〈Others〉

A protocol to assay Anti-*Yersinia pseudotuberculosis*-derived mitogen human IgG by ELISA

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ABSTRACT Our department has been continuing a research on the diagnosis of *Yersinia pseudotuberculosis* infections. This pathogen manifests in multifarious forms, including systemic vasculitis resembling Kawasaki Disease. Recently, a previously established ELISA assay for this infection was transferred from its originator at National Center for Child Health and Development. However, the protocol had to be modified for a different facility and equipment. Described herein is the protocol, successfully adapted for use at our laboratory. This protocol is contributing to the aforementioned research by improving analysis throughput of our laboratory.

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INTRODUCTION

Yersinia pseudotuberculosis (Yp), is a zoonotic pathogen found in rodents and avians that also infect humans. Yp was recognized as a cause of re-emerging human endemic disease in Okayama prefecture in the 1980's by Sato *et al.*[1)] Human infection of Yp manifests in various forms, such as enteritis, sepsis, acute renal failure and Kawasaki Disease (KD)- like vasculitis. Abe *et al.* found that Yp produces a super antigen *Yersinia pseudotuberculosis*-derived mitogen (YPM)[2)], suggestive of mediating KD-like manifestation. Its genetic sequence was also revealed shortly thereafter[3)]. YPM synthesized *in vitro*, enabled ELISA assay of anti-YPM IgG in patient sera[4)]. Our department is continuing a research under the title "Utility of the Loop mediated Isothermal Amplification (LAMP) method in the diagnosis of *Yersinia pseudotuberculosis* infection (Kawasaki Medical School's Ethical committee approval No.1680-04)", which aims to compare among different detection methods including LAMP, bacterial culture and serological methods. Therefore, in-house availability, rather than outsourced, of this previously established ELISA assay was needed. This assay was recently transferred to our laboratory by courtesy of Dr. Jun Abe, Department of Division of Advanced Medicine for Virus Infections, National Center for Child Health and Development (NCCHD), originator of this assay. However, due to

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difference in facility and equipment, minor changes had to be implemented to adapt the assay to our laboratory. Described herein is the protocol of this assay, adjusted for our laboratory.

MATERIALS

YPM antigen

Synthesis of YPM antigen, based on the DNA sequence reported by Ito *et al.*[3], was outsourced to BEX Co., Ltd. (Tokyo, Japan). Stock antigen solution was diluted to a 2 μg/mL working solution using phosphate buffered saline (PBS), which was then used for coating the 96-well plates.

Control Sera

Properly collected control sera were provided by Dr. Abe. These sera are to be replaced by patient sera previously collected under appropriate consent for the aforementioned ongoing study.

Buffers / Solutions

The following buffers/solutions were prepared. Unless otherwise specified, Milli-Q water (Merck Millipore Ltd., MA, USA) for water, and PBS supplied by the central research institute of Kawasaki Medical School were used.

Diluted Sulfuric Acid (Stop solution)

2N sulfuric acid ($\approx$ 5.28%) to be used as a stop solution of the chromogenic reaction was prepared by diluting Concentrated Sulfuric Acid (CSA, 36N $\approx$ 95%) (Kanto Chemical Co., Inc., Tokyo, Japan). To avoid accidents due to dilution heat, dilution was carried out by titrating CSA, drop by drop, into water under continuous agitation. Dilution heat released by diluting sulfuric acid from 100% to $100 \times n$% is known to be represented by the following equation (http://sulfuric-acid.biz/calculation-2.html):

$$97209 \times (1 - n) \times (5.4428 - 3.6445 \times n)^{-1} \quad [\text{cal / mol} \cdot \text{H}_2\text{SO}_4]$$

Thus, assuming the density of CSA as 1.8286, specific heat of diluted sulfuric acid as 0.84 kcal/kg (typical value for 20% sulfuric acid), the dilution heat released by dilution is estimated as 153 kcal/kg・H_2SO_4; which corresponds to a 17.69℃ rise in temperature.Thus the diluted product would not be hotter than a typical hot bath, even if the dilution started from room temperature. Furthermore, this is obviously an overestimation to the safer side, since the more diluted sulfuric acid is, the more its specific heat rises. Based on this estimate, dilution was carried out at room temperature without aggressive cooling.

A 500mL medium bottle with a stirrer bar was weighed and filled with Milli-Q water to approximately 85%. The volume of water was determined as the increment in weight. One seventeenth volume of CSA was slowly titrated into the medium bottle using a pipette, drop by drop, under continuous stirring on a magnetic stirrer.

Actual temperature rise was lower than the aforementioned estimate; on one occasion, starting at 27.2℃and ending at 36.6℃(a 9.4℃ rise).

Blocking solution (1% Bovine Serum Albumin (BSA) in PBS)

10g of BSA fraction V (Cat No. 01863-48, Nacalai Tesque, Inc., Kyoto, Japan) was weighed and dissolved into a fresh bottle of 500 mL PBS (Cat No. 13397-85, Nacalai Tesque), and left overnight to achieve complete dissolution. The solution was filter sterilized using Sartolab RF 1000 Filter System (Item No. 180C3----------E, Sartorius AG, Göttingen, Germany); the residue in the emptied bottle was washed by another 500 mL bottle of PBS and combined by filtered sterilizing in to the same bottle. The sterilized solution was homogenized by gentle swirling and

kept at 4℃ until use. This solution was used as a blocking solution. Furthermore, this solution was used as a dilution buffer by supplementing with 0.05% Tween-20. 30 mL aliquots were prepared under a biocleanbench for this usage.

10% Tween-20

Approximately 5 mL undiluted Tween-20 (Ref No. H5151, Promega, WI, USA) was transferred into a preweighed 50 mL centrifugal tube. The mass of transferred Tween-20 was determined as the incremental weight. Assuming the density of Tween-20 as 1.110, nine times as much water as the volume of transferred Tween-20 was added to the tube. Following thorough vortexing, the tube was left standing on a tube rack overnight and shaded, until the foam disappeared. Using a 50 mL syringe, and a 0.22 μM filter (Millex GV, Merck Millipore Ltd.), the diluted Tween-20 was filter sterilized into another fresh 50mL centrifugal tube and kept as a stock solution. This stock solution was supplemented into PBS and 1% BSA in PBS, diluted 200-fold (0.05%), as mentioned hereafter, when carrying out the assay.

Wash buffer (0.05% Tween-20 in PBS)

Under a biocleanbench, approximately 1/200 volume of 10% Tween-20 was supplemented to stock PBS bottles to serve as wash buffer (e.g. 2.5 mL to a 500 mL PBS stock).

Dilution buffer (DB) (0.05% Tween-20 in 1% BSA)

Blocking solution aliquots were supplemented with 1/200 volume 10% Tween-20 (e.g. 150 μL to a 30 mL blocking solution aliquot) under a biocleanbench to obtain dilution buffer for diluting serum specimens.

Computer software

The following software were used for this study on a personal computer equipped with an Intel® Core ™ i7-2600 processor 3.4GHz, 16GB memory, and running Microsoft Windows 7 Professional (64-bit) (Dell Technologies, TX, USA).

- ActiveState Perl version 5.20.2, binary build 2001 [298913] (http://www.ActiveState.com).
- R version 3.6.1[5] and dr4pl (Dose Response Data Analysis using the 4 Parameter Logistic (4pl) Model) package version 1.1.11 (https://cran.r-project.org/package=dr4pl).
- GNUPLOT version 4.6 Patchlevel 7 (http://www.gnuplot.info)

RESULTS

After careful consideration and preliminary experiments, the following protocol was established.

All plate washing steps consisted of three washes with 300 μL/well of wash buffer per wash following unsealing the plate; the seal could be reused by applying it in the exact position it was first applied.

1) Plate coating

Recombinant YPM stock solution was freshly diluted to 2 μL/mL using PBS; 5 mL per plate of this dilution is required for coating. The left side half of the wells of NUNC MaxiSorp™ high protein-binding capacity 96 well ELISA plates (Cat No. 442404, Thermo Fisher Scientific Inc., MA, USA), A1~H6, were coated with YPM by applying 100 μL of the diluted YPM solution; 100 μL of PBS was applied to the remaining wells, A7~H12, to serve as control wells. Though manual pipetting is acceptable, the authors prefer and recommend using an automatic dispenser to increase reproducibility and to save time. The plate was sealed with SureSeal ST (BM Equipment Co., Ltd., Tokyo, Japan), and incubated at 25℃ overnight.

2) Blocking

Following a washing step, 300 μL of blocking

solution was applied to all wells for blocking; the plate was sealed again and incubated at 25℃ for 2 hrs.

3) Specimen preparation

During the 2-hr incubation for blocking, DB was aliquoted into 1.5 mL microcentrifuge tubes: for quantification standards, 495, 900 and seven 500 μL aliquots; for negative control, 600 μL; for each positive control and unknown serum, a set of 1200 and 400 μL. Specimens were diluted as follows: standards, 100-fold (5 μL original standard + 495 μL DB) and 1,600-fold (60 μL 100-fold standard + 900 μL DB), serially diluted 2-fold (400 μL dilution + 400 μL DB) down to 204,800-fold; negative control, 400-fold (1.5 μL control serum + 600 μL DB); positive control and unknown serum, 800-fold (1.5 μL serum + 1,200 μL DB) and 1,600-fold (400 μL 800-fold dilution + 400 μL DB).

4) Specimen application

The plate underwent another washing step following the 2-hr incubation for blocking. Using a micropipette, 100 μL of the diluted specimens were applied to each designated well (in duplicates as illustrated in Fig. 1) of the plate. After all specimens were applied, the plate was sealed again and incubated at 25℃ for another 2 hrs.

During the 2-hr incubation, 3 μL of Polyclonal Rabbit Anti-Human IgG/HRP (Dako Denmark A/S, Glostrup, Denmark) was diluted into 12 mL of dilution buffer for the following step. This 4000-fold secondary antibody dilution was pre-poured in a buffer reservoir when 8-channel micropipette was preferred.

5) Application of HRP conjugated secondary antibody

Following another washing step, 100 μL/well

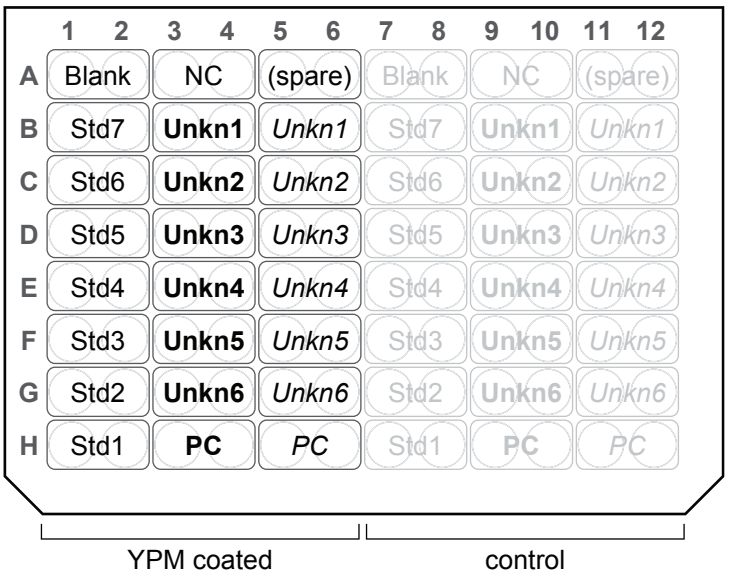

Fig. 1. Specimen application layout.
NC, negative control; PC, positive control; Std, standard; Unkn, unknown specimen; YPM, *Yersinia pseudotuberculosis* mitogen. Dilution buffer is used as blank; Std1 to Std7 are serial dilutions of standard serum, from 3,200-fold to 204,800-fold; NC is diluted to 400-fold; specimens and PC in **Bold face** are diluted to 800-fold; ***Italicized***, 1,600-fold.

of the diluted secondary antibody was applied to each well using an 8-channel micropipette or an automatic dispenser, and the plate was sealed again. The plate was incubated at 25℃ for 1 hr, followed by another washing step.

During the 1-hr incubation, KPL TMB Microwell Peroxidase Substrate System (2-Component System) (SeraCare Life Sciences, Inc., MA, USA) was brought to room temperature and 11 mL of peroxidase substrate was prepared for the following step. The prepared substrate and stop solution were pre-poured in buffer reservoirs, respectively.

6) Chromogenic reaction

Using an 8-channel micropipette and a clock or a metronome, 100 μL/well of peroxidase substrate was applied to the wells, at a constant tempo. The color change was continuously monitored under room temperature by the naked eye; as soon as a color change (to slight blue) was observed in the well with the thickest standard applied, 50 μL/well of stop solution was applied to the wells in identical order and tempo (to ensure virtually identical reaction time among the wells) as was the substrate; the blue color turns yellow after applying stop solution.

The plate was sealed after the stop solution was applied to all wells.

7) Plate read

Plate(s) were read using Varioskan Flash (Thermo Fisher Scientific Inc., MA, USA), at wavelengths 450 nm (signal) and 620 nm (background); bandwidth set at 5 nm, measurement time at 100 ms. Results were exported to a USB external storage media and subjected to further analysis on our lab PC as described hereafter.

Computer program scripts were programmed by the authors to process absorbance data exported as tab separated text data. The main script, written in Perl, 1) parses the tab separated text file, imports the absorbance of the wells, 2) averages the values for wells intended as duplicates, calculates and subtracts background values, yielding a representative absorbance value for each specimen, 3) passes the representative values of standard serial dilutions, along with their dilution factors to the R dr4pl package to calculate the parameters for a 4-parameter logistic (4PL) curve fit to use as the standard curve from which antibody titer of specimens are estimated from, 4) estimates the antibody titer of each specimen from absorbance and the standard curve using the bisection method, 5) outputs the results to a text file, generates and runs a GNUPLOT script to generate a PDF and a PNG file of the fit standard curve and data points, antibody titer estimates, and the four parameters for the fit standard curve plotted against it. The scripts were written for CUI (character user interface) based non-interactive operation, to save time and to ensure reproducibility and verifiability of data processing procedures, as we expect to do mass data processing of our pooled sera. The scripts can process data from four plates in tens of seconds.

DISCUSSION

The major factors that required consideration were 1) incubation temperature and time, 2) different spectrometer and software.

The incubation steps' temperatures and time were revised. The original transferred protocol required incubations at room temperature and 37℃. However, as central air conditioning in the laboratory is only available for limited hours, overnight incubation at unstable room temperature was best avoided. Incubators in the central research institute were available for incubation at 37℃, but as these incubators were conditioned for cell culture purposes, the 5% carbon dioxide environment could affect the assay by altering the pH of the plate well contents. Therefore, all incubation temperatures

but one was fixed to 25℃, used as normal room temperature, carried out in an air incubator solely dedicated for this assay. The rule of thumb, that 1-hour incubation at 37℃is equivalent to 2-hour incubation at 25℃, was taken into account when revising the incubation time. The only exception was the chromogenic reaction, which takes 10 minutes at most. It requires monitoring by the naked eye, and therefore carried out at ambient room temperature.

The original transferred protocol used a spectrometer manufactured by Bio-Rad Laboratories (CA, USA), and manufacturer software provided with the spectrometer for antibody titer estimation. However, the spectrometer available in the central research institute is a VarioskanFlash manufactured by Thermo Fisher Scientific Inc. The instrument is shared among many researchers, thus analyses using exported data was preferred. Therefore, an alternative to estimate the antibody titer from exported spectrometer measurement data had to be devised. Data exported from the spectrometer were processed by self-developed computer program scripts. Major issues to overcome were the following: analyzing the data format of the exported tab separated text data, computing a 4PL curve fit from seven dilutions of standard sera to serve as a standard curve, estimating the antibody titers of the test sera from the absorbance data according to the standard curve.

Fortunately, the exported data format was straightforward; thus easily processed by Perl regular expressions. The Perl script extracts the Run name, reads absorbance values as list data, and ignores the rest. The authors also created a helper pre-processing Perl script that splits export data from multiple plates into data files for each individual plate.

Using a fixed specimen applying layout (Fig. 1), the specimens' dilution factors were provided as constant "magic numbers" in the script. The Perl script calls the following R software command, to generate a fit 4PL standard curve($abs_{1\text{-}7}$ represent actual absorbance for each standard solution at 2-fold serial dilution factors of 3,200 to 204,800):

```
library(dr4pl)
coef(dr4pl(OD~conc,data=data.frame
(conc=c(1/128,1/64,1/32,1/16,1/8,1
/4,1/2),OD=c(abs7, abs6, abs5, abs4,
abs3, abs2, abs1))))
```

The return value is a 2-line text output from R, in which the four parameters that specify the standard curve are displayed on the second text line. An example is as follows:

```
UpperLimit    EC50     Slope LowerLimit
2.54786312 0.13182308 1.28652603 0.06525388
```

Using the aforementioned parameters, the standard curve is represented by the following function (x for antibody titer, y for absorption value):

$$y(x) = UpperLimit + \frac{LowerLimit - UpperLimit}{1 + \left(\frac{x}{EC50}\right)^{Slope}}$$

Since this function increases monotonically, antibody titers of specimens were estimated by solving the equation with absorption values of each specimen assigned to y, using the bisection method, starting with $x = 0$ and $x = 2$. Though this estimation was mostly successful, a workaround was needed to deal with cases when the result did not converge but alternated between two values of trace difference due to the limited accuracy of floating point arithmetic. In such cases, the y (x) deduced from the standard curve from the two x's corresponding to both ends of the bisection were slightly smaller or larger than the given absorption value. This situation was successfully circumvented by setting an extremely small threshold value, 1×10^{-180}, for the difference between the deduced

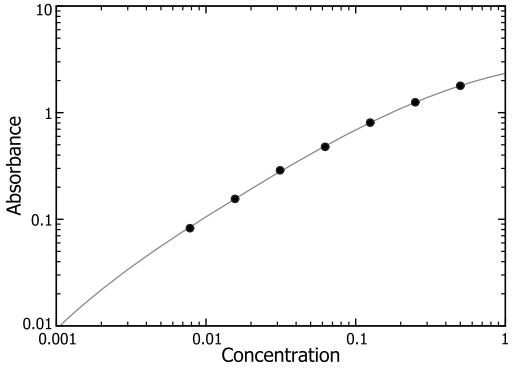

Fig. 2. A standard curve plotted as fitted 4-parameter logistic curve.
The fitted standard curve is plotted in gray; actual data points for serial dilutions of standard serum are represented as black filled circles.

y (x)'s corresponding to both ends after each iteration; a difference smaller than this threshold was defined as an additional ending condition (*de facto* convergence) for the iterations. Final antibody titer estimates of each specimen were obtained by multiplying the dilution factors to the solved x.

Standard curves of each assay were visualized by GNUPLOT software for confirmation of each assay by the human eye. Furthermore, since GNUPLOT can output images in various formats, the output can easily be reused for publication purposes as done in Fig. 2 (output as a PDF file).

In conclusion, we have successfully adapted the anti-YPM ELISA assay transferred from NCCHD to our laboratory. Furthermore, the analysis throughput and versatility of analyzed data were improved by effective use of self-developed computer programs.

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