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TOXO-IFA SYSTEM: A SIMPLE TOOL FOR SCREENING OF REACTIVITY OF HYBRIDOMAS DEVELOPED FOR DENGUE DIAGNOSIS

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ABSTRACT

A simple and alternative system for screening of dengue virus nonstructural-1 (NS1) glycoprotein mouse monoclonal antibody (mAb)secreting hybridoma using larvae of non-heamatophagous mosquito Toxorhynchites splendens larvae. Monoclonal antibody secreting hybridoma has been screened with known dengue virus specific mAbs in virus infectedTx. splendens mosquito larvae using immunofluorescent assays (IFA).Using this Toxo-IFA method, hybridoma clones have been screened in three weeks of duration. From the present study, this method could be used as an effective tool for screening of hybridomas developed for dengue diagnosis.

Keywords: Hybridoma, Dengue, Monoclonal antibody, *Toxorhynchites splendens*

I. Introduction

Hybridoma technology has been proved as an efficient methodology for monoclonal antibody (mAb) production and used frequently in medical industry. Monoclonal antibodies also play a significant role in disease diagnosis. Different monoclonal antibodies are currently available for detection of viral, bacterial and fungal pathogens, also used to detect various antigens in body fluids and tissues[1,2].

Screening and selection of hybridoma is an important and laborious step in mAb development and validation. It is very common that only the least number of fused clones will be stable and survive, after the myeloma and spleenocytes fusion process. Hence, every individual fused cell has to be confirmed for its stability and as well as capability of secreting the desired mAb[3].

This process includes, sub-cloning, limiting dilution and ELISA screening. Usually, it takes several weeks (4–6 weeks) to recover a lesser number (<10)of antibody secreting hybridomas. Depending on the source,it costs an approximate $500 to $10,000 for screening a single clone[4]. Various methods were reported already to bypass the above processes and identify the desired mAb secreting clones, especially different expensive cytometrytechniques, however their efficiency may not be sufficient [5-6]. The conventional Indirect-ELISA screening process also consumes considerable time and cost.Hence, a simple alternative technique is essential to reduce the cost and time for screening the mAb secreting hybridoma clones.

Toxorhynchites mosquito has been used for the isolation of field strains of dengue and other arboviruses. This mosquito have more advantages like, large size and easy to handle, both male and female adults do not feed vertebrate blood in nature, it will not be naturally infected and most importantly it could not act as disease vector[7-8]. In addition, Centre for Research in Medical Entomology (CRME), Madurai has already reported the potential use of *Tx. splendens* larvae in the TFA (*Toxorhynchites*-fluorescent antibody) system to detect Japanese encephalitis and dengue virus infection [9].

An alternative and simple Toxo – IFA system is proposed in the present report to screen the antibody secreting hybridoma clones using dengue virus inoculated early third instar*Tx.*

splendens larvae. So far, scientists have used the virus inoculated *Tx. splendens* larvae to confirm the presence of virus from suspected samples [10-11]. This present work will pave the new way to explore the possibility of Toxo – IFA system for routine screening of monoclonal antibody secreting hybridomas against arboviruses as well as the detection of immunogens.

II. Materials and Methods

a) Tx. splendens larvae

Tx. splendens mosquitoes were obtained from Mosquito Breeding Wing, Centre for Research in Medical Entomology, Indian Council of Medical Research, Madurai, Tamil Nadu, India.

b) Virus Inoculation

Dengue serotype 1 (P-23086),a prototype virus was obtained from National Institute of Virology (NIV), Pune, India. *Tx. splendens* larvae were immobilized on ice for few minutes and Dengue virus serotype 1 was inoculated intracerebrally with different dilution (1: 10, 1: 100, 1: 1000 and Undiluted) (Table 1) and incubated at 28°C for 7 days as per the standard methodology. During incubation period, *Tx. splendens* larvae were fed with larvae of other species of mosquitoes and the larvae inoculated with sterile Bovine albumin phosphate buffered saline (BAPS)(pH 7.4)was used as control (20 larvae). Virus inoculated and control mosquito larvae were maintained at 28 ± 1°C and relative humidity of 80%. Prior to the experiment, there were 80(20 larvae in each group),*Tx. splendens* larvae were infected with Dengue virus and stored in -80°C for further screening experiments.

c) Toxo – IFA

After incubation period, head squashes of virus infected (experimental) and control larvae were made on the teflon coated microscopic slides, air dried and fixed with chilled acetone for 5 min. Monoclonal antibody (15 µl diluted1:5 in PBS) or in cell culture fluid (20 µl) was added on the smear and incubated in a humid chamber at 37°C for 30 min, followed by 4 times wash with 1x PBS (20 µl). After wash,incubated for 30 min in15 µl of 1: 100 dilution of goat anti-mouse IgG FITC conjugate (Sigma- Aldrich) and washed with 1x PBS for 4 times. The smears were mounted in 90% glycerol (pH 8.5) and examined under fluorescent microscope. Commercial

dengue virus specific monoclonal antibodies (Abcam, ab41616) were used as a positive control throughout the study.

d) Indirect ELISA with *Tx. splendens* larvae

Supernatants of *Tx. splendens* larvae (50 µL) (prepared from *Tx. splendens* larval body part ground and centrifuged at 10000 rpm at 4°C) were coated with 96 well plates (M9410, Sigma-Aldrich) using sodium carbonate-bicarbonate buffer (pH-9.6)and incubated overnight at 4°C. The wells were blocked with 2% BSA (37520, ThermoFisherScietific), for 2 h at 37°C. After six times washing with 1x PBS containing 0.05% Tween 20 (PBS-T). The wells were incubated with 50 µL hybridoma supernatants (100 µL)/ monoclonal antibody (50 µL). The wells were washed six times with PBS-T and the bound antibodies were detected using goat anti mouse IgG conjugated with horseradish peroxidase as secondary antibody (A4416,Sigma-Aldrich) at a 1:10,000 dilutions for 1 h at 37°C. Plate was again washed three times with PBS-T and 100 µL of Tetramethylbenzidine (TMB) (N301, ThermoFisherScietific) substrate was added to the wells. Optical density was measured at 450 nm using an ELISA plate reader (Biorad).

III. Results and Discussion

a) Toxo – IFA with known mAbs

From the second post-infection day (PID) to 8th day, six*Tx. splendens* larvae were used for making head squash on the teflon coated slides along with the positive and negative control. Cell culture fluid of a selected fused positive hybridoma clone was used as a primary antibody and the commercial NS1 monoclonal antibody (1:5) was used as a positive control(Figure 1D). The Goat anti-mouse IgG FITC conjugate was used as secondary antibody and the slides were visualized on fluorescence microscope. The Toxo- IFA results showed that, Dengue 1 virus inoculated *Tx. splendens* larvae expressed dengue NS1 antigen which binds to the monoclonal antibody. The antigen antibody binding was visualized by the FITC conjugate (Figure 1A). *Tx. splendens* larvae inoculated with1:100 dilution of Dengue 1 virus (from the stock of $\sim 10^{-2}$) and 7 days of incubation period showed the high intensity for positivity, hence the same volume of the inoculum and incubation period was kept as a constant for the screening of hybridoma clones (Table 1).

Table 1. The detection of Dengue virus antigen in virus infected *Tx. splendens* larvae on Toxo-IFA System and Indirect ELISA(n=20).

Inoculum (Dengue Virus)		**Post-infection day**						
Day		2	3	4	5	6	7	8
Undiluted	ELISA	-	-	-	-	-	-	-
	IFA	-	-	-	-	-	-	-
1:10	ELISA	-	-	-	-	-	-	-
	IFA	-	-	-	-	-	-	-
1:100	ELISA	-	-	-	+	++	++	+
	IFA	-	-	-	++	+++	++++	+++
1:1000	ELISA	-	-	-	-	++	++	++
	IFA	-	-	-	+	++	++	+++
Negative Control	ELISA	-	-	-	-	-	-	-
	IFA	-	-	-	-	-	-	-

- Negative

+ Positive (Only the 1:100 dilution showed best results and other dilutions showed larval mortality or low positivity, number of plus indicate the intensity of positive reactions)

The control*Tx. splendens* larval heads were processed as a negative control and the results showed no fluorescence (Figure 1E).The body parts of the dengue virus inoculated *Tx. splendens* larvae were homogenized and the supernatant was used as an antigen source for the indirect ELISA. The results were correlated with the Toxo – IFA.

b) Toxo-IFA screening with unscreened fused hybridomas

Thirty two positive fused cells were chosen for Toxo-IFA screening process along with the conventional ELISA screening method (Figure 2). Initially, 7 fused cells showed positive

results in ELISA. These clones were selected for further screening using the limiting dilution method. Out of 7 fused cells, only 4 hybridoma clones were selected through the limiting dilution method. Each clone was further confirmed using ELISA, where only 3 NS1 mAb-secreting positive clones could be selected. It took 6 weeks to obtain clones using other conventional screening method.

On the other hand, among the 32 fused cells, 3 hybridomas were selected based on Toxo-IFA system results. Subsequently, these three hybridomas were confirmed by ELISA assay (Figure 1B and 1C). These positive hybridomas were further screened though limiting dilution and 3 NS1 mAb secreting positive clones have been effectively obtained after limiting dilution. Moreover, it took only three weeks to complete the screening process. Toxo-IFA screened NS1 mAb secreting clones and conventional ELISA screened NS1 mAb secreting clones were able to show the same stability and better mAbyield. Toxo-IFA screened clones showed higher Dengue NS1 antigen detection range up to 0.25 µg/mL (Figure 3A), whereas the conventional screened clones showed the detection range up to 0.5 µg/mL (Figure 3B). The purified recombinant NS1 antigen developed through our earlier studies was used to determine the NS1 antigen detection range [12]. The detection difference was notable, because the clones showed higher detection range will be able to detect even the lowest level of dengue virus presence on diagnosis purpose.Morover, the antigen detection limits were analysedusing the cell culture supernatant and certainly sensitivity will be higher if purified mAb was used[13].

Theabove resultsclearly demonstrate the usefulness of Toxo – IFA system for screening of hybridomas and the final confirmation of monoclonal antibody reactivity. Generally, after the hybridoma fusion, the fused clones will be screened and selected further using limiting dilution and ELISA [14]. Though the Toxo – IFA system cannot replace the existing ELISA based screening method, this methodcould be used as analternative method byconsidering its advantage. Seven positive hybridomas were identified on initial stage of screening using conventional Indirect-ELISA, however after the final screening only 3 stable clones were found secreting the desiredNS1 mAb. Hence, Toxo-IFA system could very well be used for the screening of hybridomas in monoclonal antibody production technology.

Figure 1

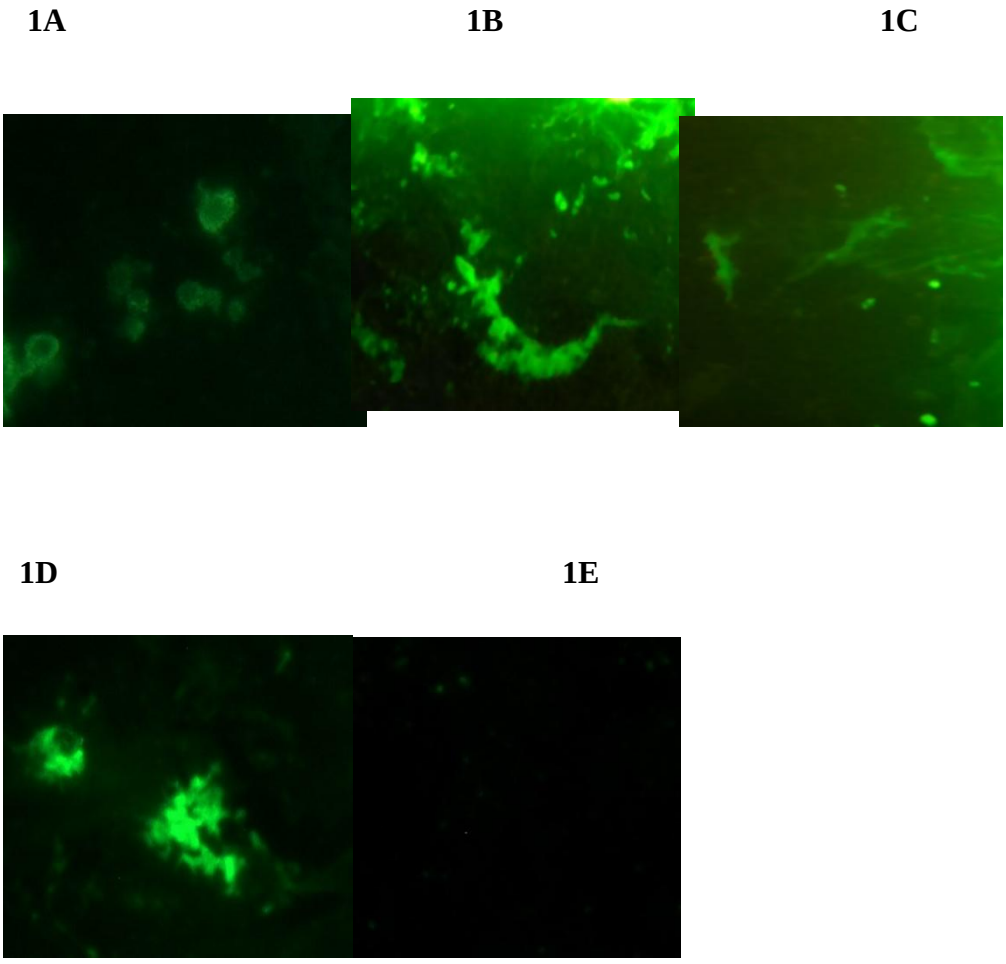

Figure 1A, 1B, 1C and 1D - Fluorescence indicates the binding of mAbs to the Dengue NS1 antigen

Figure 1D - Positive control (Commercial Dengue NS1 mAb)

Figure 1E - Negative Control (Control*Tx. splendens* larvae)

Figure 2 Schematic representation of the methodology used for screening the Dengue NS1 mAbsecreting hybridomas throughToxo-IFA and ELISA

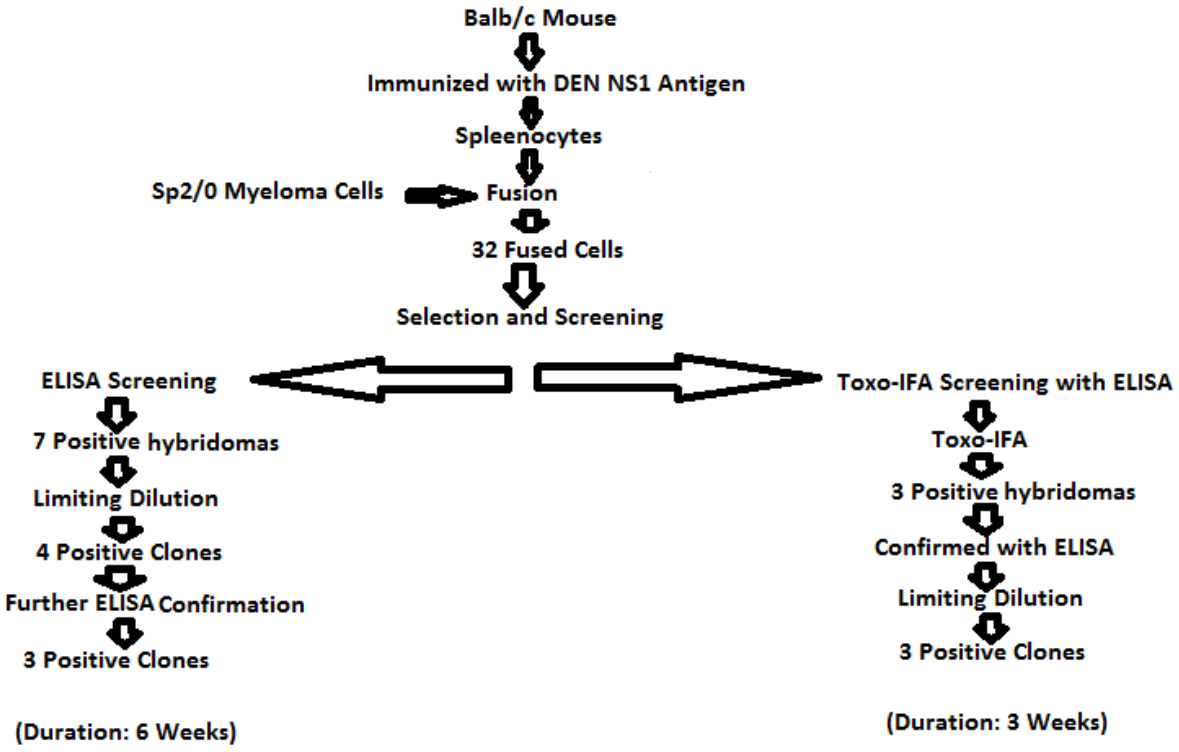

Figure 3A Detection of dengue NS1 antigen using indirect ELISA

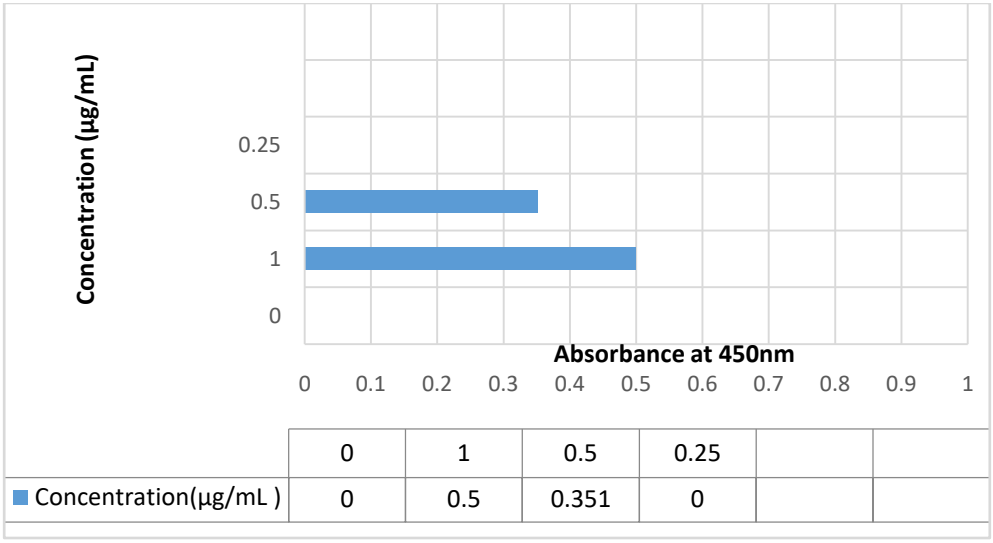

Figure 3A Hybridomas screened through indirect ELISA can able to detect NS1 antigen up to 0.5 µg/mL.

Figure 3B Detection of dengue NS1 antigen using Toxo-IFA system

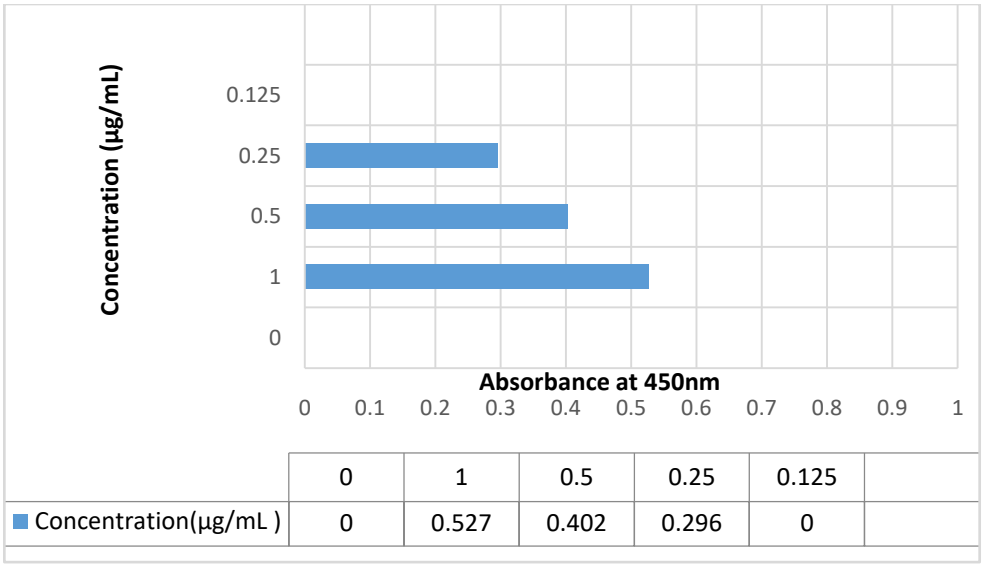

Figure 3B Hybridomas screened through Toxo-IFA can able to detect NS1 antigen up to 0.25 µg/mL.

IV. Conclusion

The results of the present study demonstrated a simple and alternative system for screening of dengue NS1 mouse monoclonal antibody using larvae of non- heamatophagous mosquito *Tx. splendens*. In a short duration (3 weeks), there were 3 NS1 monoclonal antibody secreting hybridoma clones have been successfully screened using Toxo-IFA system and this could be an effective method of screening hybridomas developed for arboviruses.

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References

1. Ghosh S and Ansar W (2013), "Monoclonal Antibodies: A tool in clinical research", Ind J Clin Med, vol. 4, pp. 9–21.
2. Kohler, G and Milstein, C (1975), "Continuous cultures of fused cell secreting antibodies of predefined specificity", Nature, vol. 256, pp. 459–497.
3. Chiarella, P and Fazio, V.M (2008), "Mouse monoclonal antibodies in biological research: strategies for high-throughput production", Biotechnol. Lett, vol. 30, no. 8. pp. 1303–1310.
4. Ogunniyi, AO, Story, CM, Papa, E, Guillen, E and Love, JC (2009), "Screening individual hybridomas by microengraving to discover monoclonal antibodies", Nat Proto, vol. 4, no. 5, pp. 767–782.
5. Rouiller, Y, Bielser, J-M, Brühlmann, D, Jordan, M, Broly, H and Stettler, M (2016), "Screening and assessment of performance and molecule quality attributes of industrial cell lines across different fed-batch systems", Biotechnol. Prog, vol. 32, no. 1, pp. 160–170.
6. Misaghi, S, Shaw, D, Louie, S, Nava, A, Simmons, L, Snedecor, B, Poon, C, Paw, JS, Gilmour-Appling, L and Cupp, JE (2016), "Slashing the timelines: Opting to generate high-titer clonal lines faster via viability-based single cell sorting", Biotechnol. Prog, vol. 32, no. 1, pp. 198–207.
7. Leon R, (1981),"The use of Toxorhynchites mosquitoes to detect and propagate dengue and other arboviruses", American J Trop Med and Hygiene, vol. 30, no. 1, pp. 177 – 183.
8. Norazizah S and Sazaly A, (2006), "Effects of dengue 2 virus inoculation on Toxorhynchites splendens larvae", J Entomol, vol. 3, pp. 89-94.
9. Thenmozhi, V, Paramasivan, R, Samuel, P. P, Kamaraj, T, Balaji, T, Dhananjeyan, KJ, Venkatasubramani, K, Leo, SVJ, Babu, RS, Govindarajan, R and Tyagi, BK (2013), "Japanese encephalitis virus isolation from mosquitoes during an outbreak in 2011 in Alappuzha district, Kerala", J Vect Born Dis, vol.50, no.3, pp. 229-231.
10. Paramasivan R, Thenmozhi V, Hiriyan J, Dhananjeyan K, Tyagi BK and Dash AP (2006), "Serological and entomological investigations of an outbreak of dengue fever in

certain rural areas of Kanyakumari district, Tamil Nadu", Ind J Med Res, vol. 123, no. 5, pp. 697-701.

11. Sabchareon, A, Sirivichayakul, C, Limkittikul, K, Chanthavanich, P, Suvannadabba, S, Jiwariyavej, V, Dulyachai, W, Pengsaa, K, Margolis, HS and Letson, GW (2012), "Dengue Infection in Children in Ratchaburi, Thailand: A Cohort Study. I. Epidemiology of Symptomatic Acute Dengue Infection in Children, 2006–2009", PLoSNeg Trop Dis, vol. 6, no 7, pp. e1732.
12. Sankar, SG, Dhanajeyan, KJ, Paramasivan, R, Thenmozhi, V, Tyagi, BK and Vennison, SJ (2013), "High-Level Expression of Functionally Active Dengue-2 Non-Structural Antigen 1 Production in Escherichia coli", BioMed Res Inter, vol. 2013, no. 343195, pp. 1–6.
13. Kathiresan, E, Paramasivan, R, Thenmozhi, V, Das, A, Dhananjeyan, K.J, Sankar, S.G, Jerald Leo, S.V, Rathnapraba, S and Vennison, S.J, (2017), "Development and multi-use applications of dengue NS1 monoclonal antibody for early diagnosis", RSC Adv, vol. 7, pp. 1927–1931.
14. Zhang, Z, Liu, H, Guan, Q, Wang, L and Yuan, H, (2017), "Advances in the Isolation of Specific Monoclonal Rabbit Antibodies", Front. Immuno, vol. 8. https://doi.org/10.3389/fimmu.2017.00494