Purified Monoclonal Antibody of Anti-Flavivirus Group Antigen Clone D1-4G2-4-15 for the Development of Japanese Encephalitis Virus Surface Proteins in Plants

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ABSTRACT

Production of *Japanese encephalitis virus* (JEV) surface proteins in plants requires specific monoclonal antibodies to some part of antigens of JEV as a tool of detection. This research is to develop anti-flavivirus group antigen clone D1-4G2-4-15 monoclonal antibodies specific to the envelope protein of viral particle of genus *Flavivirus* in serum-free media by inside adaptation culture method. The monoclones were grown quickly in serum-free media and purified from cell culture supernatant by precipitation with 50% saturated ammonium sulfate and fractionated by affinity chromatography. Then, the product was tested for antibody specificity by typing ELISA. The test results were positive against inactivated Japanese encephalitis vaccines and *Dengue virus* type 1. The stability tests were performed after storage at -20°C for 10 weeks. The reactivity of antibodies against the *Dengue virus* type 1 were detectable at a dilution of 1:1000. The cost of antibody production is lower than culturing cells in serum media. This technique is feasible for laboratory because of its simplicity. The monoclonal antibodies derived from this procedure are stable and specific to *Flavivirus* antigens. It can be used to characterize not only the JEV envelope protein production in plants but also further protein production of other flaviviruses.

Keywords: Japanese encephalitis virus, D1-4G2-4-15, serum-free media, purification.

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INTRODUCTION

Japanese encephalitis virus (JEV) is an important cause of virus encephalitis in Asia. It was estimated that there were approximately 68,000 cases of Japanese encephalitis (JE) patients and 15,000 deaths each year (Campbell et al., 2011). Japanese encephalitis vectors are *Culex tritaeniorhynchus* mosquitoes and the amplifying vertebrate hosts are particularly pigs and water birds (De Wispelaere, Frenkiel, & Desprès, 2015). Pigs are infected with JEV having asymptomatic appearance, act as reservoirs, while carrying JEV in their blood. Mosquitoes that bite these pigs become infected, having high level of JEV in their bodies and can transmit the viruses to humans. JEV is a member of the *Flavivirus* genus in the *Flaviviridae* family, an enveloped virus of ~ 50 nm in diameter with a positive-sense ssRNA. JEV genome encoded a single reading frame cleaved by host and viral proteases into ten proteins. There are three structural proteins including envelope (E), pre-membrane (prM), core (C) and seven nonstructural (NS) proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Among all three structural proteins, the envelope protein is the most studied and most important one. Envelope protein is a major structural protein that contains numerous epitopes including neutralization epitopes, membrane fusion, attachment of the virus and virus entry to the host cells (Kant Upadhyay, 2013). It is the major target of humoral immune responses (Misra & Kalita, 2010).

Monoclonal antibodies (mAbs) are very specific and homogeneous immunoglobulins known as "hybridomas". They have been widely used in immunotherapeutics, immunoaffinity chromatography, immunodiagnostics, and development of immunoassays. In recent technology, hybridoma is produced *in vitro* by cell culture method. The production and culturing of hybridoma cells require various supplementations including fetal bovine serum (FBS), amino acids, growth factors and vitamins (Köhler & Milstein, 1975; Spriggs, Aggarwal, Deng, Soderstrom, & Donovan, 2011; van der Valk et al., 2018). FBS is the preferred animal serum for cell culture enhancement as it contains various elements and abundance of high protein content, enzymes, growth factors and other chemical components for promoting cell health and growth (Even, Sandusky, & Barnard, 2006). However, FBS in cell culture have several disadvantages: serum has various unknown varying and undefined medium supplement (Bauman, Granja, & Barrias, 2018). Moreover, its qualitative changes of the composition, batch display quantitative, and possible source of contamination of, for example, bacteria, viruses, fungi and mycoplasma are encountered (Yao & Asayama, 2017). These disadvantages in the use of FBS in cell culture allowed the researchers to develop methods of cell culture in serum-free media.

Purification is an important method for antigen specific antibodies used in research and pharmaceutical industry as it allows the functional manipulation of target antigens. The purification of the therapeutic antibody products, equivalent to 50-80% of the total process costs (A. M. Azevedo et al., 2009; Ana M Azevedo et al., 2009; Silva, Fernandes-Platzgummer, Aires-Barros, & Azevedo, 2014). In general, selecting the appropriate purification approach is based on the desired antibodies for example, application for the recovery and purity of IgG, usually either by precipitation or chromatography method (Arora, Saxena, & Ayyar, 2017). Purification by

chromatography is widely used to purify proteins at high price because of the high purity of the product. The purification of the protein can be processed under varying parameters such as flow rate of the mobile phase, pH and type of the buffer, length of gradient elution, and the ionic strength (Yang, Gurgel, & Carbonell, 2009). An ammonium sulfate precipitation, a downstream process during the first stage with high recovery of antibodies, is usually practical, straight forward and successful purification procedure (Mariam et al., 2015).

In this study, researchers focused on 2 aims: (i) to develop a cell culture system to produce the anti-flavivirus group antigen clone D1-4G2-4-15 monoclonal antibodies specifically to the antigen of the genus *Flavivirus* in serum-free media (ii) to purify anti-flavivirus group antigen clone D1-4G2-4-15 from a serum-free media, including the determination of the specific activities to *Flavivirus* antigens and its stability. The monoclonal antibody derived from this research can be used to study the properties of the Japanese encephalitis virus surface protein in plants.

MATERIALS AND METHODS

D1-4G2-4-15 (ATCC® HB-112™) Cell Culture and Expansion

Frozen cells of D1-4G2-4-15 (Gentry, Henchal, McCown, Brandt, & Dalrymple, 1982; Henchal, Gentry, McCown, & Brandt, 1982) were rapidly thawed in a water bath at 37°C and transferred the entire contents from the cryovial into a 6-well tissue culture plate (costar®3516, USA) containing pre-warmed complete Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco™, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Gibco™), 2 mM L-glutamine (Invitrogen), 100 I.U./ml penicillin (Invitrogen) and 100 µg/ml streptomycin (Invitrogen) of which was called the complete RPMI 1640 medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and sub-cultured during an exponential growth phase approximately 3–5 days post-thawed, at a seeding density of 5×10^5 viable cells/ml. They were sub-cultured at a minimum of 3 passages before expansion.

Cells were expanded by sub-culturing in the complete RPMI 1640 using T75 and T162 cell culture flasks. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and grown for 5 days until viable cell density reached 1×10^6 viable cells/ml.

Adaptation of D1-4G2-4-15 Cells to Serum-Free Media Cell Culture by Inside Adaptation

The viability and cell density were determined using a Neubauer hemocytometer until the amount of cells reached $\geq 1 \times 10^6$ viable cells/ml in T162 cell culture flask in complete RPMI 1640 medium. The D1-4G2-4-15 cells were harvested and washed 2 times using CD Hybridoma medium supplement with final concentration of 8 mM L-glutamine (Invitrogen) which was called the complete CD Hybridoma medium. The cells were centrifuged at 200g for 5 minutes and sub-cultured to 6×10^5 viable cells/ml in fresh pre-warmed complete CD Hybridoma medium. Cells were incubated at 37°C in a humidified, 5% CO_2 incubator and grown for 5 days. Then, the cells were monitored and passed for 3 – 5 passages until consistent growth was achieved. After the cells were sub-cultured, the supernatant was harvested from cell

culture by centrifugation at 200g for 10 minutes and supernatant was stored at -20°C for testing their antibody activities.

Ammonium Sulfate Precipitation

The supernatant from D1-4G2-4-15 in serum-free media cell culture was transferred to a beaker containing a stir bar, placed on the magnetic stirrer and saturated ammonium sulfate was slowly added to the final concentration of 50% saturation at 4°C and continued stirring overnight. Then the precipitate was centrifuged at 10,000g for 15 minutes. The pellets were resuspended in phosphate buffer saline (1X PBS (Gibco™)), pH 7.4 with the minimum volume. (Arora, Ayyar, & O'Kennedy, 2014; J. Sambrook & D. Russell, 2001; J. Sambrook & D. W. Russell, 2001)

Chromatography Purification Process

Sample purification is simplified by using the Econo-Pac® Serum IgG Purification Kit (Bio-Rad, Richmond, CA, U.S.A.). The kit is composed of Econo-Pac 10DG and DEAE Affi-Gel blue gel column. According to the manufacturer's instructions, each Econo-Pac 10DG column can process up to 3 milliliters of pellet dissolved in 1x PBS (Gibco™) per cycle and qualitative detection of ammonia sulfate by Nessler's reagent. Then, the prepared sample was applied to the DEAE Affi-Gel blue gel column. The IgG were eluted with 20 ml of application buffer and 4 ml fractions were collected. The determination of total protein in each supernatant using Bio-Rad Protein Assay was performed by measuring OD at 595 nm with a microplate reader (Bradford, 1976; Kielkopf, Bauer, & Urbatsch, 2020). Standard mouse IgGs (Sigma, USA) were used for calculation of protein fraction concentrations after the method of Bradford. The yield of anti-flavivirus group antigen clone D1-4G2-4-15 was calculated from the percentage of protein concentration ratio of the purified anti-flavivirus group antigen clone D1-4G2-4-15 to the initial amount of anti-flavivirus group antigen clone D1-4G2-4-15 in supernatant of the cell culture.

Immunoglobulin Determination

The concentration of immunoglobulin was determined by Enzyme-linked Immunosorbent Assay (ELISA). The 96-well plate was coated with goat anti-mouse IgG prepared in 18 mM carbonate buffer (pH 9.0) and incubated overnight at 4°C. Then, the coating solution was removed and the plate was washed with 1X PBS, pH 7.4 (prepared from 10X PBS containing 1.37 M NaCl, 27 mM KCl, 63 mM Na_2HPO_4, and 13.9 mM KH_2PO_4 at pH 7.4).The remaining protein-binding sites were blocked by blocking buffer and incubated for at least 1–2 h at 37°C. The plate was washed with 1X PBS (pH 7.4). The diluted mAb samples and mouse IgG for standard curve were added to each well and incubated for at least 2–3 h at 37°C and the plate was washed with 1X PBS (pH 7.4). The conjugated secondary antibodies were added and incubated for at least 1 hr. at 37°C. Then the plate was washed with 1X PBS (pH 7.4). SureBlue™ TMB Microwell Peroxidase Substrate 1-Component (KPL, A SeraCare Company) were added to each well and incubated for 15–30 min. The reactants were inactivated by adding 2 M H_2SO_4 prior to measure the absorbance at 450 nm.

Antibody Specificity

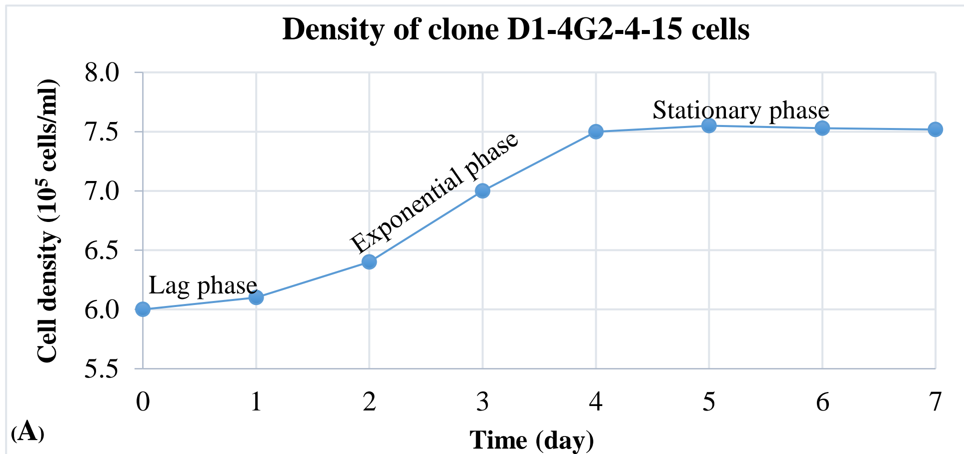
The antibody specificity to flavivirus antigen was determined by following the typing ELISA protocol of the Armed Forces Research Institute of Medical Sciences (AFRIMS) laboratory (Jarman et al., 2011). The antigens included *Dengue virus* type 1 from AFRIMS and Japanese encephalitis vaccines as follows: (I) CD.JEVAX® (Inj.), live attenuated viral vaccine: prepared by passaging Japanese encephalitis virus strain SA14-14-2 in a monolayer of primary hamster kidney cell culture and lyophilized, from Chengdu Institute of Biological Products, China, (II) IMOJEV®, live attenuated viral vaccine: prepared by a recombinant DNA technology of Yellow fever vaccine 17D-204 strain of which the pre-membrane (prM) and envelope (E) coding sequences of the Japanese encephalitis virus strain SA14-14-2 have been replaced, from Sanofi Pasteur Ltd, Australia, (III) J.E. (Beijing) – GPO, an inactivated mouse brain-based Japanese encephalitis vaccine Beijing-1 strain, from Government Pharmaceutical Organization, Thailand and (IV) JEVAX™, an inactivated Japanese encephalitis vaccine P3 strain: cultured in Vero cell, inactivated with β-propiolactone and lyophilized, from Liaoning Cheng Da Biotechnology Co., Ltd., China (Ishikawa, Yamanaka, & Konishi, 2014). These antigens were diluted 1:2 in 0.5% BSA in 20% acetone-extracted normal human serum and in 1X PBS, pH 7.4. The negative control was 1X PBS. The 96-well plates were coated with Goat anti-Mouse IgG in 6 mM carbonate buffer (pH 9.0±0.2). The plate was read on a microplate reader (SpectraMax® Plus 384, Molecular Devices LLC, US) at 492 nm absorbance .

Antibody Stability

To determine the specificity and stability of the anti-flavivirus group antigen clone D1-4G2-4-15, ten aliquots of the purified mAbs were collected and stored at -20°C for testing every week for 10 weeks by typing ELISA. The D1-4G2-4-15 mAbs was ten-fold serially diluted for testing specificity of antibody (Morgenthaler, Struck, Alonso, & Bergmann, 2006).

RESULTS AND DISCUSSION

The basal media for hybridoma cell cultures include the Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's modified Eagle's medium (DMEM), and RPMI-1640, supplemented with FBS (Carvalho et al., 2017). D1-4G2-4-15 cells were cultured in complete RPMI 1640 supplement with 10% FBS at a minimum of 3 passages before adaptation to serum-free media. The serum contains growth factors which are important in promoting the growth of cells, and other substances that help adhesion to the surfaces. In addition, serum also serves as good source of minerals, lipids, carbohydrates, enzymes and hormones etc. (Gstraunthaler, 2003) so the cells are in good health and ready to adapt for growing in serum-free media by inside adaptation when seeding at 1×10^6 viable cells/ml into the CD hybridoma medium. The morphology of growing cells was observed using an Olympus phase contrast inverted microscope at 20 x, 40 x magnification. The D1-4G2-4-15 cells were grown well similar to those cells in complete RPMI 1640 supplement with 10% FBS **(Fig. 1)**.



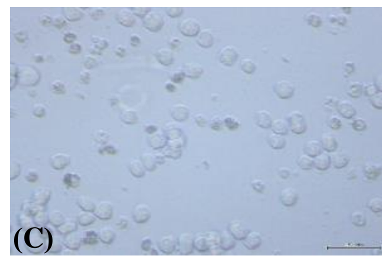


Fig. 1. D1-4G2-4-15 cells were cultured in serum-free media (A) Growth curve for D1-4G2-4-15 cells, showing cell density on day 0-7. Cells were sub-cultured during the exponential phase. (B) D1-4G2-4-15 cells, on the day 7th cultured in serum-free media, at a 20x magnification (C) D1-4G2-4-15 cells, on day 7th cultured in serum-free media, at a 40x magnification.

A suspension of cells are composed of population of a single cell and cells in groups with various ratios. Most viable cells were shiny circular shape in appearance. Size and characteristic of the cells depend on type and concentration of ingredients of the media, in particular growth regulators. Range of lag phase is on the 1st day, exponential phase is during the 2nd - 4th days and stationary phase is during the 5th – 7th days while sub-culture range is optimum at the exponential phase on the 3rd day. Previous studies in various kinds of cells growing in serum-free media such as CB. Hep-1 hybridoma cells (Aragón et al., 2013), Chinese hamster ovary cells (Costa et al., 2013), HEK 293T and Huh 7.0 cells (Paschoal et al., 2014), BHK 21 cell lines (Reddy, Reddy, & Rayulu, 2016) and hybridoma cell lines: C4B, 10C2G5, 6C5F4C7, 2D10G11 (Manna et al., 2015) have been successfully reported.

The immunoglobulin was prepared by 50% ammonium sulfate precipitation from cell supernatant of D1-4G2-4-15 in serum-free media cell culture as described (Ferreira et al., 2015). Sample purifications were simplified by using the Econo-Pac® Serum IgG Purification Kit. (Claudine Bruck, Drebin, Glineur, & Portetelle, 1986;

Claudine Bruck, Portetelle, Glineur, & Bollen, 1982; Mendelson, Wright, Evans, Porter, & Simpson, 1985). The high yield and purity of anti-flavivirus group antigen clone D1-4G2-4-15 were achieved at 96.27% and 97%, respectively. These number were closed to other works, for example, the original yield of mAbs started from cell culture supernatant was 85.4 ± 1.0% (Rajak, et al., 2012), while, the yield and purity after the process performed were 99% and 94%, respectively (Mariam, et al., 2015). Culturing cells in serum-free media produced less interfering affect in the purification system. IgG binds to ligand efficiently and the sample passes slowly and smoothly at 1 ml/min therefore the IgG obtained at higher purity. The product was tested for antibody specificity by typing ELISA as shown in **Table 1.**

Table 1. The antibody specificity of anti-flavivirus group antigen clone D1-4G2-4-15 mAbs, as tested by typing ELISA (positive cutoff is $A_{492} \geq 0.2$), are shown.

Antigen	**Anti-flavivirus group antigen clone D1-4G2-4-15**			
	Undiluted	1:10	1:100	1:1000
CD.JEVAX®Inj.	-	-	-	-
IMOJEV®	-	-	-	-
Beijing-GPO	+	+	+	+
JEVAC™	+	+	+	+
***Dengue virus* type1**	+	+	+	+

The positive outcome was determined by the absorbance measurement at 492 nm to be greater than or equal to 0.2 means (±3SD), while negative control (uninfected C6/36 cell culture fluid) is 0.03 means (±3SD), and cutoff followed Armed Forces Research Institute of Medical Science (AFRIMS) laboratory. The results of anti-flavivirus group antigen clone D1-4G2-4-15 mAbs were positive against *Dengue virus* type 1 (positive control), Beijing-GPO and JEVAC™. Both of them are inactivated Japanese encephalitis vaccines. However anti-flavivirus group antigen clone D1-4G2-4-15 mAbs produced negative results against CD.JEVAX®Inj and IMOJEV®. These latter vaccines are live attenuated Japanese encephalitis vaccine of which have some alteration on their E proteins, thus the anti-flavivirus group antigen clone D1-4G2-4-15 could not specifically recognize. The E protein monomer is organized into three structurally distinct envelope domains I, II, and III (EDI, EDII, and EDIII). All three domains are connected to the viral membrane through a helical anchor. The flavivirus E proteins belong to class-II fusion protein, which has a unique structure with a double membrane spanning the C-terminal anchor. The E structural rearrangements involve a unique portion of the transmembrane segment, which forms a hairpin-like structure and transforms into a trimer under other conditions effecting the particle infectivity. The EDI, EDII, EDIII, and helix-transmembrane domains (TMDs) of the E protein play significant roles in membrane fusion and mediate irreversible conformational changes during the fusion process. Biochemical studies have also revealed that temperature and

chemicals alter the E protein structure and inactivate the viruses, suggesting the E protein's importance during Flavivirus infection (Zhang et al., 2017). Normally, JE vaccines have been produced by killing the virus with heat shock, chemicals or radiation. The inactivated vaccine is safer and more stable than the live attenuated vaccine. They can be easily stored and delivered in a freeze-dried form, which makes them more accessible to people in developing countries. (Petrovsky & Aguilar, 2004).

The stability test of anti-flavivirus group antigen clone D1-4G2-4-15 mAbs when stored at -20 °C and the antibody specific activities against *Dengue virus* type 1 were determined once a week for 10 weeks. The results showed that the anti-flavivirus group antigen clone D1-4G2-4-15 mAbs were detectable at undiluted and three of ten-fold serial dilutions (1:10, 1:100 and 1:1000) as shown in **Fig. 2.**

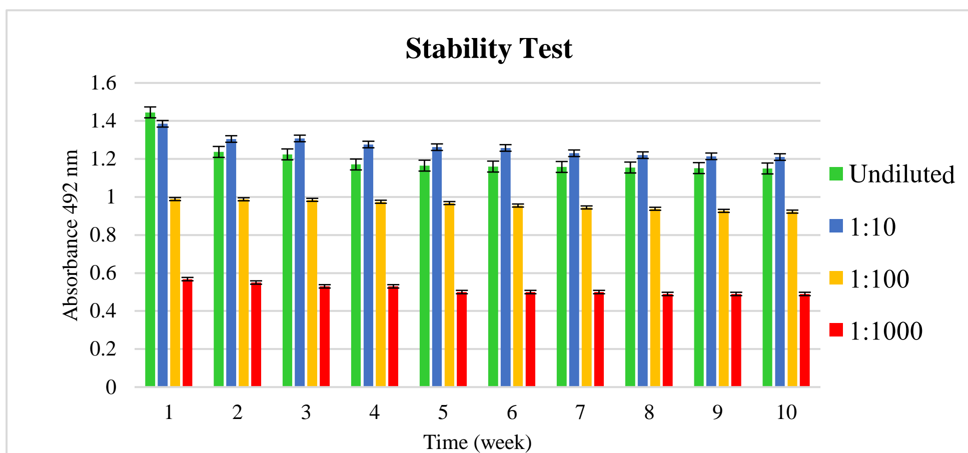


Fig. 2. Stability test of anti-flavivirus group antigen clone D1-4G2-4-15 mAbs when stored at -20°C, were determined every week for 10 weeks by typing ELISA on varying antibody dilutions [(Undiluted (■), 1:10 (■), 1:100 (■), 1:1000 (■)] (positive cutoff is ≥ 0.2).

The stability of antibodies after storing at -20°C were determined by ELISA as previously described (Bobrovnik, 2003). The results showed that the anti-flavivirus group antigen clone D1-4G2-4-15 mAbs maintained their stability for 1-10 weeks after storing at -20°C. The antibody specificities at 1:1000 dilution against *Dengue virus* type 1 antigen binding sites were stable and retained. The mean absorbance was greater than 0.49 (±3SD).

Anti-flavivirus group antigen clone D1-4G2-4-15, purchased at the concentration of 0.2 mg / ml from the Absolute Antibody Ltd. was $300 or 10552.5 baht (rate 35.175 baht/US) while the cost of total production process/0.2 mg / 8ml was 680.97 baht. In other word, the cost of all production process was 93.54 percent reduced. The cost detail and cell culture assumption are as follows: 1,300.00 baht/500 ml medium; 18,000.00 baht /500 ml fetal bovine serum and other supplement containing Medium NCTC-109 (1X), OPI (Oxaloacetic acid, Pyruvic acid, Insulin), L-

glutamine, NEAA (non-essential amino), HEPES [4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)], 7.5% $NaHCO_3$, penicillin and streptomycin are shown in **Table 2**. The total cost of complete growth medium with 10% FBS (4701.70 Baht/500 ml) switching to serum-free media, (3,145 bath/500 ml), an approximately of 33% total cost saving from all the process to produce Anti-Flavivirus Group Antigen Clone D1-4G2-4-15 mAbs were compared.

Table 2. Comparing of potential cost saving between using complete growth media containing 10% FBS versus Serum-free media in cell culture.

Medium with 10% FBS		**Serum-free media**	
500 ml medium	฿1,300.00	CD hybridoma	฿3,145.00
50 ml FBS	฿1,800.00		
Supplements	฿1,601.70		
Total	**฿4,701.70**	**Total**	**฿3,145.00**

CONCLUSION

This research aimed to develop anti-flavivirus group antigen clone D1-4G2-4-15 cell culture method to produce monoclonal antibodies specific to antigens of the genus *Flavivirus* in serum-free media by inside adaptation. The cells can be cultured in serum-free media quickly while their growth curve of the lag phase, exponential phase and stationary phase are shown on Day 1, Days 2 to 4 and Days 5 to 7, respectively. The yield of anti-flavivirus group antigen clone D1-4G2-4-15 was 96.27% with its purity of 97%. The antigen specific binding to four commercial JE virus vaccine products by ELISA was determined. The results of anti-flavivirus group antigen clone D1-4G2-4-15 mAbs were positive against Beijing-GPO and JEVAC™ of which both of them are inactivated Japanese encephalitis vaccines. The anti-flavivirus group antigen clone D1-4G2-4-15 were stabled for 1 to 10 weeks after storing at -20°C. In addition, the specific activity of anti-flavivirus group antigen clone D1-4G2-4-15 mAbs at 1:1000 dilution was maintained. Moreover, the cost of production process of anti-flavivirus group antigen clone D1-4G2-4-15 was 93.54 percent decreased when compared to the price of Absolute Antibody Ltd. at the same concentration of 0.2 mg / ml while the cost could be saved approximately 33% from all production process. The inside adaptation method for hybridoma cell culture in serum-free media is a new alternative culture method that not only save time and cost but also obtain high yield of purified mAbs.

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