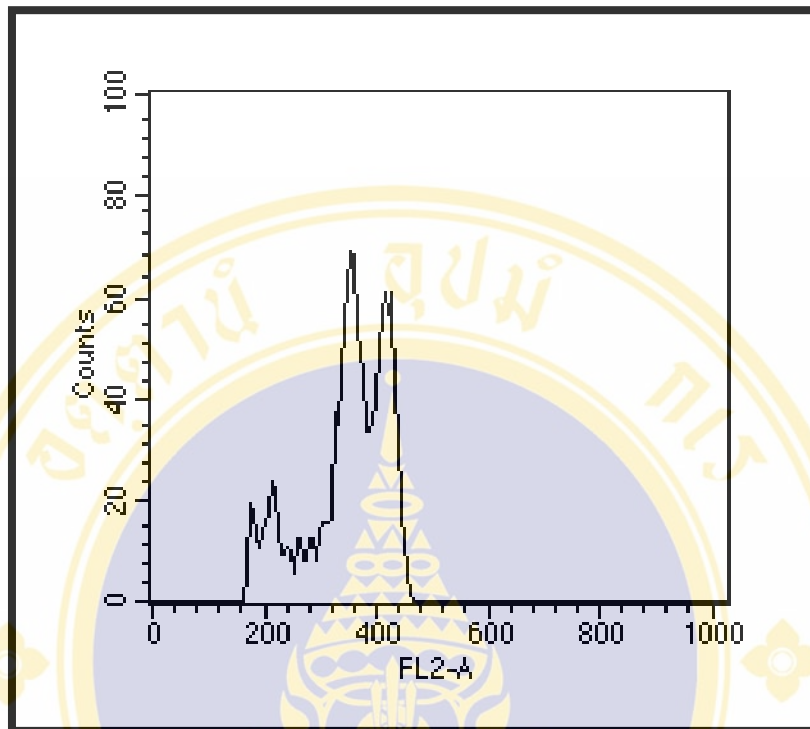


Figure 34. Flow cytometry of Vero cells, which were synchronized with 0.2%DMSO in DMEM for 96 hours.

After Vero cells were incubated in 0.2% DMSO in DMEM supplemented with 5% FBS for 96 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G₀/G₁ phase, S phase and G₂/M phase is about 13.13%, 10.19% and 76.36% respectively.

4.4 Synchronization of Vero cells into S phase

Vero cells were incubated in DMEM supplemented with 5% FBS, which contains 5 µg/ml of aphidicolin at 37°C / 5% CO₂ for 24 hours. The result (figure 35) shows that Vero cells were not synchronized into S phase. Then the time that Vero cells used to incubate in DMEM supplemented with 5% FBS, which contains 5 µg/ml of aphidicolin at 37°C / 5% CO₂ was extended to 48 hours. However the result (figure 36) indicates that Vero cells were not synchronized into S phase by this method. Then thymidine double block method (53) was utilized. Vero cells were incubated in 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 5% CO₂ for 12 hours. After that the cells were changed to incubate in DMEM supplemented with 5% FBS at 37°C / 5% CO₂ for 10 hours. Finally, the cells were incubated in 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 5% CO₂ for 12 hours. The result (figure 37) indicates that Vero cells were not synchronized into S phase. Then the time that Vero cells used to incubate in DMEM supplemented with 5% FBS at 37°C / 5% CO₂ was changed from 10 hours to 16 hours. But the time that Vero cells used to incubate in 2mM thymidine in DMEM supplemented with 0.1% FBS at 37°C / 5% CO₂ was not changed. The result (figure 38) shows that Vero cells were synchronized into S phase by this method.

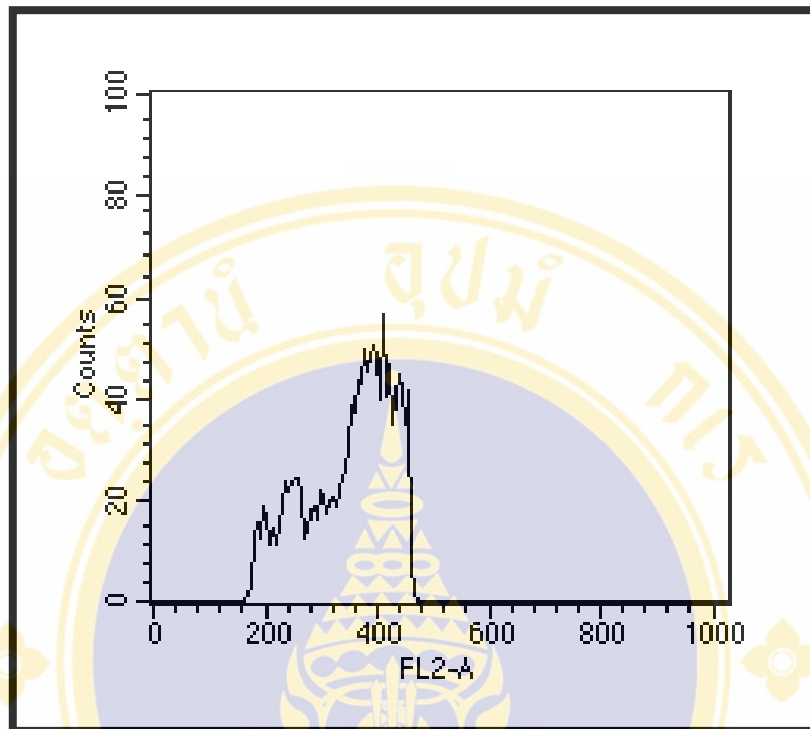


Figure 35. Flow cytometry of Vero cells, which were synchronized with 5 $\mu\text{g/ml}$ of aphidicolin in DMEM supplemented with 5%FBS for 24 hours.

After Vero cells were incubated in 5 $\mu\text{g/ml}$ of aphidicolin in DMEM supplemented with 5%FBS for 24 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 17.31%, 16.52% and 66.03% respectively.

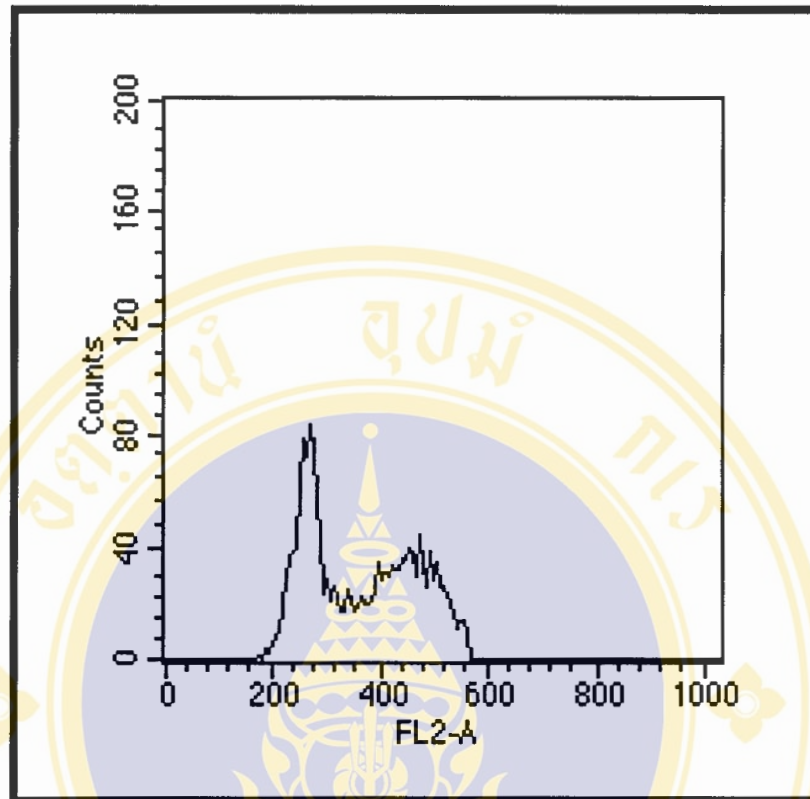


Figure 36. Flow cytometry of Vero cells, which were synchronized with 5 $\mu\text{g/ml}$ of aphidicolin in DMEM supplemented with 5%FBS for 48 hours.

After Vero cells were incubated in 5 $\mu\text{g/ml}$ of aphidicolin in DMEM supplemented with 5%FBS for 48 hours, the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 37.94%, 19.20% and 42.89% respectively.

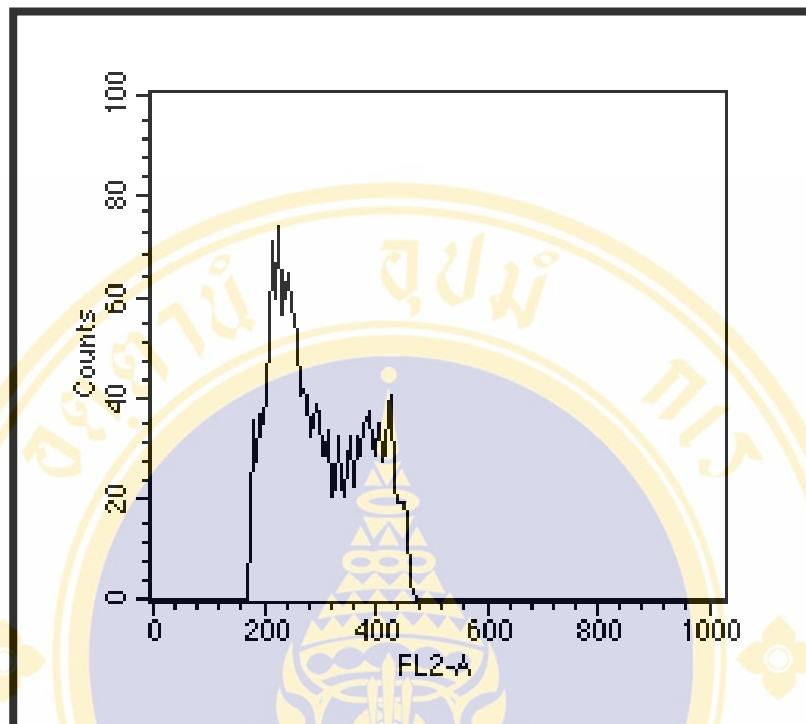


Figure 37. Flow cytometry of Vero cells, which were synchronized with thymidine double block method

Vero cells were incubated in 2 mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours. Then the cells were grown in DMEM supplemented with 5%FBS for 10 hours and incubated in 2mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours again. After that the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The flow cytometry result shows that the population of Vero cells in G₀/G₁ phase, S phase and G₂/M phase is about 27.12%, 38.86% and 34.85% respectively.

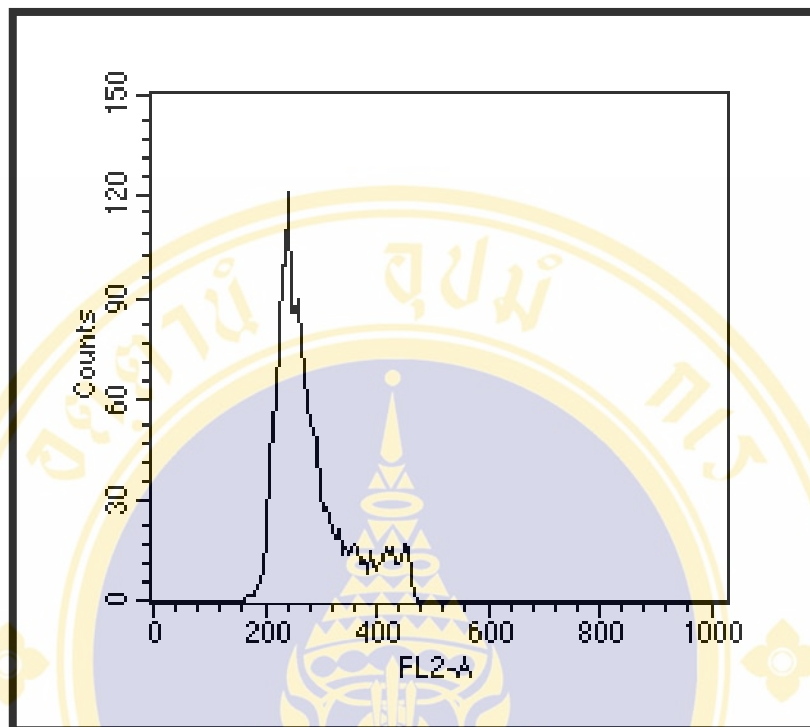


Figure 38. Flow cytometry of Vero cells, which were synchronized with thymidine double block method

Vero cells were incubated in 2 mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours. Then the cells were grown in DMEM supplemented with 5%FBS for 16 hours and incubated in 2mM thymidine in DMEM supplemented with 0.1%FBS for 12 hours again. After that the cells were trypsinized and washed twice with PBS. Then the cells were stained with propidium iodide by using Cycle test plus DNA reagent kit and analyzed by flow cytometer. The flow cytometry result shows that the population of Vero cells in G_0/G_1 phase, S phase and G_2/M phase is about 41.84%, 39.01% and 20.15% respectively.

5. Infection of synchronized HepG2 cells

HepG2 cells were synchronized into each phase of the cell cycle by using several methods. (Table 3)

Table 3. Summary of the method to synchronize HepG2 cells

Cell cycle phase	Method
G ₂	Incubation in MEM with no FBS for 36 hours
M	Incubation in 0.1 μ M paclitaxel in DMEM supplemented with 10% FBS for 24 hours
S	Thymidine double block method by incubating in 2 mM thymidine in DMEM supplemented with 0.1% FBS for 12 hours and incubating in DMEM supplemented with 10% FBS for 16 hours. Finally, the cells were incubated in 2 mM thymidine in DMEM supplemented with 0.1% FBS for 12 hours

5.1 Comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in G₂ phase

After HepG2 cells were synchronized into G₂ phase by serum starvation method, as described in table 3, the cells were infected with dengue serotype 2 at moi 1 and incubated at 37°C / 10% CO₂ for 1 hour. The medium containing virus was collected at 19 hours to 22 hours. Then the sample was titered by the plaque assay method. Figure 39 shows the result of comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in G₂ phase. Figure 39a shows little difference of viral production from unsynchronized HepG2 cells and HepG2 cells in G₂ phase in term of titer of the infectious virus. From this result, dengue serotype 2 is produced from HepG2 cells in G₂ phase more than from unsynchronized HepG2 cells. But the amount of cells when the cells were infected is not equal between the cells in G₂ phase and unsynchronized cells. The amount of the unsynchronized cells is more than the amount of the cells in G₂ phase because the cells that grow in MEM, which has the nutrient less than in DMEM and has no FBS, grow slower. Then the results were calculated in term of the titer of infectious virus per cells. Figure 39b shows the difference of viral production from unsynchronized HepG2 cells and HepG2 cells in G₂ phase in term of titer of the infectious virus per cell. From this picture, the viral production from HepG2 cells in G₂ phase is more than from unsynchronized HepG2 cells about 2.3 fold at 22 hours.

5.2 Comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in M phase

After HepG2 cells were synchronized into M phase by incubating in paclitaxel, as described in table 3, the cells were infected with dengue serotype 2 at moi 1 and incubated at 37°C / 10% CO₂ for 1 hour. The medium containing virus was collected at 19 hours to 22 hours. Then the sample was titered by the plaque assay method. Figure 40 shows the result of comparison of dengue serotype 2 production between unsynchronized

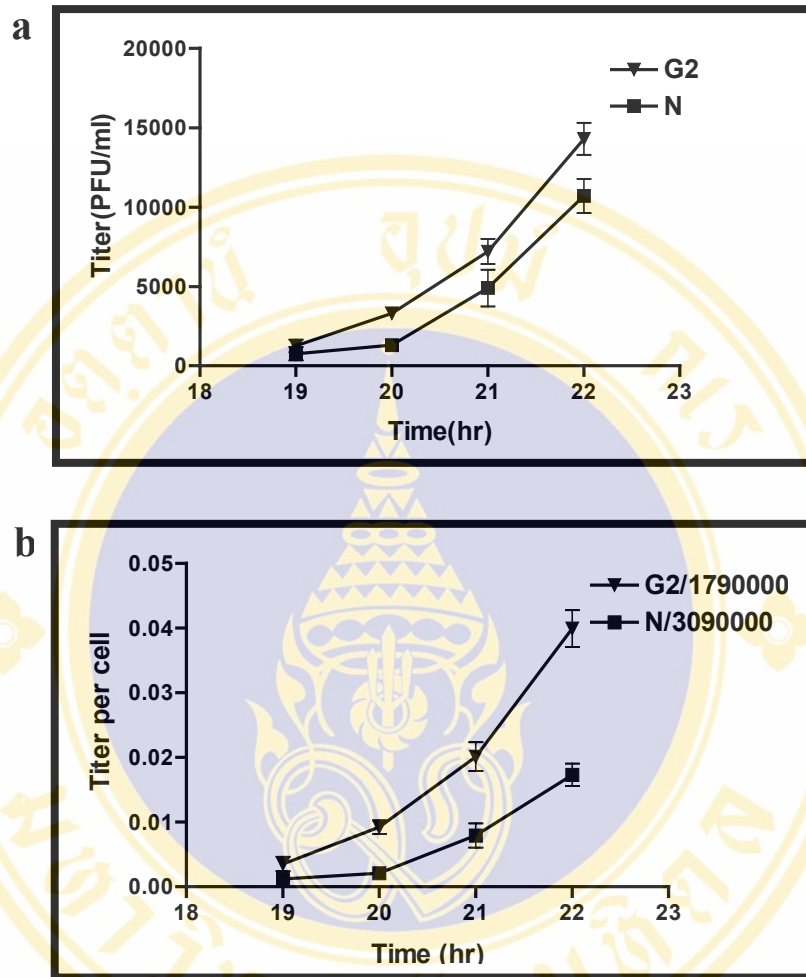


Figure 39. Comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in G₂ phase

Dengue serotype 2 is produced from HepG2 cells in G₂ phase more than from unsynchronized HepG2 cells. Figure 39a shows the difference of viral production from HepG2 cells in G₂ phase and unsynchronized HepG2 cells in term of the titer of infectious virus whereas figure 39b shows in term of the titer per cell.

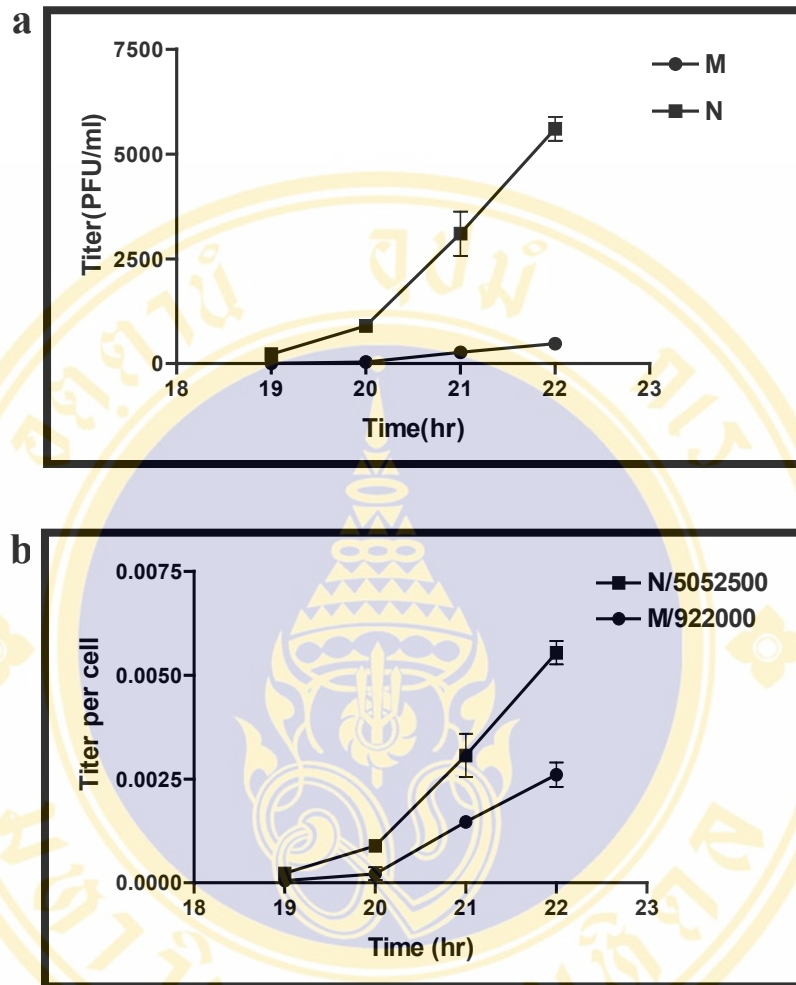


Figure 40. Comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in M phase

Dengue serotype 2 is produced from HepG2 cells in M phase less than from unsynchronized HepG2 cells. Figure 40a shows the difference of viral production from HepG2 cells in M phase and unsynchronized HepG2 cells in term of the titer of infectious virus whereas figure 40 shows in term of the titer per cell.

HepG2 cells and HepG2 cells in M phase. Figure 40a shows a difference of viral production from unsynchronized HepG2 cells and HepG2 cells in M phase in term of titer of the infectious virus but figure 40b shows in term of titer of the infectious virus per cell. From figure 40a, there is no difference between the viral production from unsynchronized HepG2 cells and HepG2 cells in M phase at 19 hours but there is a big difference at 22 hours. From this figure, viral production from HepG2 cells in M phase is much lower than from unsynchronized HepG2 cells. From figure 40b, viral production from HepG2 cells in M phase is lower than from unsynchronized HepG2 cells. But this difference is not much different as in term of titer of the infectious virus in figure 40a because the number of cells in M phase is lower than unsynchronized cells. These results show that dengue serotype 2 produces from HepG2 cells in M phase less than from unsynchronized HepG2 cells about 2.1 fold at 22 hours.

5.3 Comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in S phase

After HepG2 cells were synchronized into S phase by using thymidine double block method, as described in table 3, the cells were infected with dengue serotype 2 at moi 1 and incubated at 37°C / 10% CO₂ for 1 hour. The medium containing virus was collected at 19 hours to 22 hours. Then the sample was titered by the plaque assay method. Figure 41 shows the result of comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in S phase. Figure 41a shows a difference of viral production from unsynchronized HepG2 cells and HepG2 cells in S phase in term of titer of the infectious virus but figure 41b shows in term of titer of the infectious virus per cell. Figure 41 shows that there is no difference between viral production from HepG2 cells in S phase and unsynchronized HepG2 cells at 19 and 20 hours whereas there is a big difference between viral production from HepG2 cells in S phase and unsynchronized HepG2 cells at 22 hours. These results demonstrate that dengue serotype 2 produces from HepG2 cells in S phase lower than from unsynchronized HepG2 cells about 3.3 fold at 22 hours.

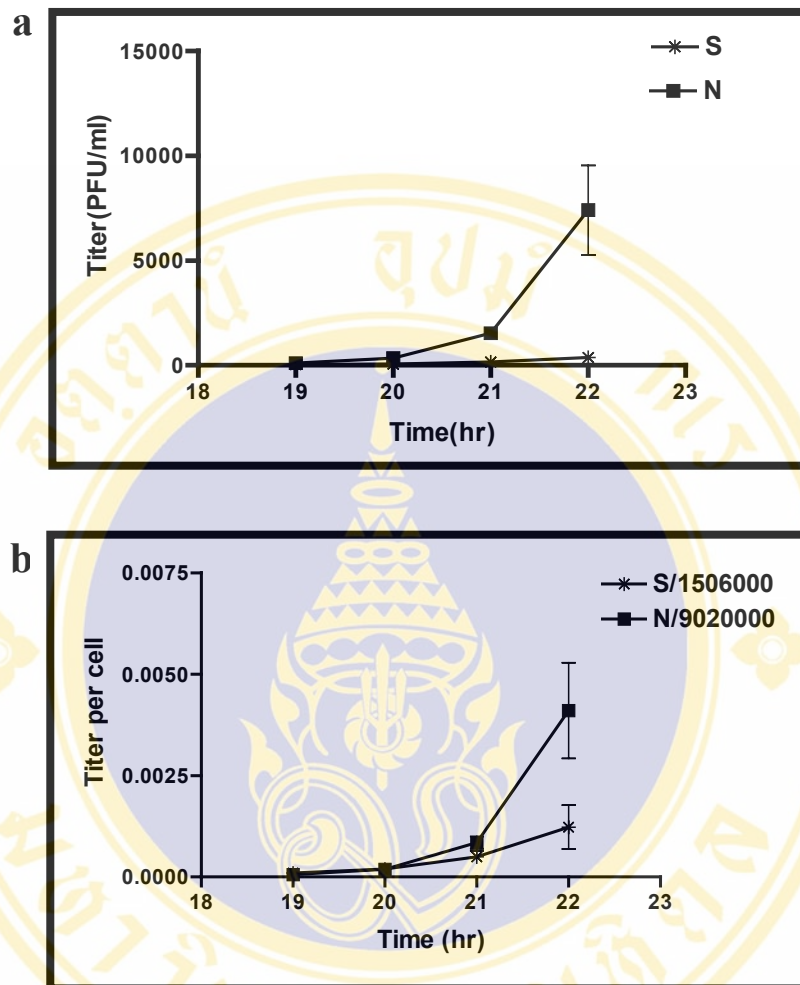


Figure 41. Comparison of dengue serotype 2 production between unsynchronized HepG2 cells and HepG2 cells in S phase

Dengue serotype 2 is produced from HepG2 cells in S phase less than from unsynchronized HepG2 cells. Figure 41a shows the difference of viral production from HepG2 cells in S phase and unsynchronized HepG2 cells in term of the titer of infectious virus whereas figure 41b shows in term of the titer per cell.

5.4 Comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in G₂ phase

After HepG2 cells were synchronized into G₂ phase by serum starvation method, as described in table 3, the cells were infected with dengue serotype 3 at moi 1 and incubated at 37°C / 10% CO₂ for 1 hour. The medium containing virus was collected at 19 hours to 22 hours. Then the sample was titered by the plaque assay method. Figure 42 shows the result of comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in G₂ phase. Figure 42a shows a difference of viral production from unsynchronized HepG2 cells and HepG2 cells in G₂ phase in term of titer of the infectious virus but figure 42b shows in term of titer of the infectious virus per cell. Both figure 42a and 42b show that there is a slight difference between viral production from unsynchronized HepG2 cells and HepG2 cells in G₂ phase at 19 hours whereas there is a big difference between viral production from unsynchronized HepG2 cells and HepG2 cells in G₂ phase at 22 hours. These results show that dengue serotype 3 produces from HepG2 cells in G₂ phase more than from unsynchronized HepG2 cells about 6.1 fold at 22 hours.

5.5 Comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in M phase

After HepG2 cells were synchronized into M phase by incubating in paclitaxel, as described in table 3, the cells were infected with dengue serotype 3 at moi 1 and incubated at 37°C / 10% CO₂ for 1 hour. The medium containing virus was collected at 19 hours to 22 hours. Then the sample was titered by the plaque assay method. Figure 43 shows the result of comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in M phase. Figure 43a shows a difference of viral production from unsynchronized HepG2 cells and HepG2 cells in M phase in term of titer of the infectious virus but figure 43b shows in term of titer of the infectious virus per cell. Figure 43a shows a slight difference between viral production from unsynchronized HepG2 and HepG2 cells in M phase at 19 hours whereas viral production from

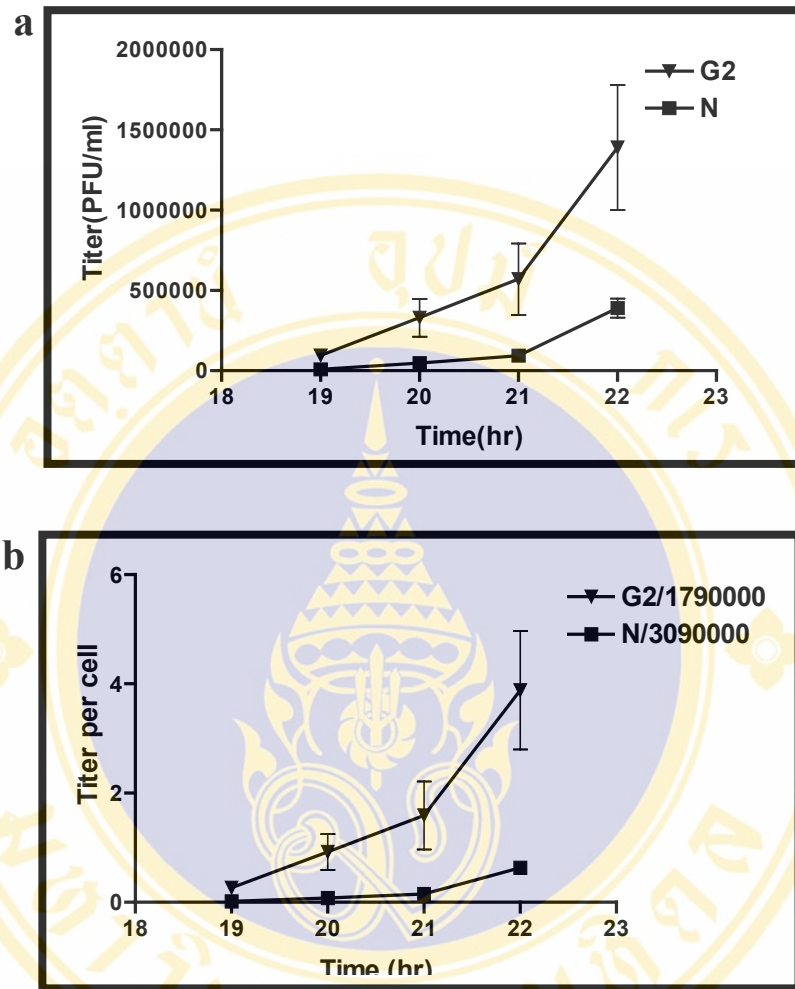


Figure 42. Comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in G₂ phase

Dengue serotype 3 is produced from HepG2 cells in G₂ phase more than from unsynchronized HepG2 cells. Figure 42a shows the difference of viral production from HepG2 cells in G₂ phase and unsynchronized HepG2 cells in term of the titer of infectious virus whereas figure 42b shows in term of the titer per cell.

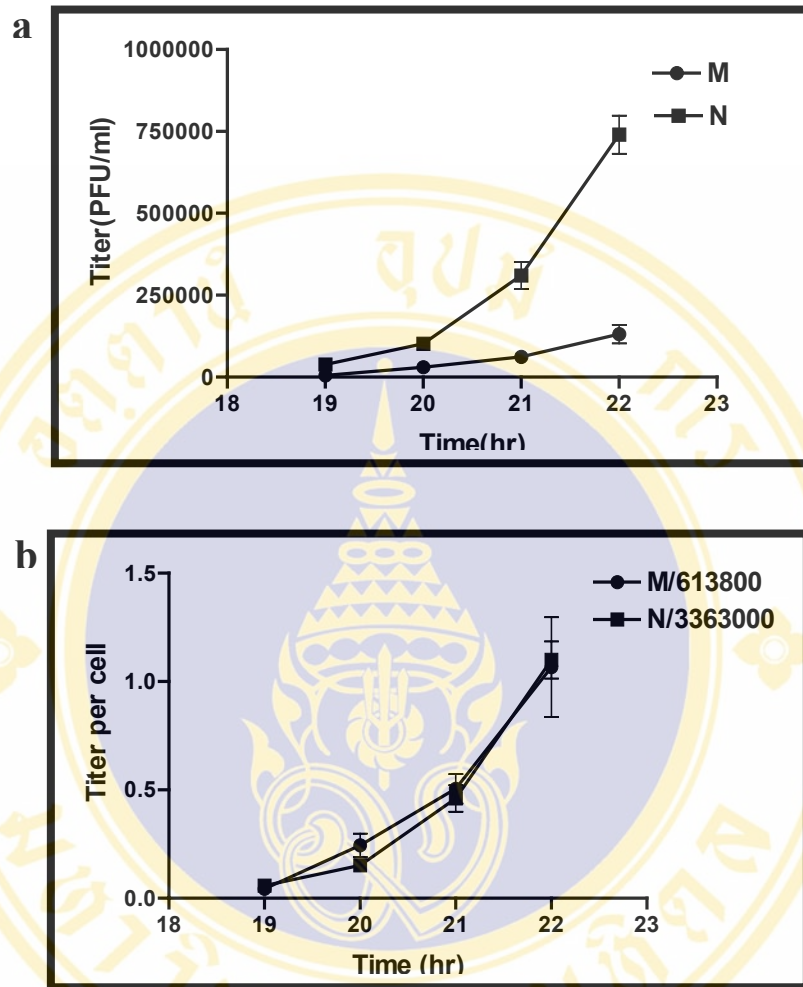


Figure 43. Comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in M phase

Dengue serotype 3 is produced from HepG2 cells in M phase and unsynchronized HepG2 cells equally. Figure 43a shows the difference of viral production from HepG2 cells in M phase and unsynchronized HepG2 cells in term of the titer of infectious virus whereas figure 43b shows in term of the titer per cell.

unsynchronized HepG2 is much higher than from HepG2 cells in M phase at 22 hours. But the number of cells of HepG2 cells in M phase is much less than the number of cells of unsynchronized HepG2 cells. Then when the result was calculated in term of titer of the infectious virus per cell (figure 43b), viral production from unsynchronized HepG2 cells and HepG2 cells in M phase is not different. This result indicates that dengue serotype 3 produces equally from HepG2 cells in M phase and unsynchronized HepG2 cells.

5.6 Comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in S phase

After HepG2 cells were synchronized into S phase by using thymidine double block method, as described in table 3, the cells were infected with dengue serotype 3 at moi 1 and incubated at 37°C / 10% CO₂ for 1 hour. The medium containing virus was collected at 19 hours to 22 hours. Then the sample was titered by the plaque assay method. Figure 44 shows the result of comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in S phase. Figure 44a shows a difference of viral production from unsynchronized HepG2 cells and HepG2 cells in M phase in term of titer of the infectious virus but figure 44b shows in term of titer of the infectious virus per cell. From figure 44a, there is no difference between viral production from HepG2 cell in S phase and unsynchronized HepG2 cells at 19 hours, whereas there is the difference at 22 hours. But when the result was change to compare in term of titer of the infectious virus per cell (figure 44b), the viral production from HepG2 cells in S phase is equal to the viral production from unsynchronized HepG2 cells. This result shows that dengue serotype 3 produce from HepG2 cells in S phase and unsynchronized HepG2 cells equally.

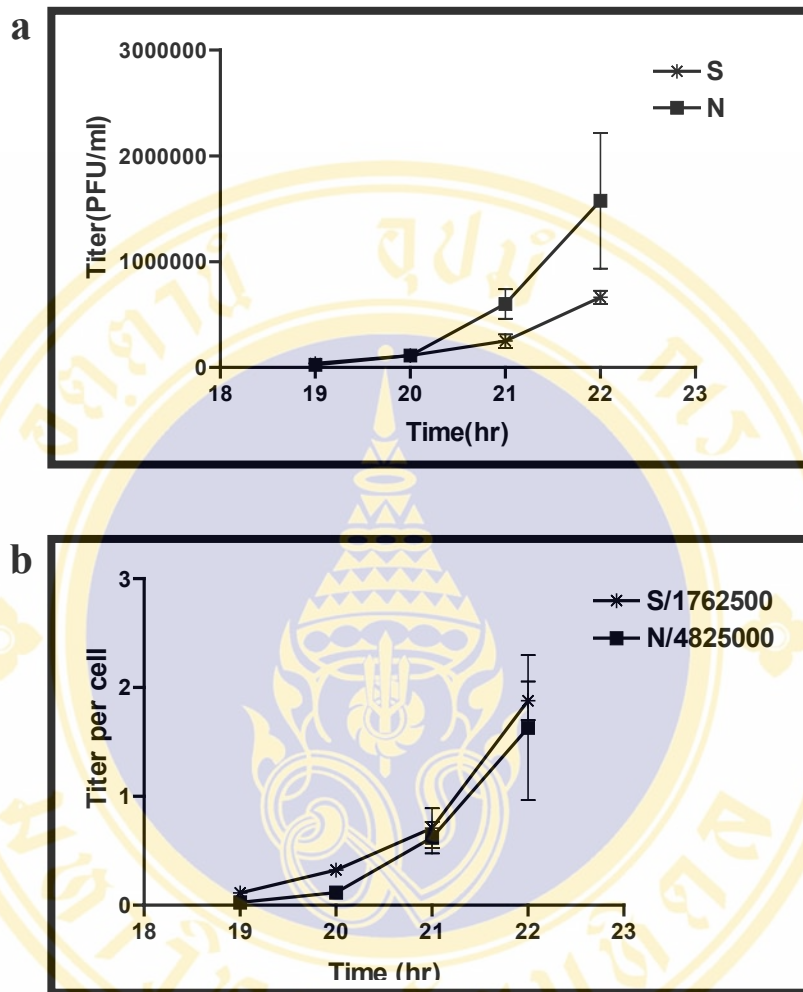


Figure 44. Comparison of dengue serotype 3 production between unsynchronized HepG2 cells and HepG2 cells in S phase

Dengue serotype 3 is produced from HepG2 cells in S phase and unsynchronized HepG2 cells equally. Figure 44a shows the difference of viral production from HepG2 cells in S phase and unsynchronized HepG2 cells in term of the titer of infectious virus whereas figure 44b shows in term of the titer per cell.

5.7 Summary of the infection of synchronized HepG2 cells

The infection of HepG2 cells in each phase of the cell cycle is summarized in Table 4.

Table 4. Summary of the infection of HepG cells in each phase of the cell cycle

Phase of the cell cycle	dengue serotype 2	dengue serotype 3
G ₂	↑	↑
M	↓	↔
S	↓	↔

6. Infection of synchronized Vero cells

Vero cells were synchronized into each phase of the cell cycle by using several methods. (Table 5)

Table 5. Summary of the method to synchronize Vero cells

Cell cycle phase	Method
G ₀	Incubation in MEM with no FBS for 36 hours
M	Incubation in 0.1 μ M paclitaxel in DMEM supplemented with 5% FBS for 24 hours
S	Thymidine double block method by incubating in 2 mM thymidine in DMEM supplemented with 0.1% FBS for 12 hours and incubating in DMEM supplemented with 5% FBS for 16 hours. Finally, the cells were incubated in 2 mM thymidine in DMEM supplemented with 0.1% FBS for 12 hours

6.1 Comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in G₀ phase

After Vero cells were synchronized into G₀ phase by serum starvation method, as described in table 4, the cells were infected with dengue serotype 2 at moi 1 and incubated at 37°C / 5% CO₂ for 1 hour. The medium containing virus was collected at 16 hours to 19 hours. Then the sample was titered by the plaque assay method. Figure 45 shows the result of comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in G₀ phase. Figure 45a shows a difference between viral production from unsynchronized Vero cells and Vero cells in G₀ phase in term of titer of the infectious virus but figure 45b shows in term of titer of the infectious virus per cell. From figure 45a, the viral production from unsynchronized Vero cells is higher than from Vero cells in G₀ phase but in term of titer of the infectious virus per cell (figure 45b), the viral production from unsynchronized Vero cells is not highly different from Vero cells in G₀ phase. Especially at 18 hours, it's quite same between viral production from Vero cells in G₀ phase and unsynchronized Vero cells. This result indicates that dengue serotype 2 production from Vero cells in G₀ phase is about 0.7 fold of dengue serotype 2 production from unsynchronized Vero cells at 19 hours.

6.2 Comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in M phase

After Vero cells were synchronized into M phase by incubating in paclitaxel, as described in table 4, the cells were infected with dengue serotype 2 at moi 1 and incubated at 37°C / 5% CO₂ for 1 hour. The medium containing virus was collected at 17 hours to 20 hours. Then the sample was titered by plaque assay method. Figure 46 shows the result of comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in M phase. Figure 46a shows a difference of viral production from unsynchronized Vero cells and Vero cells in M phase in term of titer of the infectious virus but figure 46b shows in term of titer of the infectious virus per cell. From figure 46a, the viral production from unsynchronized Vero cell is slightly higher than from Vero

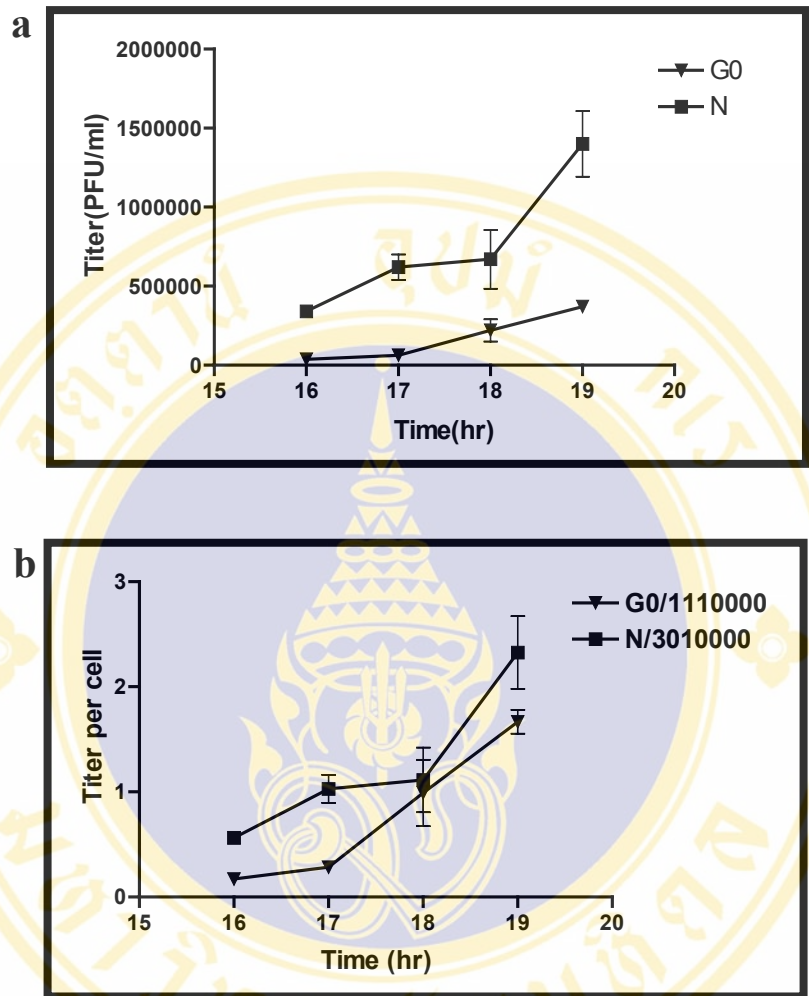


Figure 45. Comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in G₀ phase

Dengue serotype 2 is produced from Vero cells in G₀ phase not much less than from unsynchronized Vero cells. Figure 45a shows the difference of viral production from Vero cells in G₀ phase and unsynchronized Vero cells in term of the titer of infectious virus whereas figure 45b shows in term of the titer per cell.

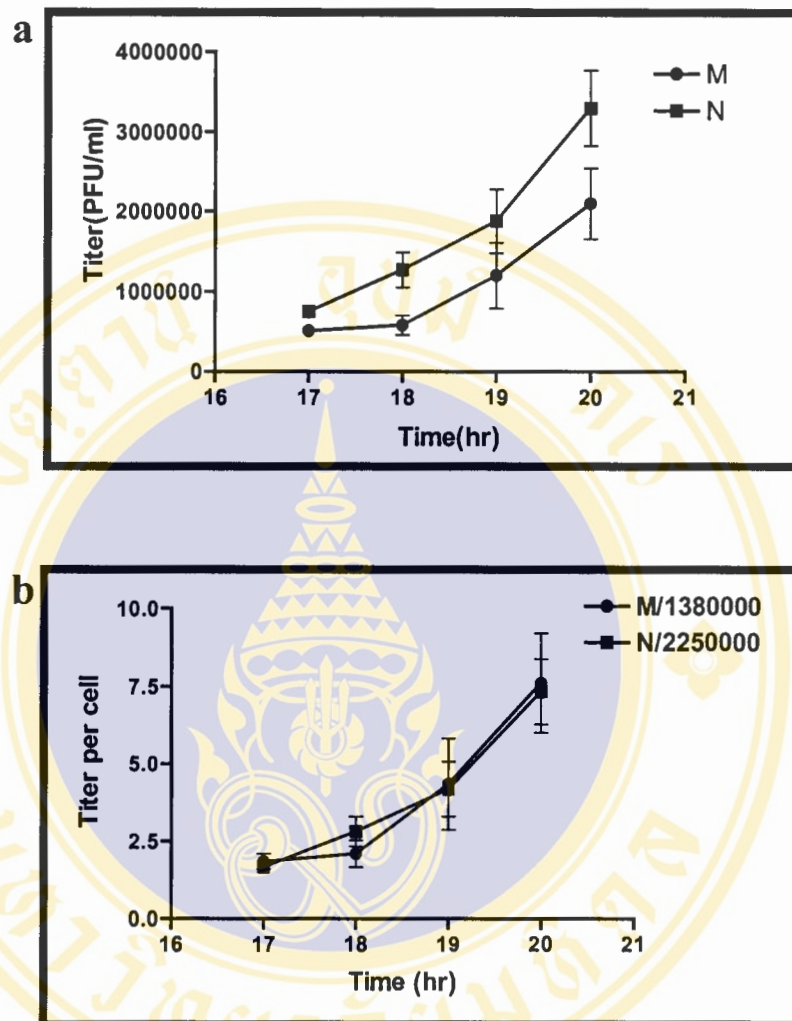


Figure 46. Comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in M phase

Dengue serotype 2 is produced from Vero cells in M phase and unsynchronized Vero cells equally. Figure 46a shows the difference of viral production from Vero cells in M phase and unsynchronized Vero cells in term of the titer of infectious virus whereas figure 46b shows in term of the titer per cell.

cells in M phase. But when the result was changed to calculate in term of titer of the infectious virus per cell (figure 46b), there is no difference between the viral production from Vero cells in M phase and from unsynchronized Vero cells. This result indicates that dengue serotype 2 production from Vero cells in M phase and from unsynchronized Vero cells is equal.

6.3 Comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in S phase

After Vero cells were synchronized into S phase by using thymidine double block method, as described in table 4, the cells were infected with dengue serotype 2 at moi 1 and incubated at 37°C / 5% CO₂ for 1 hour. The medium containing virus was collected at 17 hours to 20 hours. Then the sample was titered by the plaque assay method. Figure 47 shows the result of comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in S phase. Figure 47a shows a difference of viral production from unsynchronized Vero cells and Vero cells in S phase in term of titer of the infectious virus but figure 47b shows in term of titer of the infectious virus per cell. From these results, there is slightly difference between the viral production from Vero cells in S phase and unsynchronized Vero cells but the viral production at other time point is much different. These results show that dengue serotype 2 production from Vero cells in S phase is lower than from unsynchronized Vero cells about 1.4 fold at 20 hours.

6.4 Comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in G₀ phase

After Vero cells were synchronized into G₀ phase by serum starvation method, as described in table 4, the cells were infected with dengue serotype 3 at moi 1 and incubated at 37°C / 5% CO₂ for 1 hour. The medium containing virus was collected at 16 hours to 19 hours. Then the sample was titered by the plaque assay method. Figure 48 shows the result of comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in G₀ phase. Figure 48a shows a difference of viral production from unsynchronized Vero cells and Vero cells in G₀ phase in term of titer of the infectious virus but figure 48b shows in term of titer of the infectious virus per cell.

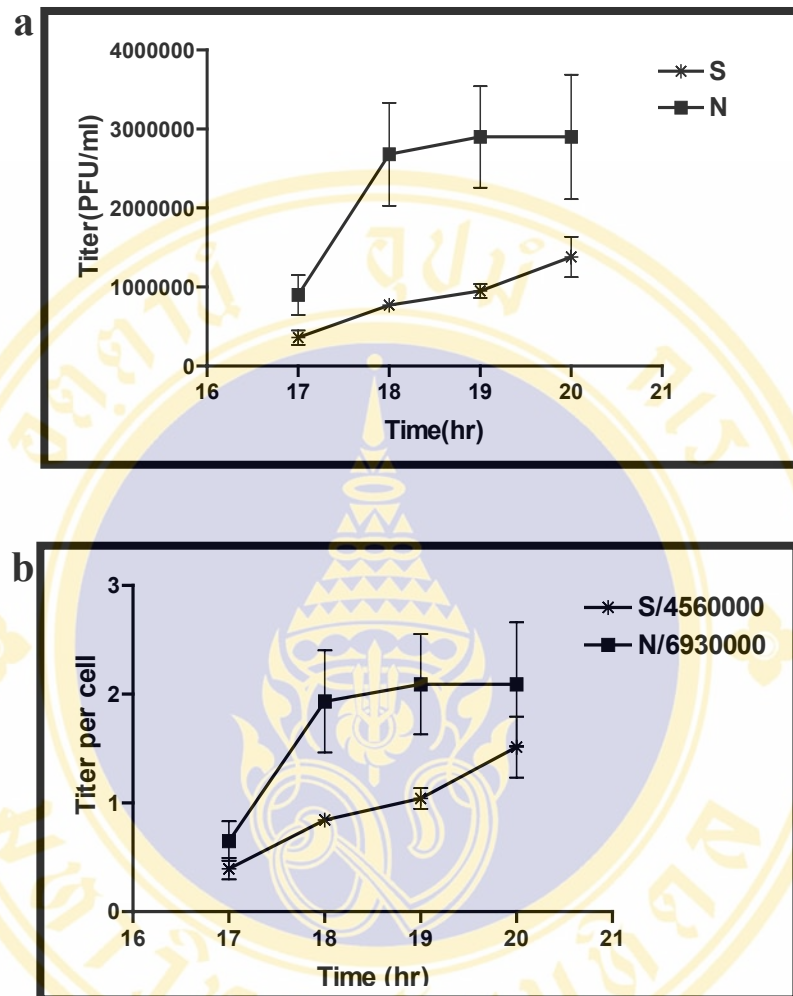


Figure 47. Comparison of dengue serotype 2 production between unsynchronized Vero cells and Vero cells in S phase

Dengue serotype 2 is produced from Vero cells in S phase less than from unsynchronized Vero cells. Figure 47a shows the difference of viral production from Vero cells in S phase and unsynchronized Vero cells in term of the titer of infectious virus whereas figure 47b shows in term of the titer per cell.

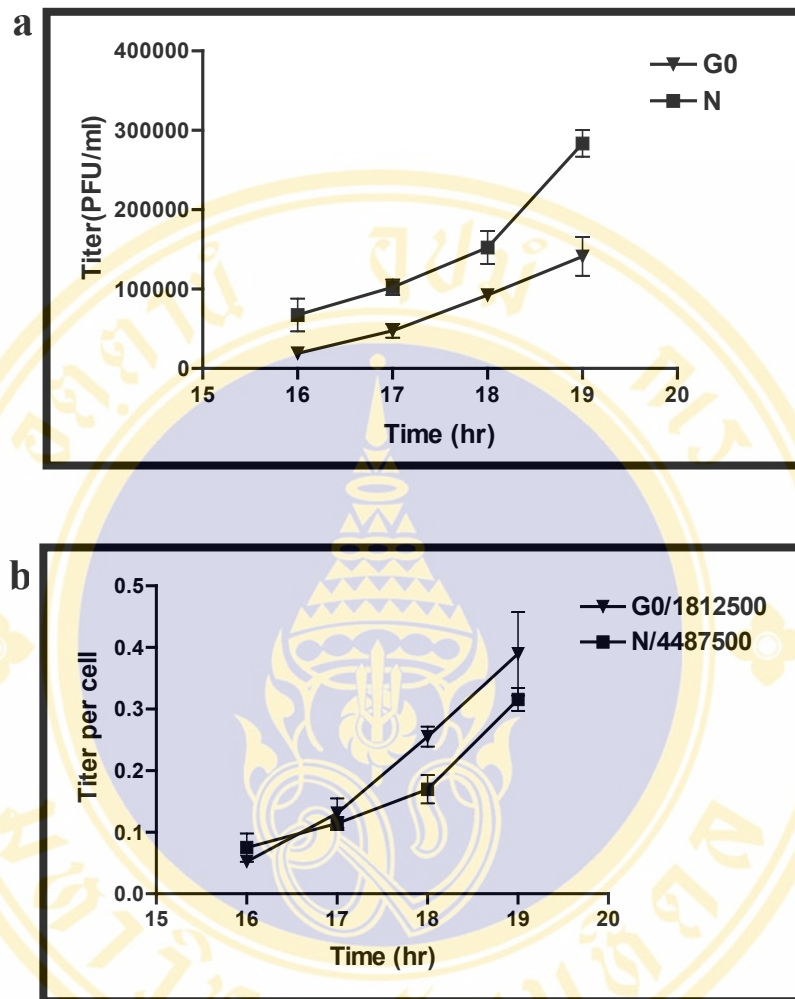


Figure 48. Comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in G₀ phase

Dengue serotype 3 production from Vero cells in G₀ phase and unsynchronized Vero cells is not different. Figure 48a shows the difference of viral production from Vero cells in G₀ phase and unsynchronized Vero cells in term of the titer of infectious virus whereas figure 48b shows in term of the titer per cell.

From figure 48a, the viral production from Vero cells in G₀ phase is lower than the viral production from unsynchronized Vero cells. But when the result was calculated in term of titer of the infectious virus per cell (figure 48b), the viral production from Vero cells in G₀ phase and unsynchronized Vero cells is quite equal. This result indicates that dengue serotype 3 produces from Vero cells in G₀ phase and unsynchronized Vero cells equally.

6.5 Comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in M phase

After Vero cells were synchronized into M phase by incubating in paclitaxel, as described in table 4, the cells were infected with dengue serotype 3 at moi 1 and incubated at 37°C / 5% CO₂ for 1 hour. The medium containing virus was collected at 16 hours to 19 hours. Then the sample was titered by the plaque assay method. Figure 49 shows the result of comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in M phase. Figure 49a shows a difference of viral production from unsynchronized Vero cells and Vero cells in M phase in term of titer of the infectious virus but figure 49b shows in term of titer of the infectious virus per cell. From both figure 49a and 49b, the viral production from Vero cells in M phase and unsynchronized Vero cells is not different. These results demonstrate that dengue serotype 3 produces equally from Vero cells in M phase and unsynchronized Vero cells.

6.6 Comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in S phase

After Vero cells were synchronized into S phase by using thymidine double block method, as described in table 4, the cells were infected with dengue serotype 3 at moi 1 and incubated at 37°C / 5% CO₂ for 1 hour. The medium containing virus was collected at 16 hours to 19 hours. Then the sample was titered by the plaque assay method. Figure 50 shows the result of comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in S phase. Figure 50a shows a difference of viral production from unsynchronized Vero cells and Vero cells in S phase in term of titer of the infectious virus but figure 50b shows in term of titer of the infectious virus per cell.

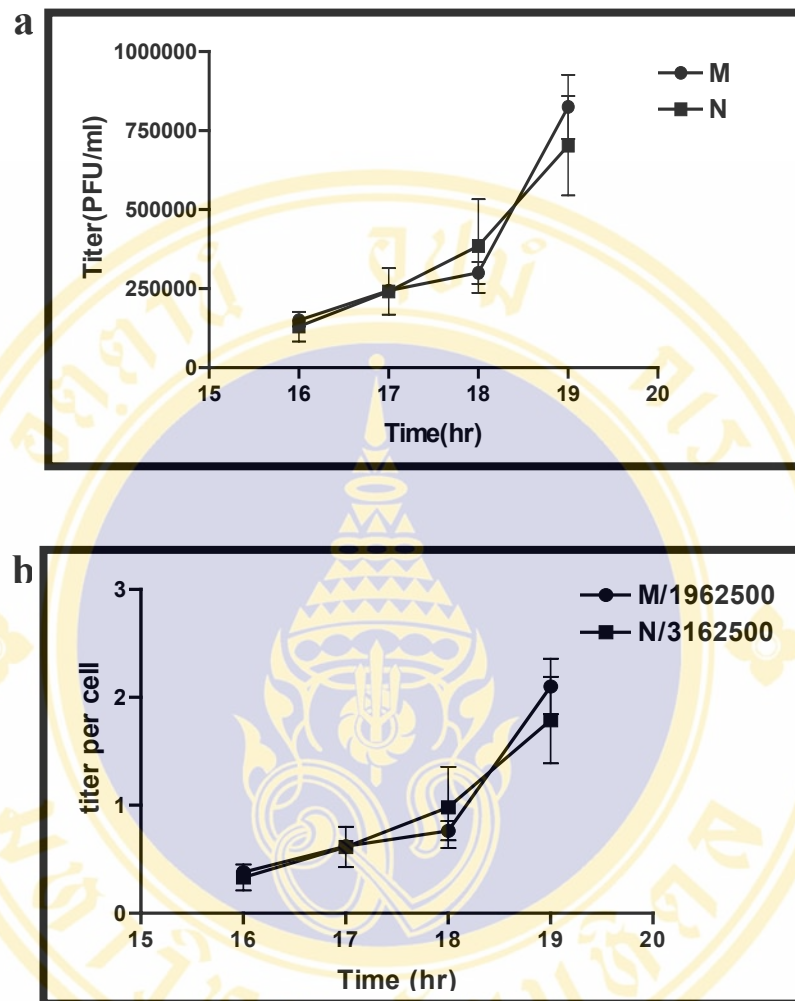


Figure 49. Comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in M phase

Dengue serotype 3 production from Vero cells in M phase and unsynchronized Vero cells is not different. Figure 49a shows the difference of viral production from Vero cells in M phase and unsynchronized Vero cells in term of the titer of infectious virus whereas figure 49b shows in term of the titer per cell.

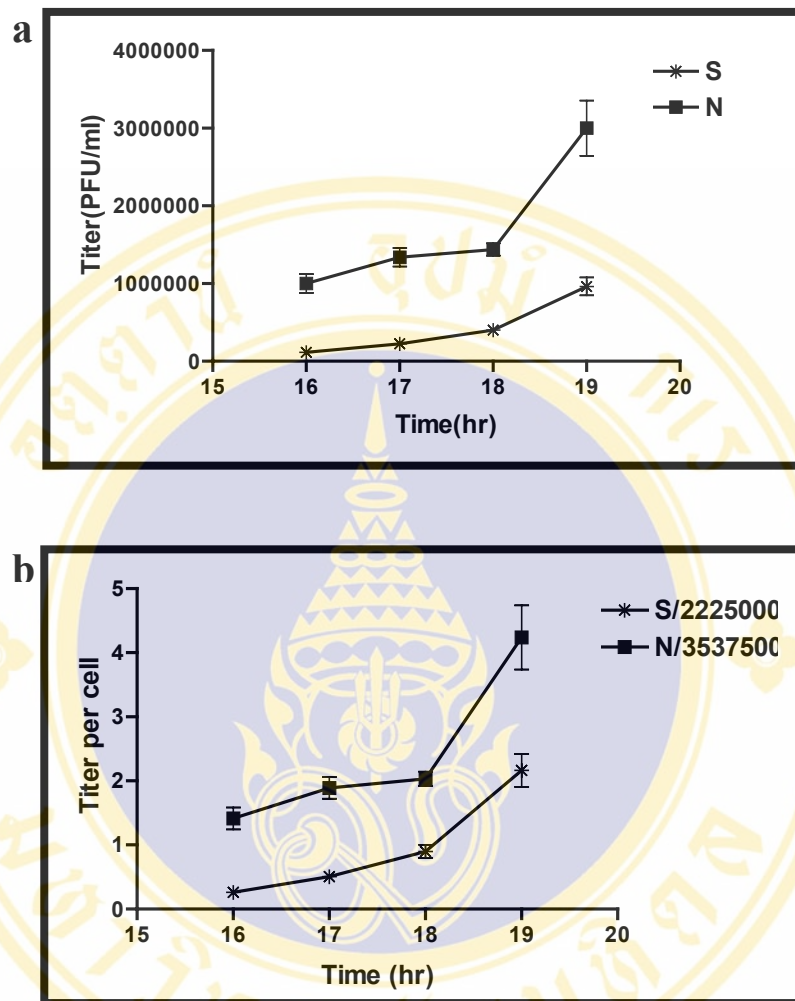


Figure 50. Comparison of dengue serotype 3 production between unsynchronized Vero cells and Vero cells in S phase

Dengue serotype 3 produces from Vero cells in S phase less than from unsynchronized Vero cells. Figure 50a shows the difference of viral production from Vero cells in S phase and unsynchronized Vero cells in term of the titer of infectious virus whereas figure 50b shows in term of the titer per cell.

From both figure 50a and 50b, the viral production from Vero cells in S phase is lower than the viral production from unsynchronized Vero cells. These results indicate that dengue serotype 3 produces from Vero cells in S phase lower than from unsynchronized Vero cells about 2 fold at 19 hours.

6.7 Summary of the infection of synchronized Vero cells

The infection of Vero cells in each phase of the cell cycle is summarized in Table 6.

Table 6. Summary of the infection of Vero cells in each phase of the cell cycle

Phase of the cell cycle	dengue serotype 2	dengue serotype 3
G ₀	↓	↔
M	↔	↔
S	↓	↓

From the result in section 5 and 6, dengue serotype 2 and 3 produce from HepG2 cells and Vero cells in each phase of the cell cycle differently. There are two possible ways that cause the difference of the production of dengue serotype 2 and 3 between synchronized and unsynchronized cells. Firstly, the receptors of dengue virus that express on the surface of HepG2 cells and Vero cells may express depending on the cell cycle of the cells. Another possible way is that the environment in the cells may cause the difference between the infection of synchronized and unsynchronized cells. The way to answer what is the factor, which cause the difference of dengue production from the cells among each phase of the cell cycle, is to determine the percent infectivity of dengue in the cells in each phase.

7. Comparison of percent infectivity in HepG2 cells

7.1 Comparison of percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells and HepG2 cells in G₂ phase

Unsynchronized HepG2 cells and HepG2 cells in G₂ phase were infected with dengue virus serotype 2 at 37°C / 10%CO₂ for 1 hour. DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 10% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 51) shows that percent infectivity of dengue serotype 2 in HepG2 cells in G₂ phase is 1.16% whereas percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells is 0.76% and the P value is about 0.0291. Then the percent infectivity of dengue serotype 2 from unsynchronized HepG2 cells and HepG2 cells in G₂ phase is significantly different. From this result, dengue serotype 2 can infect HepG2 cells in G₂ phase higher than unsynchronized HepG2 cells.

7.2 Comparison of percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells and HepG2 cells in M phase

Unsynchronized HepG2 cells and HepG2 cells in M phase were infected with dengue virus serotype 2 at 37°C / 10%CO₂ for 1 hour. DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 10% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 52) shows that percent infectivity of dengue serotype 2 in HepG2 cells in M phase is 0.43% whereas percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells is 0.76% and the P value is about 0.1645.

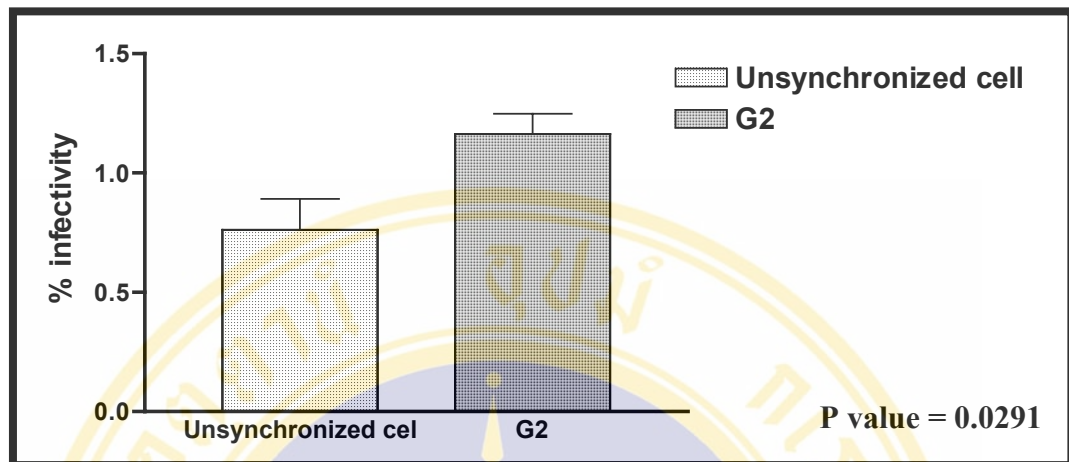


Figure 51. Comparison of percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells and HepG2 cells in G₂ phase

The percent infectivity of dengue serotype 2 in HepG2 cells in G₂ phase is 1.16% whereas percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells is 0.76% and the P value is about 0.0291. Dengue serotype 2 can infect HepG2 cells in G₂ phase higher than unsynchronized HepG2 cells.

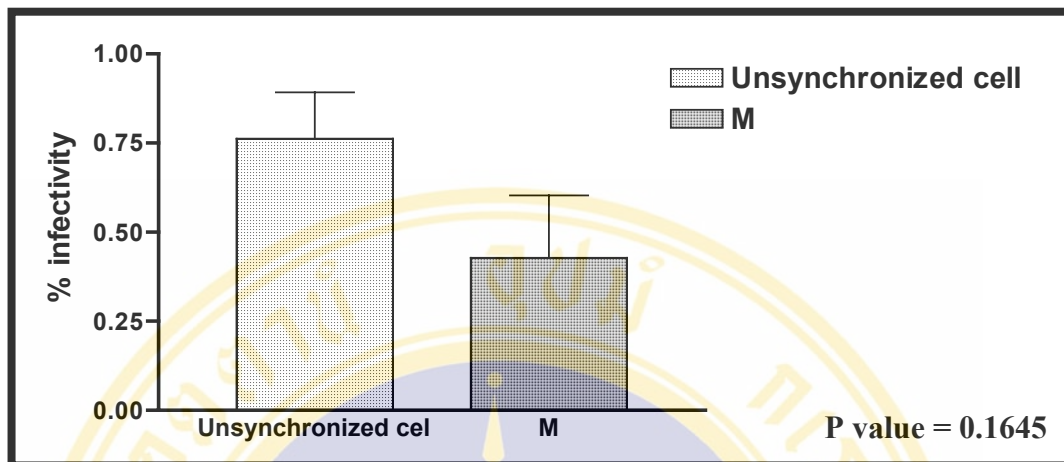


Figure 52. Comparison of percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells and HepG2 cells in M phase

The percent infectivity of dengue serotype 2 in HepG2 cells in M phase is 0.43% whereas percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells is 0.76% and the P value is about 0.1645. Dengue serotype 2 can infect HepG2 cells in M phase lower than unsynchronized HepG2 cells.

Then the percent infectivity of dengue serotype 2 from unsynchronized HepG2 cells and HepG2 cells in M phase is not significantly different. From this result, dengue serotype 2 can infect HepG2 cells in M phase lower than unsynchronized HepG2 cells.

7.3 Comparison of percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells and HepG2 cells in S phase

Unsynchronized HepG2 cells and HepG2 cells in S phase were infected with dengue virus serotype 2 at 37°C / 10%CO₂ for 1 hour. DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 10% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 53) shows that percent infectivity of dengue serotype 2 in HepG2 cells in S phase is 0.15% whereas percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells is 0.76% and the P value is about 0.0017. Then the percent infectivity of dengue serotype 2 from unsynchronized HepG2 cells and HepG2 cells in S phase is significantly different. From this result, dengue serotype 2 can infect HepG2 cells in S phase lower than unsynchronized HepG2 cells.

7.4 Comparison of percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells and HepG2 cells in G₂ phase

Unsynchronized HepG2 cells and HepG2 cells in G₂ phase were infected with dengue virus serotype 3 at 37°C / 10%CO₂ for 1 hour. DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 10% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 54) shows that percent infectivity of dengue serotype 3 in HepG2 cells in G₂ phase is 0.66% whereas percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells is 0.1% and the P value is about 0.0002.

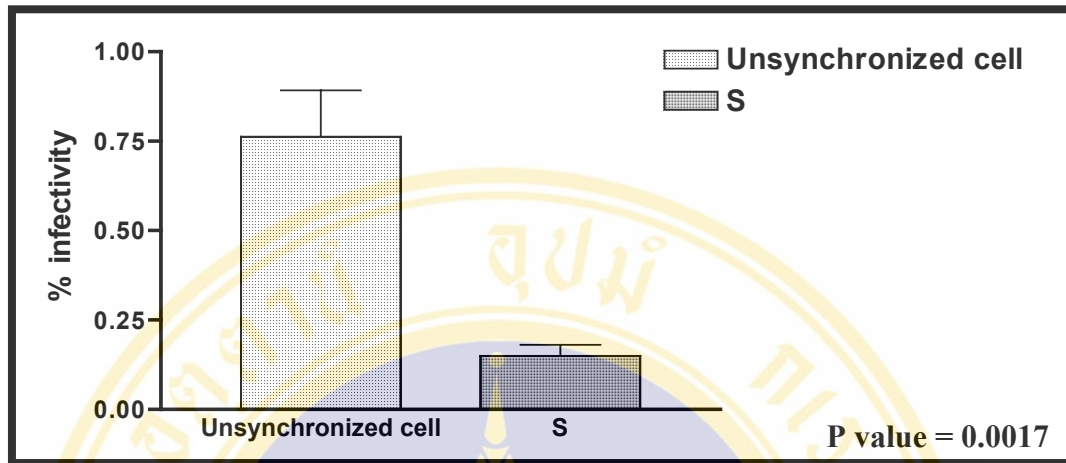


Figure 53. Comparison of percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells and HepG2 cells in S phase

The percent infectivity of dengue serotype 2 in HepG2 cells in S phase is 0.15% whereas percent infectivity of dengue serotype 2 in unsynchronized HepG2 cells is 0.76% and the P value is about 0.0017. Dengue serotype 2 can infect HepG2 cells in S phase lower than unsynchronized HepG2 cells.

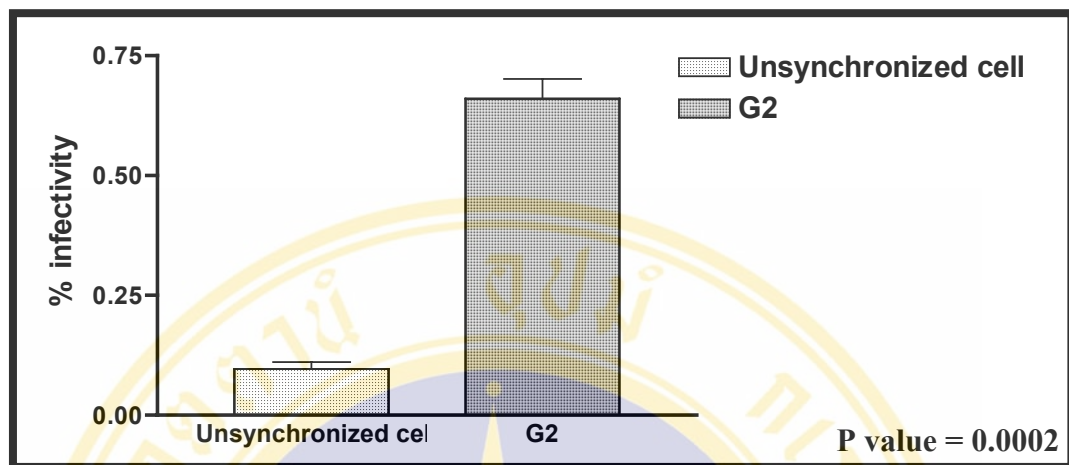


Figure 54. Comparison of percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells and HepG2 cells in G₂ phase

The percent infectivity of dengue serotype 3 in HepG2 cells in G₂ phase is 0.66% whereas percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells is 0.1% and the P value is about 0.0002. Dengue serotype 3 can infect HepG2 cells in G₂ phase higher than unsynchronized HepG2 cells.

Then the percent infectivity of dengue serotype 3 from unsynchronized HepG2 cells and HepG2 cells in G₂ phase is significantly different. From this result, dengue serotype 3 can infect HepG2 cells in G₂ phase higher than unsynchronized HepG2 cells.

7.5 Comparison of percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells and HepG2 cells in M phase

Unsynchronized HepG2 cells and HepG2 cells in M phase were infected with dengue virus serotype 3 at 37°C / 10%CO₂ for 1 hour. DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 10% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 55) shows that percent infectivity of dengue serotype 3 in HepG2 cells in M phase is 0.58% whereas percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells is 0.1% and the P value is about 0.0323. Then the percent infectivity of dengue serotype 3 from unsynchronized HepG2 cells and HepG2 cells in M phase is significantly different. From this result, dengue serotype 3 can infect HepG2 cells in M phase higher than unsynchronized HepG2 cells.

7.6 Comparison of percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells and HepG2 cells in S phase

Unsynchronized HepG2 cells and HepG2 cells in S phase were infected with dengue virus serotype 3 at 37°C / 10%CO₂ for 1 hour. DMEM supplemented with 10% FBS was added and infected cells were incubated at 37°C / 10%CO₂. The infected HepG2 cells were incubated for 16 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 10% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 56) shows that percent infectivity of dengue serotype 3 in HepG2 cells in S phase and unsynchronized HepG2 cells is 0.1% the P value is about 0.8593.

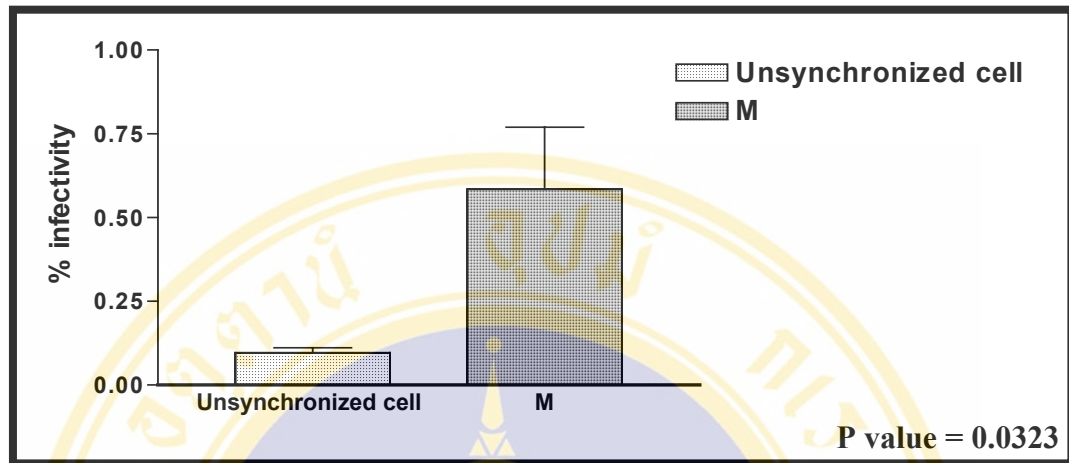


Figure 55. Comparison of percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells and HepG2 cells in M phase

The percent infectivity of dengue serotype 3 in HepG2 cells in M phase is 0.58% whereas percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells is 0.1% and the P value is about 0.0323. Dengue serotype 3 can infect HepG2 cells in M phase higher than unsynchronized HepG2 cells.

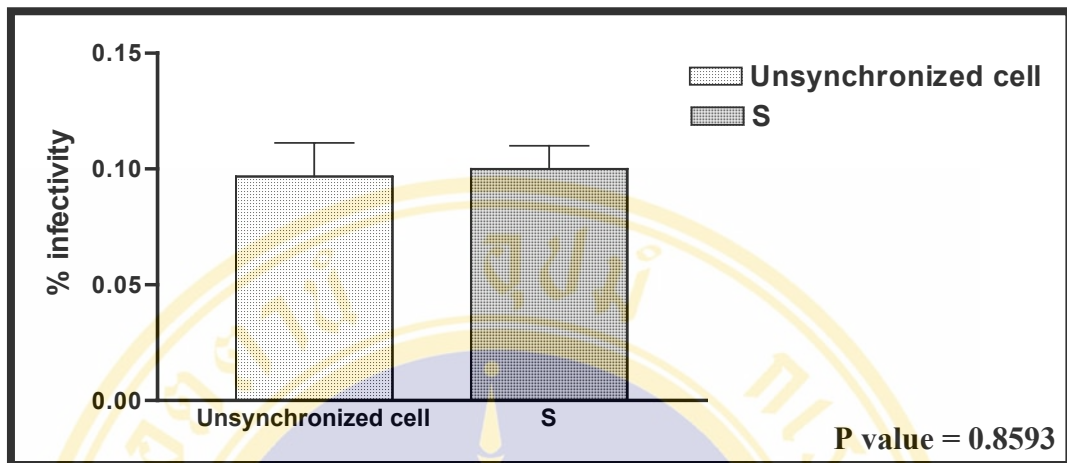


Figure 56. Comparison of percent infectivity of dengue serotype 3 in unsynchronized HepG2 cells and HepG2 cells in S phase

The percent infectivity of dengue serotype 3 in HepG2 cells in S phase and unsynchronized HepG2 cells is 0.1% the P value is about 0.8593. Dengue serotype 2 can infect HepG2 cells in S phase and unsynchronized HepG2 cells equally.

Then the percent infectivity of dengue serotype 2 from unsynchronized HepG2 cells and HepG2 cells in S phase is not significantly different. From this result, dengue serotype 2 can infect HepG2 cells in S phase and unsynchronized HepG2 cells equally.

7.7 Summary of the percent infectivity in HepG2 cells

The percent infectivity of dengue serotype 2 and 3 in HepG2 cells are summarized in figure 57 and figure 58 respectively. From figure 57, the percent infectivity of DEN-2 in HepG2 cells in G₂ phase, M phase, S phase and unsynchronized HepG2 cells is 1.16%, 0.43%, 0.15% and 0.76% respectively and P value is less than 0.0001. From figure 58, The percent infectivity of DEN-3 in HepG2 cells in G₂ phase, M phase, S phase and unsynchronized HepG2 cells is 0.66%, 0.58%, 0.10% and 0.10% respectively and P value is 0.0012. From these two figures, the percent infectivity of dengue serotype 2 and 3 in HepG2 cells is significantly different among each phase of the cells cycle.

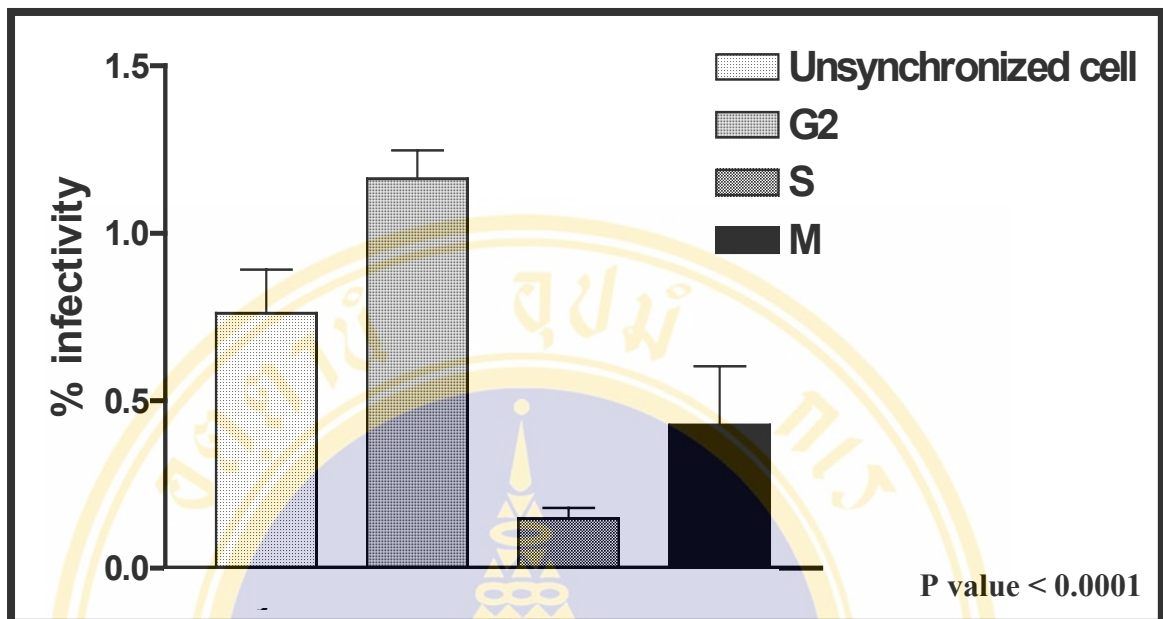


Figure 57. The comparison of percent infectivity of HepG2 cells in each phase of the cell cycle compared with unsynchronized HepG2 cells, which were infected with dengue serotype 2

The percent infectivity of dengue serotype 2 in HepG2 cells is significantly different among each phase of the cell cycle. The percent infectivity in G₂ phase is higher whereas in M phase and S phase is lower than the percent infectivity in unsynchronized HepG2 cells.

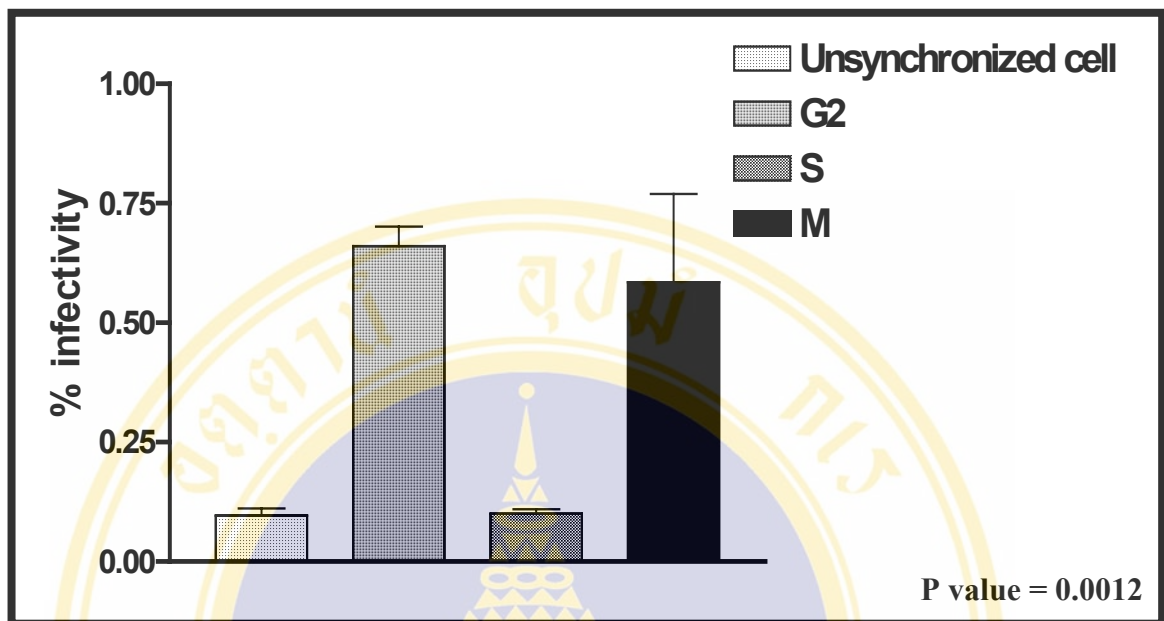


Figure 58. The comparison of percent infectivity of HepG2 cells in each phase of the cell cycle compared with unsynchronized HepG2 cells, which were infected with dengue serotype 3

The percent infectivity of dengue serotype 3 in HepG2 cells is significantly different among each phase of the cell cycle. The percent infectivity in G₂ phase and M phase are higher whereas in S phase is lower than the percent infectivity in unsynchronized HepG2 cells.

8. Comparison of percent infectivity in Vero cells

8.1 Comparison of percent infectivity of dengue serotype 2 in unsynchronized Vero cells and Vero cells in G₀ phase

Unsynchronized Vero cells and Vero cells in G₀ phase were infected with dengue virus serotype 2 at 37°C / 5%CO₂ for 1 hour. DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 5% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 59) shows that percent infectivity of dengue serotype 2 in Vero cells in G₀ phase is 12.74% whereas percent infectivity of dengue serotype 2 in unsynchronized Vero cells is 24.2% and the P value is about 0.0704. Then the percent infectivity of dengue serotype 2 from unsynchronized Vero cells and Vero cells in G₀ phase is not significantly different. From this result, dengue serotype 2 can infect Vero cells in G₀ phase lower than unsynchronized Vero cells.

8.2 Comparison of percent infectivity of dengue serotype 2 in unsynchronized Vero cells and Vero cells in M phase

Unsynchronized Vero cells and Vero cells in M phase were infected with dengue virus serotype 2 at 37°C / 5%CO₂ for 1 hour. DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 5% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 60) shows that percent infectivity of dengue serotype 2 in Vero cells in M phase is 17.52% whereas percent infectivity of dengue serotype 2 in unsynchronized Vero cells is 24.2% and the P value is about 0.3231. Then the percent infectivity of dengue serotype 2 from unsynchronized Vero cells and Vero cells in M phase is not significantly different. From this result, dengue serotype 2 can infect Vero cells in M phase lower than unsynchronized Vero cells.

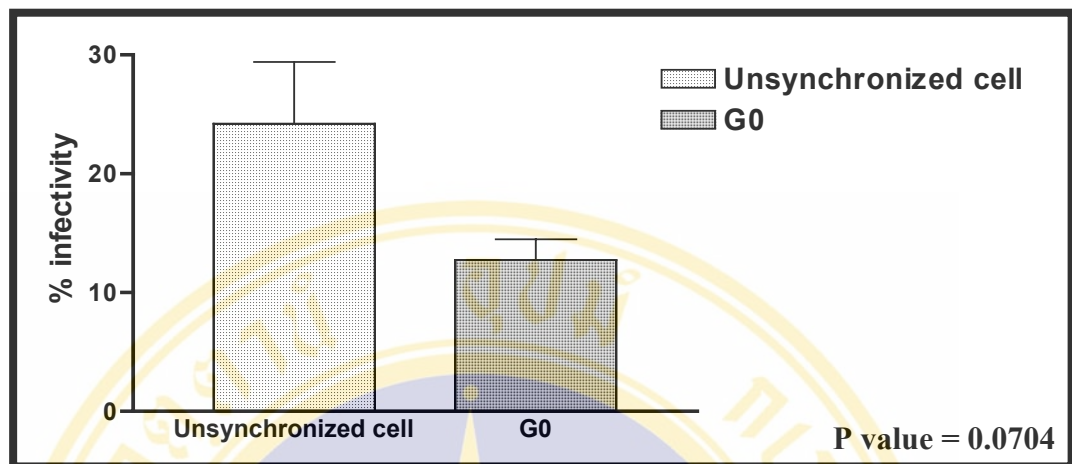


Figure 59. Comparison of percent infectivity of dengue serotype 2 in unsynchronized Vero cells and Vero cells in G₀ phase

The percent infectivity of dengue serotype 2 in Vero cells in G₀ phase is 12.74% whereas percent infectivity of dengue serotype 2 in unsynchronized Vero cells is 24.2% and the P value is about 0.0704. Dengue serotype 2 can infect Vero cells in G₀ phase lower than unsynchronized Vero cells.

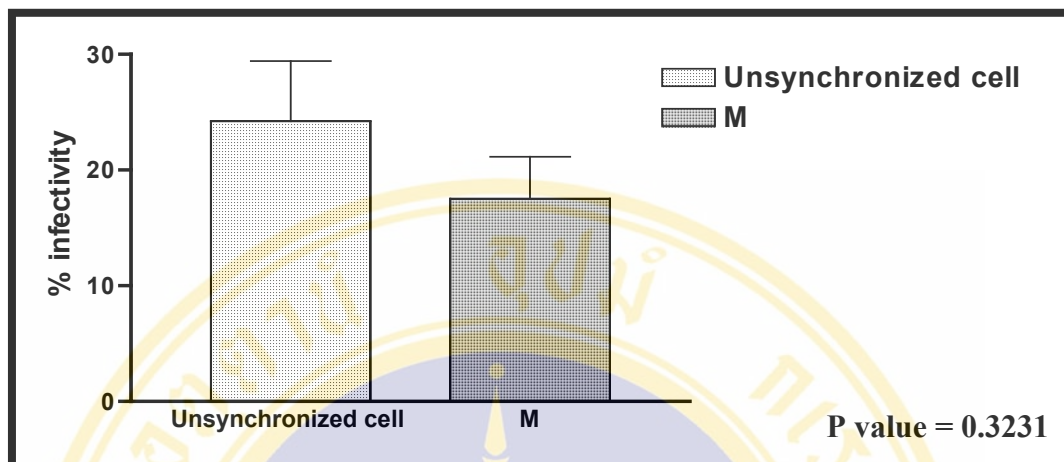


Figure 60. Comparison of percent infectivity of dengue serotype 2 in unsynchronized Vero cells and Vero cells in M phase

The percent infectivity of dengue serotype 2 in Vero cells in M phase is 17.52% whereas percent infectivity of dengue serotype 2 in unsynchronized Vero cells is 24.2% and the P value is about 0.3231. Dengue serotype 2 can infect Vero cells in M phase lower than unsynchronized Vero cells.

8.3 Comparison of percent infectivity of dengue serotype 2 in unsynchronized Vero cells and Vero cells in S phase

Unsynchronized Vero cells and Vero cells in S phase were infected with dengue virus serotype 2 at 37°C / 5%CO₂ for 1 hour. DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 5% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 61) shows that percent infectivity of dengue serotype 2 in Vero cells in S phase is 17.39% whereas percent infectivity of dengue serotype 2 in unsynchronized Vero cells is 24.2% and the P value is about 0.3263. Then the percent infectivity of dengue serotype 2 from unsynchronized Vero cells and Vero cells in S phase is not significantly different.

8.4 Comparison of percent infectivity of dengue serotype 3 in unsynchronized Vero cells and Vero cells in G₀ phase

Unsynchronized Vero cells and Vero cells in G₀ phase were infected with dengue virus serotype 3 at 37°C / 5%CO₂ for 1 hour. DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 5% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 62) shows that percent infectivity of dengue serotype 3 in Vero cells in G₀ phase is 13.21% whereas percent infectivity of dengue serotype 3 in unsynchronized Vero cells is 19.70% and the P value is about 0.0616. Then the percent infectivity of dengue serotype 3 from unsynchronized Vero cells and Vero cells in G₀ phase is not significantly different. From this result, dengue serotype 3 can infect Vero cells in G₀ phase lower than unsynchronized Vero cells.

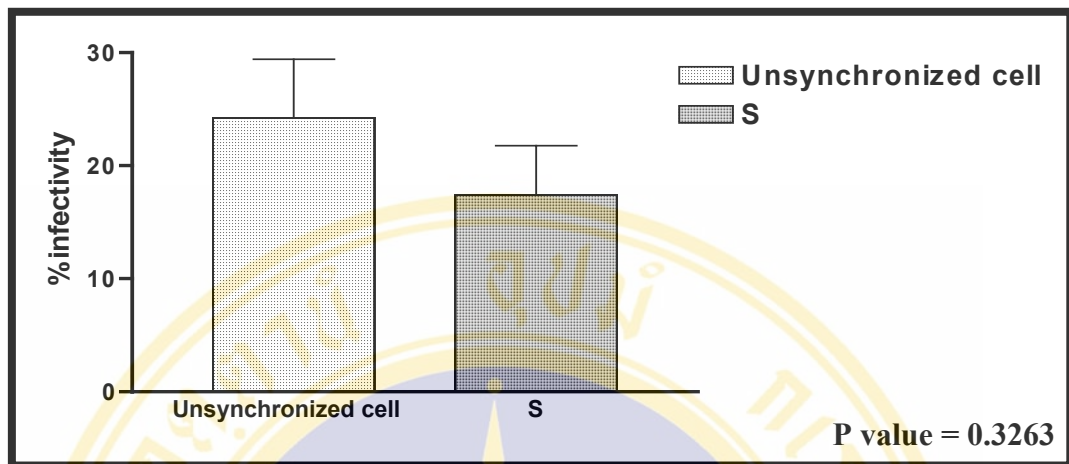


Figure 61. Comparison of percent infectivity of dengue serotype 2 in unsynchronized Vero cells and Vero cells in S phase

The percent infectivity of dengue serotype 2 in Vero cells in S phase is 17.39% whereas percent infectivity of dengue serotype 2 in unsynchronized Vero cells is 24.2% and the P value is about 0.3263. From this result, dengue serotype 2 can infect Vero cells in S phase lower than unsynchronized Vero cells.

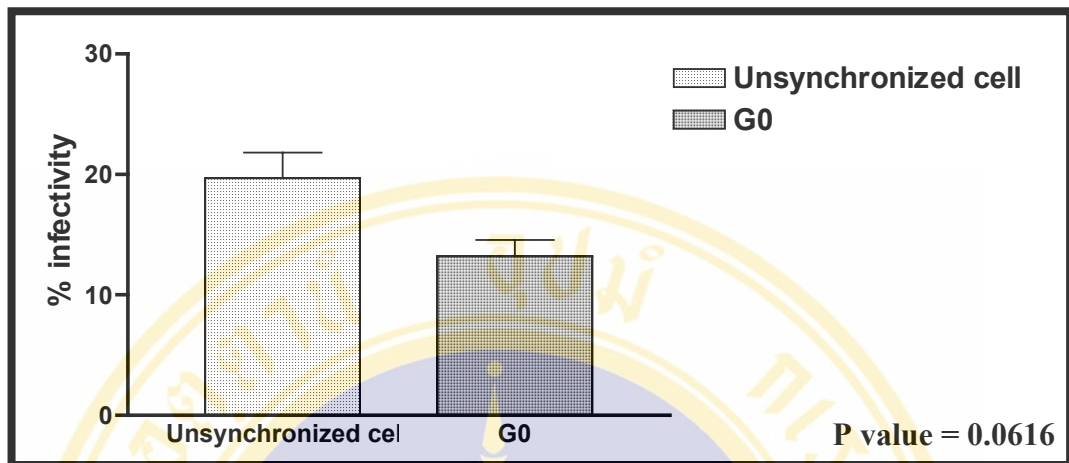


Figure 62. Comparison of percent infectivity of dengue serotype 3 in unsynchronized Vero cells and Vero cells in G₀ phase

The percent infectivity of dengue serotype 3 in Vero cells in G₀ phase is 13.21% whereas percent infectivity of dengue serotype 3 in unsynchronized Vero cells is 19.70% and the P value is about 0.0616. Dengue serotype 3 can infect Vero cells in G₀ phase lower than unsynchronized Vero cells.

8.5 Comparison of percent infectivity of dengue serotype 3 in unsynchronized Vero cells and Vero cells in M phase

Unsynchronized Vero cells and Vero cells in M phase were infected with dengue virus serotype 3 at 37°C / 5%CO₂ for 1 hour. DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 5% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 63) shows that percent infectivity of dengue serotype 3 in Vero cells in M phase is 23.33% whereas percent infectivity of dengue serotype 3 in unsynchronized Vero cells is 19.70% and the P value is about 0.4041. Then the percent infectivity of dengue serotype 3 from unsynchronized Vero cells and Vero cells in M phase is not significantly different. From this result, dengue serotype 3 can infect Vero cells in M phase higher than unsynchronized Vero cells.

8.6 Comparison of percent infectivity of dengue serotype 3 in unsynchronized Vero cells and Vero cells in S phase

Unsynchronized Vero cells and Vero cells in S phase were infected with dengue virus serotype 3 at 37°C / 5%CO₂ for 1 hour. DMEM supplemented with 5% FBS was added and infected cells were incubated at 37°C / 5%CO₂. The infected Vero cells were incubated for 2 hours. After that the infected cells were trypsinized and diluted in DMEM supplemented with 5% FBS. The confluent Vero cells in 6 well plates were added with serially diluted infected cells at 37°C / 5%CO₂ for 1 hour. The first overlay mixture solution was gently added as same as the plaque assay method. The result (figure 64) shows that percent infectivity of dengue serotype 3 in Vero cells in S phase is 19.35% and percent infectivity of dengue serotype 3 in unsynchronized Vero cells is 19.70%. The P value is about 0.8774. Then the percent infectivity of dengue serotype 3 from unsynchronized Vero cells and Vero cells in S phase is not significantly different. From this result, dengue serotype 3 can infect Vero cells in S phase and unsynchronized Vero cells quite equally.

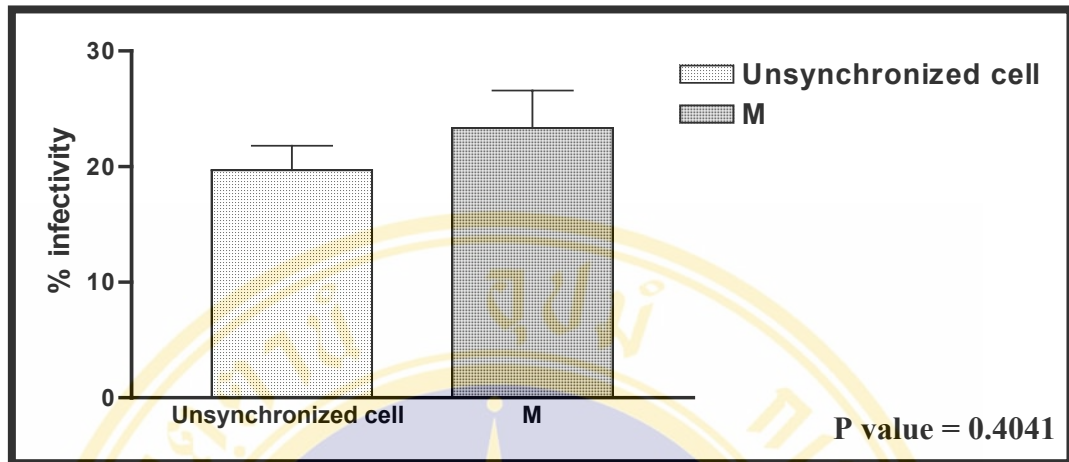


Figure 63. Comparison of percent infectivity of dengue serotype 3 in unsynchronized Vero cells and Vero cells in M phase

The percent infectivity of dengue serotype 3 in Vero cells in M phase is 23.33% whereas percent infectivity of dengue serotype 3 in unsynchronized Vero cells is 19.70% and the P value is about 0.4041. dengue serotype 3 can infect Vero cells in M phase higher than unsynchronized Vero cells.

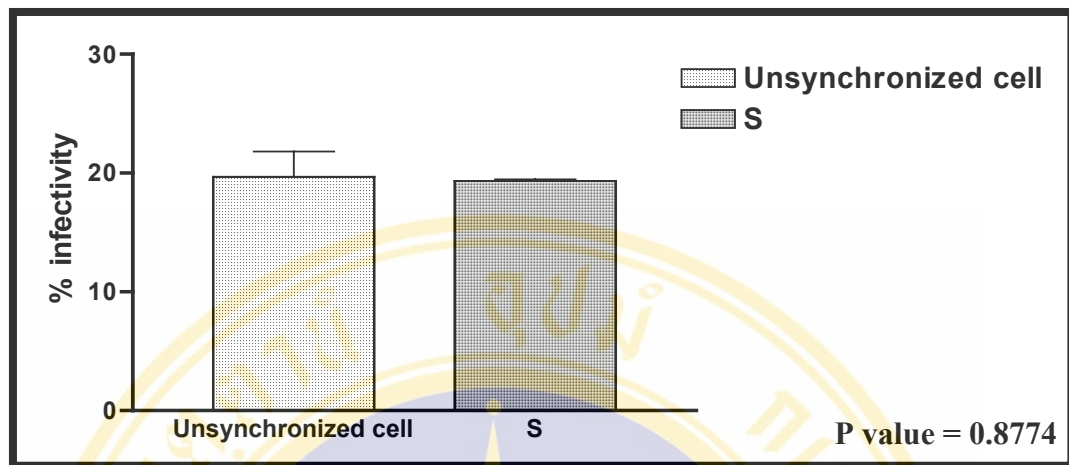
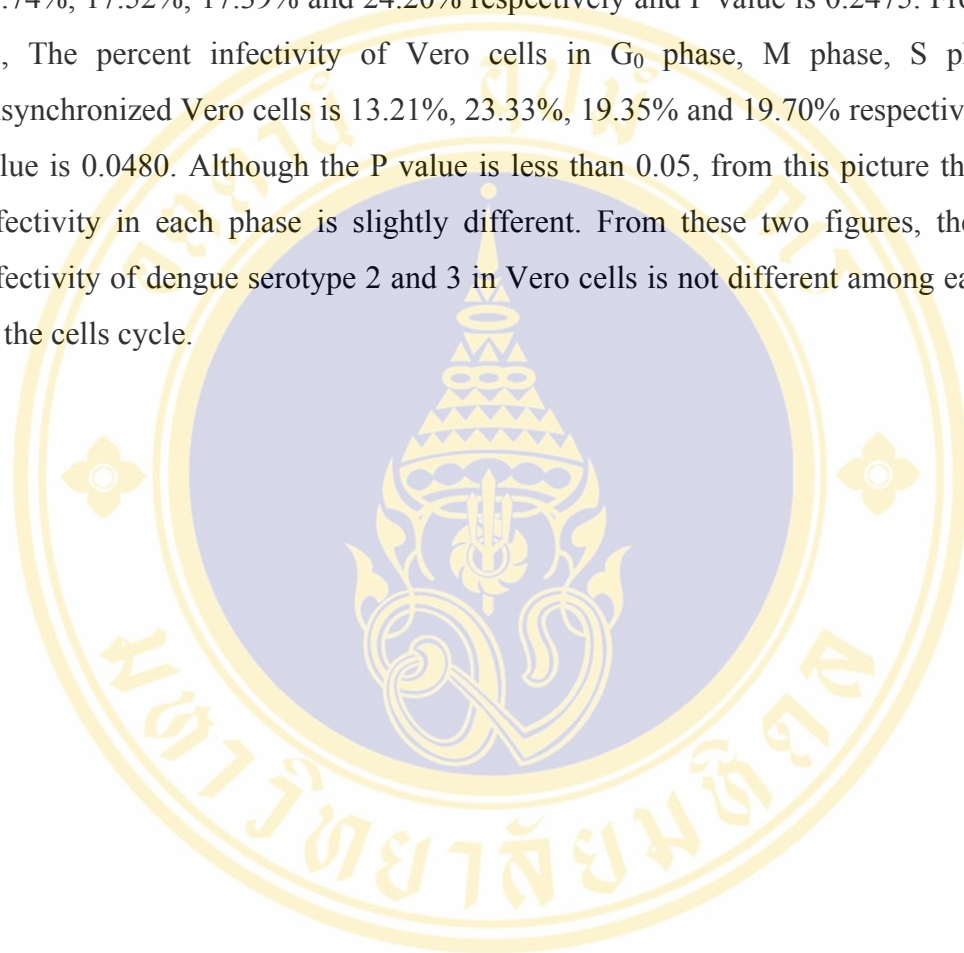


Figure 64. Comparison of percent infectivity of dengue serotype 3 in unsynchronized Vero cells and Vero cells in S phase

The percent infectivity of dengue serotype 3 in Vero cells in S phase is 19.35% whereas percent infectivity of dengue serotype 3 in unsynchronized Vero cells is 19.70% and the P value is about 0.8774. Dengue serotype 3 can infect Vero cells in S phase and unsynchronized Vero cells quite equally.

8.7 Summary of the percent infectivity in Vero cells

The percent infectivity of dengue serotype 2 and 3 in Vero cells are summarized in figure 65 and figure 66 respectively. From figure 65, The percent infectivity of DEN-2 in Vero cells in G₀ phase, M phase, S phase and unsynchronized Vero cells is 12.74%, 17.52%, 17.39% and 24.20% respectively and P value is 0.2475. From figure 66, The percent infectivity of Vero cells in G₀ phase, M phase, S phase and unsynchronized Vero cells is 13.21%, 23.33%, 19.35% and 19.70% respectively and P value is 0.0480. Although the P value is less than 0.05, from this picture the percent infectivity in each phase is slightly different. From these two figures, the percent infectivity of dengue serotype 2 and 3 in Vero cells is not different among each phase of the cells cycle.



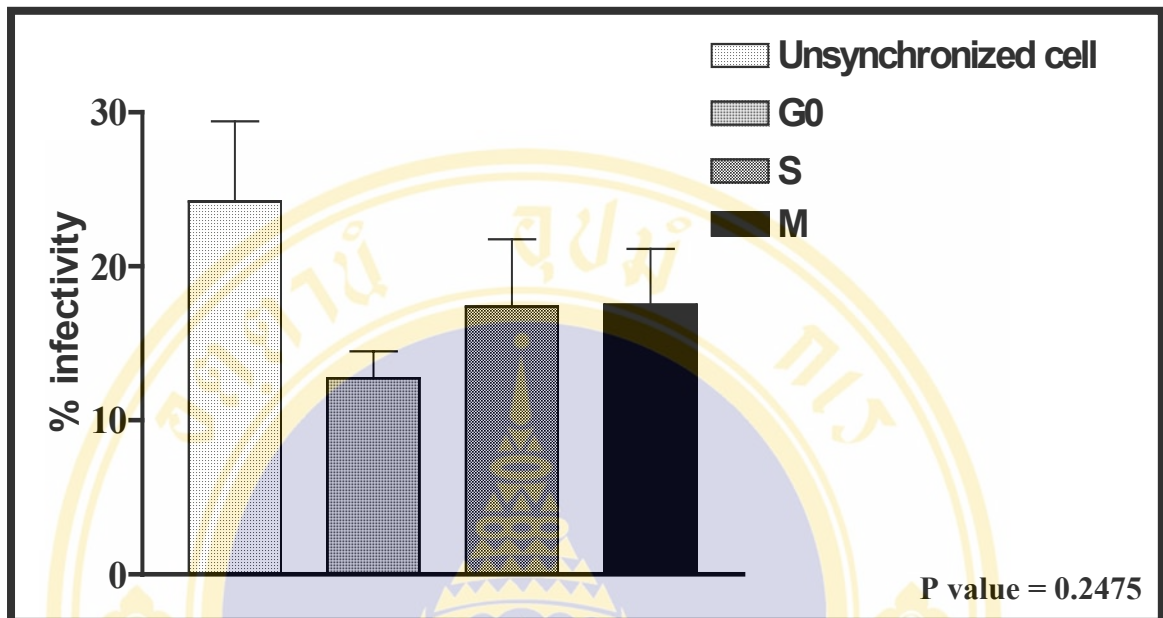


Figure 65. The comparison of percent infectivity of Vero cells in each phase of the cell cycle compared with unsynchronized Vero cells, which were infected with dengue serotype 2

The percent infectivity of dengue serotype 2 in Vero cells is not significantly different among each phase of the cell cycle. The percent infectivity in G₀ phase, S phase and M phase are not different to the percent infectivity in unsynchronized Vero cells.

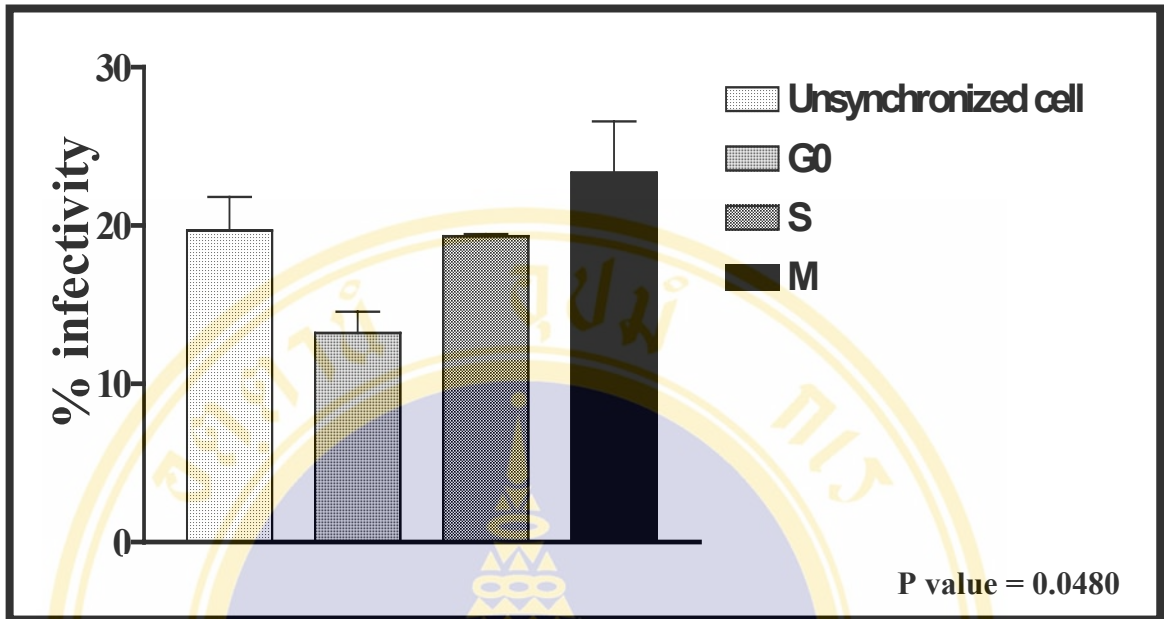
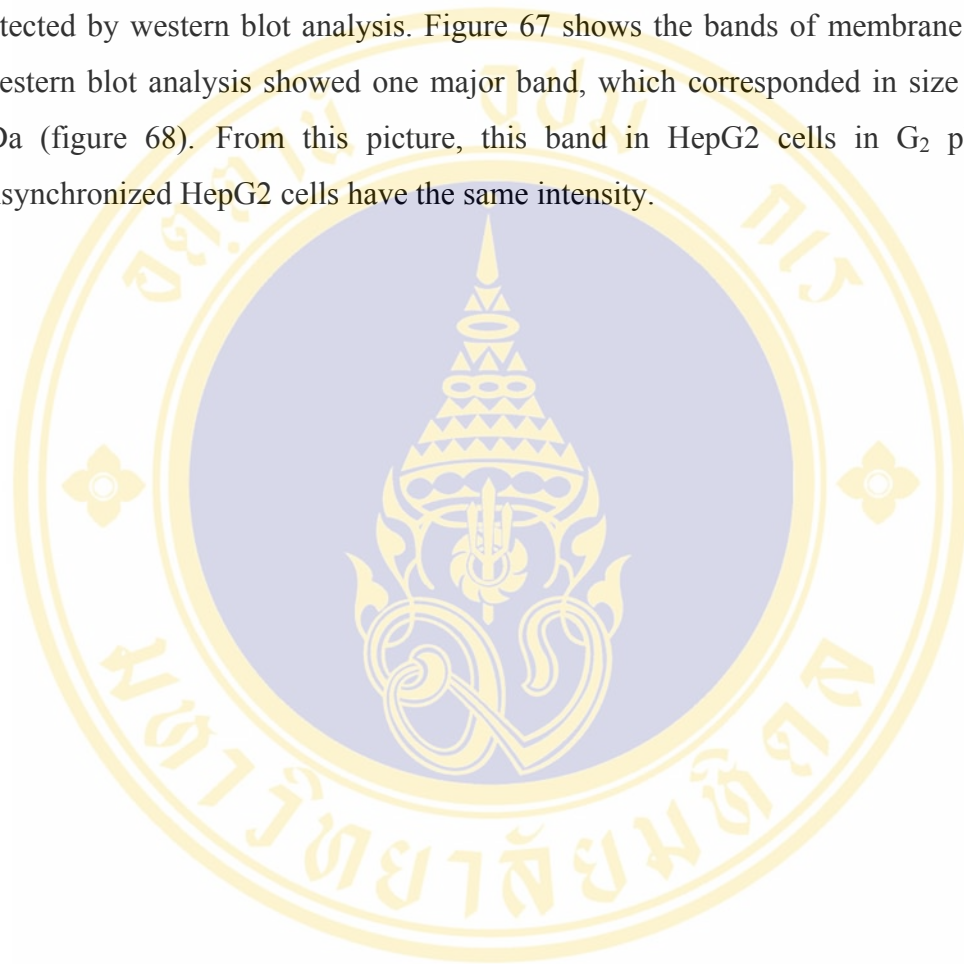


Figure 66. The comparison of percent infectivity of Vero cells in each phase of the cell cycle compared with unsynchronized Vero cells, which were infected with dengue serotype 3

The percent infectivity of dengue serotype 3 in Vero cells is slightly different among each phase of the cell cycle. The percent infectivity in G₀ phase is lower whereas in M phase is higher than in unsynchronized Vero cells. The percent infectivity in S phase is equal to the percent infectivity in unsynchronized Vero cells.

9. Comparison of the expression of GRP78 on membrane proteins of HepG2 in G₂ phase and unsynchronized HepG2 cells

The membrane proteins of HepG2 cells in G₂ phase and unsynchronized HepG2 cells were extracted and separated by SDS-PAGE. The expression of GRP78 was detected by western blot analysis. Figure 67 shows the bands of membrane proteins. Western blot analysis showed one major band, which corresponded in size about 82 kDa (figure 68). From this picture, this band in HepG2 cells in G₂ phase and unsynchronized HepG2 cells have the same intensity.



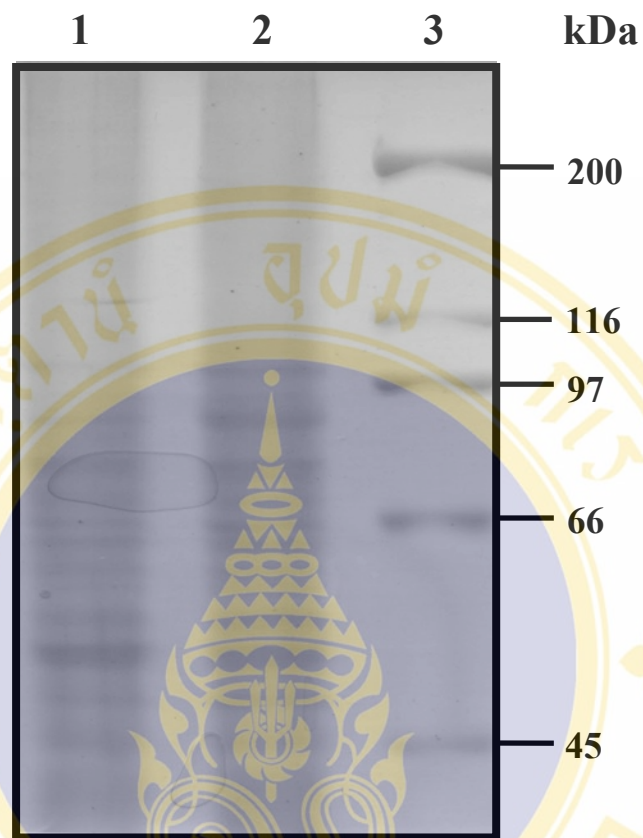


Figure 67. SDS-PAGE of the membrane proteins of HepG2 cells

This figure shows SDS-PAGE profile of the membrane proteins of HepG2 cells in G₂ phase and unsynchronized HepG2 cells.

Lane1: The membrane proteins of HepG2 cell in G₂ phase

Lane2: The membrane proteins of unsynchronized HepG2 cells

Lane3: Standard molecular weight marker

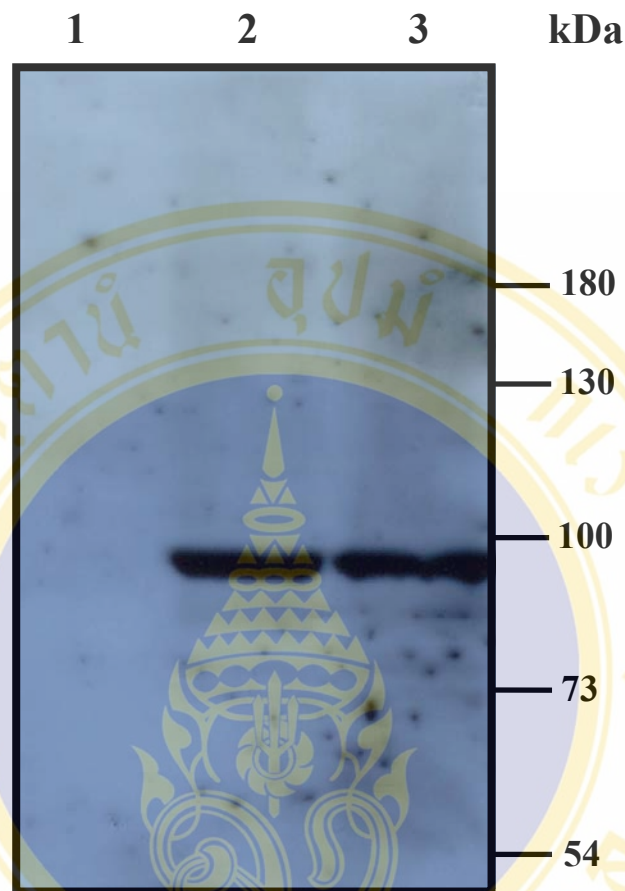


Figure 68. Western blot analysis of GRP78

This figure represents western blot analysis, which is corresponding to SDS-PAGE in figure 67. Membrane proteins of unsynchronized HepG2 cells and HepG2 cells in G₂ phase were detected by using rabbit anti-GRP78 antibody and anti-rabbit IgG as primary and secondary antibody respectively.

Lane1: Standard molecular weight marker

Lane2: The membrane proteins of unsynchronized HepG2 cells

Lane3: The membrane proteins of HepG2 cells in G₂ phase

CHAPTER V

DISCUSSION

1. Dengue production from HepG2 and Vero cells

From the results of dengue viral production from Vero cells and HepG2 cells (figure 6 and figure 7), all 4 serotypes of dengue virus are produced from Vero cells quicker than from HepG2 cells. Moreover all 4 serotypes of dengue virus produced from Vero cells higher than from HepG2 cells which be because the environment in both cells is different. Perhaps Vero cells have the condition in the cells that is good for dengue replication whereas the condition in HepG2 cells is also good for dengue replication but less than in Vero cells. Although the results show that dengue can infect Vero cells, which is a nontarget cell, better than HepG2 cells, which is a target cell, but both cells are the cell culture and the cell culture may different from the nature cells.

From both cells, DEN-4 produced faster than other serotypes. This finding was in good agreement with the phylogenetic tree for the envelope proteins (Figure 69) (56). From this tree, the envelope protein from dengue serotype 4 is the serotype that is distinctly different from other serotypes. Then the envelope protein of dengue serotype 4 may bind to the receptors on the cell surface better than the envelope protein of other serotypes and lead to the infection earlier than other serotypes. This perhaps causes that the dengue serotype 4 production that is earlier than other serotypes.

From these results, it seems like that the production of dengue virus is specific for serotype in both HepG2 cells and Vero cells. But in these experiments, dengue viruses were used only one stain of serotype because there is only one stain of each dengue serotype in our laboratory. If there are many stains of each dengue serotype, the experiments to determine that the production of dengue is specific for stain or serotype of dengue virus can be done.

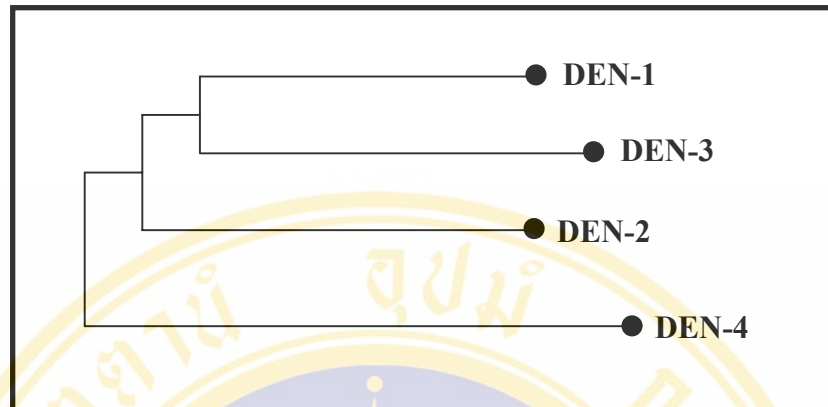


Figure 69. Dendrogram of the envelope protein from DEN-1 (Nauru), DEN-2 (Jamaica), DEN-3 (H87) and DEN-4 (814669). (56)

The sequences were aligned by the progressive-alignment method and classified by the NJ method.

2. The synchronization of HepG2 and Vero cells

From the result of synchronization of HepG2 cells, the stages of the cell cycle that HepG2 cells can be arrested in are G₂ phase, M phase and S phase. Whereas Vero cells can be arrested in G₀ phase, M phase and S phase. Both HepG2 cells and Vero cells can be arrested in M phase and S phase by using paclitaxel and thymidine double block method respectively. Paclitaxel stabilizes microtubules and prevents the microtubule reorganization required for progression mitosis, halting cell cycle progression at M phase (51). For S phase, thymidine double block method was used. Before using this method, aphidicolin was used to synchronize the cells into S phase. Aphidicolin can block the action of DNA polymerase- α , trapping cells during DNA synthesis at S phase (39). But it cannot synchronize the cells into S phase. Then thymidine double block method was performed.

Historically, the use of excess thymidine was the first widely accepted, and remains one of the most effective methods for inducing synchrony (57). By treatment with two sequential “thymidine block” a synchronous population of cells can be obtained at the beginning of S phase, and the method can be effectively utilized to synchronize both suspension and monolayer cells. This method has to adjust the time depending on the time in each phase of the cell cycle. For HepG2 and Vero cells, the time in each phase of the cell cycle was not known. Then the method that was used followed the method that used to synchronize HeLa cells (Gary et al.), which have the doubling time about 24 hours, similar to HepG2 and Vero cells. For HeLa cells, M phase is probably about 45-60 minutes long, giving a G₂ period of about 2.5-3 hours. The cells were grown in 2mM thymidine containing medium for 12 hours. During this period, the G₂/M cells will progress into G₁ and then with the original G₁ population acquire a biochemical state equivalent to G₁/S phase border cells. (G₂M = 3.6 hours + G₁ = 8.4 hours, total = 12 hours) Any cells in S phase upon addition of thymidine will become blocked in S phase. Then release of the cells from the first thymidine block is performed. The cells were incubated in normal medium for 16 hours. During this period the cells recover from the thymidine block (approximately 1-2 hours) and progress through the cell cycle, dividing and entering G₁ of the next cell cycle. Entry into G₁ of the next cell will commence with the cells that were blocked at the end of S phase (the leading-edge cells). This will take about 5-6 hours following release from

thymidine block (1-2 hours recovery + 4 hours progression through G₂/M). Entry into G₁ of the next cell cycle will end with the lagging-edge cells (those arrested at G₁/S) progressing through the cell cycle and dividing (between 16-18 hours). The leading edge cells will be at the G₁/S phase border of the next cell cycle at the end of the 16 hours. The lagging-edge cells will be in G₂/M at the end of the 16 hours. After these 16 hours, the cells were reincubated in 2 mM thymidine containing medium for 12 hours. Cells in G₂/M or G₁ would progress and arrest at the G₁/S phase border.

By using serum starvation method, HepG2 cell was arrested in G₂ phase whereas Vero cell was arrested in G₀ phase. Normally, after the cells were deprived the growth factors, they will enter the quiescence state or G₀ phase. But HepG2 cell was not arrested in G₀ phase after serum starvation. One possibility is that HepG2 cell is a human hepatoma cell line. It did not stop dividing when growth factors are depleted. The cancer cells may make growth factors themselves and may have an abnormal growth factor signaling system. Moreover, the cancer cells that stop dividing do so at random points in the cycle instead of at checkpoints (58). When HepG2 cells were incubated in MEM with no FBS for 36 hours, maybe the G₁ checkpoint did not work. But the G₂ checkpoint can work. Then HepG2 cells were arrested in G₂ phase. It is better if HepG2 cell was synchronized into G₀ phase because liver cells in adult are almost in G₀ phase (59) and they divide once a year. Then if HepG2 cells can be synchronized into G₀ phase and the infection of dengue virus in HepG2 cells in G₀ phase is different from the infection in HepG2 cells in other phases, that means other studies about the infection in liver cells by using the cell culture fail. Moreover, other studies cannot explain the infection also because normal liver cells are arrested in G₀, which the infection is different from the cells in other phases. Then HepG2 cells should be tried to synchronize into G₀ phase by using other methods such as isoleucine starvation (54). Other liver cell lines such as HuH7, PLC or Hep3B should be used to do the experiments and determine that whether there is the difference among each cell line. The best experiment should be done with the primary liver cells because it is the real system. But it is difficult to get the normal liver cells because of the moral reason.

Yeh et al. (50) synchronized HepG2 cells into G₀ phase by incubating in DMEM without FBS for 30 hours. But the result shows that HepG2 cells were not arrested in G₀ phase even incubating in DMEM without FBS for 36 hours. One possibility that

can explain is that HepG2 cell that was in this experiment has the different passage when compared to the passage of HepG2 cell that used in that experiment. When the cells were grown with several passages, the cells might change and the G₁ checkpoint of the cells did not work. Then HepG2 cells that used in this experiment were not arrested in G₀ phase.

For Vero cell, it is a monolayer cell line. When they are confluent, they will not grow in upper layer. Then the contact inhibition can occur. Both contact inhibition and serum starvation drive Vero cells into quiescent state or G₀ phase. In the experiment, Vero cells were not synchronized into G₂ phase. Then Vero cells should be synchronized into G₂ phase by using other methods and determined the difference of dengue infection comparing to other phases.

In the experiment, there are other phases of the cell cycle that were not synchronized. For example, HepG2 cells were not synchronized into G₀ phase and G₁ phase whereas Vero cells were not synchronized into G₁ phase and G₂ phase. For G₁ phase, there is another chemical that can be used to block the cells, which is lovastatin (60). There is the study, which found that lovastatin could block the cells, including normal and tumor cells of mouse, hamster and human origins. Lovastatin can block the cells into early G₁ phase because it is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

3. Cell cycle regulated dengue infection in HepG2 and Vero cells

The production and percent infectivity results of dengue serotype 2 and 3 in HepG2 cells suggest that the receptors of dengue serotype 2 and 3 express differently in each phase of the cell cycle. But the environment in the cell may affect the infection also. For example, if the virus uses one receptor that expresses on the cell surface at the time that the environment in the cell is good for viral replication, it can replicate better than use other receptors to enter into the cell in other phases that the environments are not good for viral replication. Then both the receptor expression and the environment in the cells may affect the difference of dengue infection.

Adenovirus receptors, which are coxsackie-adenovirus receptor (CAR) and α_v -integrin, are the viral receptors that express differently among each phase of the cell cycle (39). The first encounter of Ad with cells occurs through a high-affinity

interaction of the adenovirus fiber protein with its cell surface receptor, CAR, followed by low-affinity interaction between the adenovirus penton base protein and cell surface integrins (61). Receptor binding leads to internalization of the virus into the cell by receptor-mediated endocytosis, lysis of the endosomal membrane, escape to the cytosol, trafficking along microtubule toward the nucleus, binding to the nuclear envelope, and insertion of the viral genome into the nucleus. Adenovirus increases binding in the M phase cells (figure 70) and the assessment of cell surface expression of Ad receptors demonstrated that both the high-affinity coxsackie-adenovirus receptor for Ad fiber protein and the low affinity α_v integrin receptor for Ad penton base protein showed increased cell surface expression at M phase (figure 71).

This experiment is different from our experiment because the receptors for adenovirus have been already known. But now the receptors for dengue virus in HepG2 cells are not identified. Then there is no antibody against dengue receptor to detect the expression of dengue receptors in each phase of the cell cycle directly.

From the results of the percent infectivity of dengue serotype 2 and 3 in HepG2 cells, dengue serotype 2 can infect in HepG2 cells in G₂ phase better than other phases. In contrast, the phases that dengue serotype 3 can infect better are G₂ phase and M phase. These results may suggest that dengue serotype 2 and 3 use different receptors to infect in HepG2 cells.

For Vero cells, the productions of dengue serotype 2 and 3 were different among each phase of the cell cycle whereas the percent infectivities were equal. These results may imply that the receptors of dengue serotype 2 and 3 in Vero cells express in each phase of the cell cycle similarly. But the environments in Vero cells are the cause of the difference of dengue production. Because dengue serotype 2 and 3 infect Vero cells in each phase of the cell cycle equally whereas they produce from Vero cells in each phase of the cell cycle differently.

There are other studies about the cell cycle involvement with the viral infection such as retrovirus (40;41). A retrovirus infects the cells, which progress through M

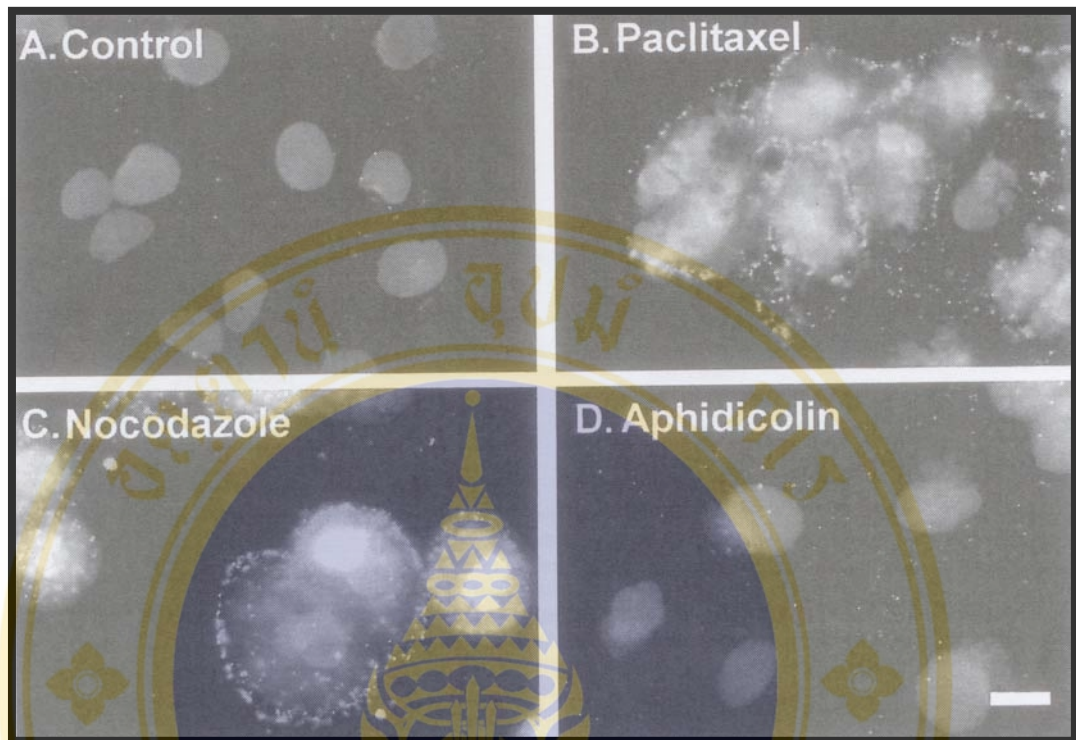


Figure 70. Fluorescence microscopy analysis of adenovirus binding following treatment with cell cycle inhibitors. (39)

A549 cells were plated and treated with cell cycle inhibitors such as paclitaxel and nocodazole to synchronize the cells into M phase and aphidicolin to synchronize the cells into S phase. After 24 hours, the cells were washed to remove the inhibitor, incubated for 1 hour in culture medium, and infected with Cy3-conjugated Ad vector (1011 particles/ml, 10 min, 37°C). After infection, cells were washed, fixed, and analyzed by fluorescence microscopy. Single optical sections show Cy3-Ad in the red channel and DAPI-stained nuclei. (A) Control. (B) Paclitaxel (0.1 μ M). (C) Nocodazole (1.0 μ M). (D) Aphidicolin (5 μ g/ml). Note that with paclitaxel and nocodazole, there is accumulation of Cy3-Ad on cells. Bar, 10 μ m.

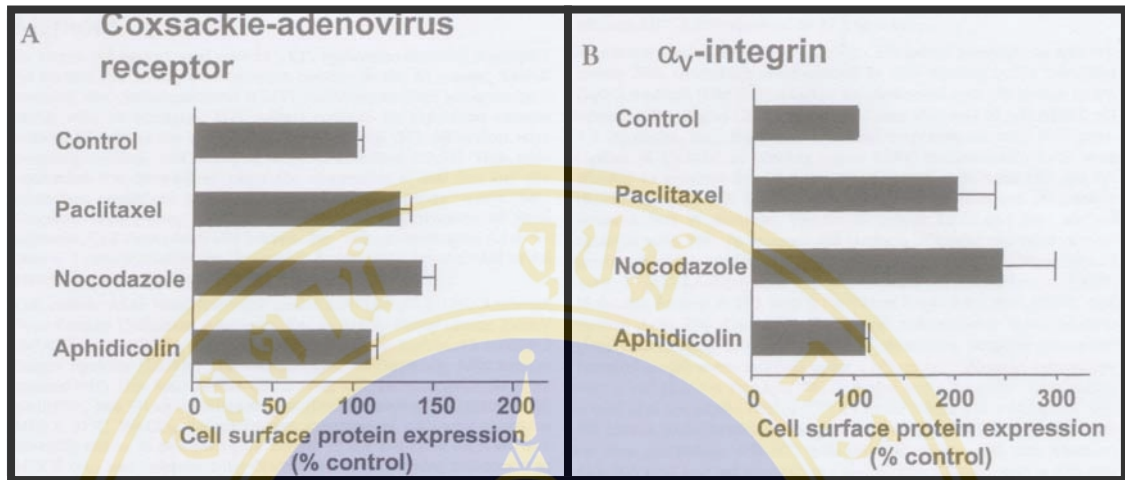


Figure 71. Flow cytometric analysis of cell surface coxsackie-adenovirus receptor (CAR) and α_v integrin expression following treatment with cell cycle inhibitors. (39)

A549 cells were treated with cell cycle inhibitors as described in figure 69. After 24 hours, the cells were stained with primary antibody (mouse anti-CAR or mouse anti- α_v integrin) or an irrelevant primary antibody (Control) and secondary antibody (fluorescence-conjugated goat anti-mouse), and analyzed by flow cytometry. Data are presented as the percent change in mean fluorescence relative to control cells.

phase better than the arrested cells because retrovirus genome has to be integrated into the host cell's nuclear DNA. Retrovirus needs to infect in M phase cells because the nuclear membrane will disassemble, which is easy for retrovirus genome to enter the nucleus.

Although dengue viral genome is RNA as same as the genome of retrovirus, the replication of dengue virus occur in cytoplasm of the cells and dengue virus does not enter into the nucleus of the cell through its life cycle (2). Then the nuclear membrane break is not necessary for dengue virus. But dengue virus has to use some factors from the cells, such as some enzymes, which may increase in some phase of the cell cycle. Then dengue can produce from Vero cells in each phase of the cell cycle differently although it can infect equally.

Another possibility is that dengue serotype 2 and 3 may enter into Vero cells by using membrane fusion, not receptor mediated endocytosis. Then it is not necessary to use the receptor to internalize into the cells. There are the examples of Flavivirus that can enter into the cell by membrane fusion such as tick-borne encephalitis (TBE). The membrane fusion of TBE is triggered by the mildly acidic pH of the endosome and is mediated by envelope protein E (62;63). Moreover, there is the study about the entry mode of dengue virus into C6/36 mosquito cells (18). The result suggested that dengue virus serotype 2, enter into C6/36 mosquito cells by membrane fusion. Then dengue virus may enter into Vero cells by using membrane fusion, which may cause the similarity of the infection in each phase of the cell cycle.

Biological membranes exist as a fluid mosaic of protein and lipid which possess various degrees of motion (64). Many fundamental properties of membranes are affected by molecular motions of the membrane components. For example, the transport process, which also includes viral internalization, has been related to membrane fluidity (65). Studies of the surface of mammalian cells in culture have indicated changes related to growth and to the cell cycle (66;67). By electron spin resonance, Lai et al. (68) showed that changes of the membrane fluidity were dependent on the cell cycle; that is, cells in mitosis had the highest membrane fluidity, whereas cells in the G₁ and early S phases had the lowest membrane fluidity.

From figure 58 and figure 66, dengue serotype 3 can infect into HepG2 cells and Vero cells in M phase more than the unsynchronized cells respectively. These results

were in good agreement with the result of electron spin resonance. Dengue serotype 3 may internalize into HepG2 cells and Vero cells by using membrane fusion. Then it can internalize into the cells in M phase better because the cells are less rigid in this phase. It may be implied that the fluidity of the cell membrane is one factor that affects the difference of dengue serotype 3 infection. But dengue serotype 2 can infect into HepG2 cells and Vero cells in M phase less than the unsynchronized cells (figure 57 and figure 65). These results were in contrast with the result of electron spin resonance, which may suggest that the fluidity of the cell membrane is not the factor that affects the difference of dengue serotype 2 infection.

4. Detection of Glucose-regulated protein 78 (GRP78)

From the result of Virus Overlay Protein Binding Assay (VOPBA) (Jindadamrongwech S., Personal Communication) shows many bands of proteins that dengue serotype 2 can bind to the membrane proteins of HepG2 cells. One band is GRP78. Then GRP78 is the candidate to be dengue receptor in HepG2 cells. GRP78 or glucose-regulated protein 78 is an endoplasmic reticulum chaperone, which is essential for the proper glycosylation, folding and assembly of many membrane bound and secreted proteins (69). In cultured cells, transcription of the GRP78 gene is induced by stresses that produce incorrectly folded or assembled proteins in the endoplasmic reticulum (70). These stresses include hypoxia, endoplasmic reticulum calcium depletion and acute glucose starvation. The effects of glucose concentration on GRP78 mRNA abundance have been investigated only with cells cultured in medium containing 4.5 mg/ml glucose and medium containing no glucose (71).

In the experiment, after HepG2 cells were incubated in MEM with no FBS for 36 hours, they were in G₂ phase. In this condition, the cells were deprived the growth factors, many nutrients and glucose because normally HepG2 cells were maintained in DMEM supplemented with 10%FBS. DMEM has glucose 4,500 mg/L whereas MEM has only 1,000 mg/L. Then the GRP78 should be expressed higher in HepG2 cells in G₂ phase.

Then the western blot was used to detect the expression of GRP78 in HepG2 cell in G₂ phase comparing to unsynchronized HepG2 cells. GRP78 expression was not different in HepG2 cells in G₂ phase and unsynchronized HepG2 cells. This means

that HepG2 cells do not increase the expression of GRP78 in the glucose starvation condition, which is in contrast with previous study (71).

Nevertheless, the experiments to determine whether GRP78 expresses differently in M phase and S phase should be done. From the results of dengue serotype 2 infection in HepG2 cells in M phase and S phase are less than in unsynchronized HepG2 cells. Then GRP78 may express in M phase and S phase less than unsynchronized HepG2 cells, which implies that GRP78 is the cell cycle dependent receptor for dengue virus serotype 2. Then the experiment to detect the expression of GRP78 in M phase and S phase comparing to unsynchronized cells should be done.

If GRP78 expresses equally in every phases of the cell cycle, it suggests that dengue virus perhaps uses several receptors to infect HepG2 cells. Then maybe there are other receptors, which dengue virus uses for infection, that express depending on the cell cycle of HepG2 cells. Another possibility is that GRP78 may be not the receptor for dengue virus. It should be more experiments to confirm whether GRP78 is the real receptor for dengue virus.

CHAPTER VI

CONCLUSION

Dengue serotype 2 and 3 infect HepG2 cells and produce from HepG2 cells differently among each phase of the cell cycle. These results imply that the receptors of dengue serotype 2 and 3 on HepG2 cells express depending on the cell cycle. However, the environment in the cells may also affect the production of the virus. Moreover the phases, which both serotypes can infect better, are different. Then dengue serotype 2 and 3 may use different receptors to infect HepG2 cells.

For Vero cells, both serotypes of dengue virus produce from Vero cells differently among each phase of the cell cycle whereas they infect Vero cells in each phase of the cell cycle equally. These results may imply that the receptors of dengue serotype 2 and 3 on Vero cells express equally among each phase of the cell cycle or both serotypes of dengue virus may enter into Vero cells by membrane fusion, not receptor mediated endocytosis.

GRP78, a candidate to be dengue serotype 2 receptor in HepG2 cells, expressed equally in HepG2 cells in G₂ phase and unsynchronized HepG2 cells. This means HepG2 cells do not increase the expression of GRP78 in the glucose starvation condition. Moreover, there should be other receptors that express depending on the cell cycle.

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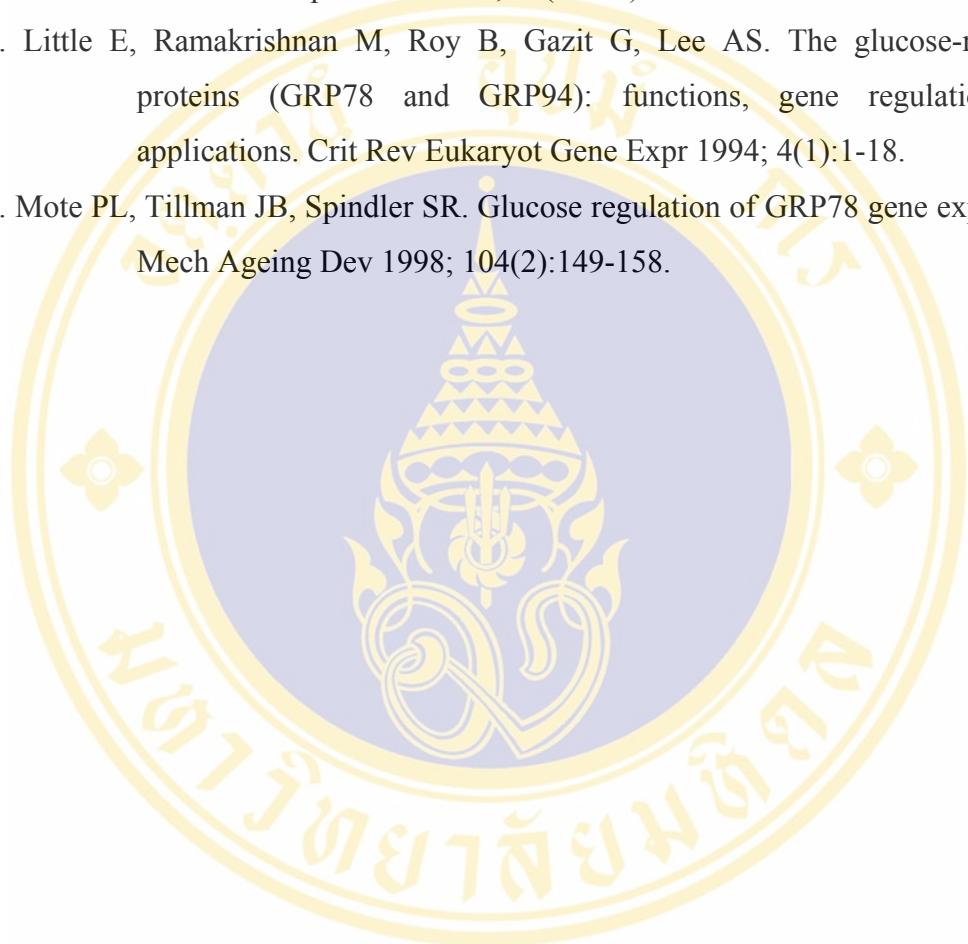
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