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Title: Standard Operating Procedure (SOP) for Quantification of IgG4 antibodies against *Plasmodium falciparum* GLURP$_{25-514}$ (RO)

Introduction:

The IgG4 level is determined by an indirect ELISA method using a single serum / plasma dilution obtained by checker-board titration experiments. The optical density (OD) of the test sample is converted into arbitrary units (A.U.) by means of a standard-curve from known concentrations of purified human myeloma IgG4.

Wells of microtiter plates are coated with either GLURP$_{25-514}$ antigen or purified human myeloma IgG4 in serial dilutions. Diluted test-samples (primary antibody) are added to the wells containing the GLURP$_{25-514}$ antigen. Specific antibodies against GLURP will bind to the antigen-coated wells. To all wells mouse anti-human IgG4 is added (secondary antibody), followed by the addition to all wells of peroxidase conjugated goat anti-mouse IgG (tertiary antibody). Bound secondary antibody is quantified by colouring with ready to use TMB (*3,3', 5,5-Tetramethylbenzidine*) substrate. Optical density (OD) is read at 450 nm with a reference at 620nm in a plate reader, and the OD value of the test-sample is converted into A.U. by means of a standard curve on each plate.

(a) Preparation of Standard curve:

For antibody quantification, each assay includes a calibration (standard) curve obtained with purified IgG4, myeloma protein of known concentration (1 mg/ml). For the standard curve, serial dilutions (2-fold) of standard IgG4 proteins are coated directly to duplicate wells in columns 1 and 2 using 100 µl / well. Concentrations of standard IgG4 are 100; 50; 25; 12.5; 6.25; 3.13; 1.56; 0.78 ng / ml.
In order to prepare the standard reference curve for 12 plates, first make a working stock of 500 ng/ml by transferring 5 µl of the standard into 9.995 ml of coating buffer (1:2000)

Then prepare 8 tubes labelled 1 to 8 for a 2-fold serial dilution of the standard.

1) To tube 1, add 8 ml of coating buffer.
2) Add 3 ml of coating buffer each to tubes 2 to 8.
3) Transfer 2 ml from the working stock (500 ng/ml) to tube 1, and mix well.
4) Transfer 3 ml from tube 1 into tube 2, and mix well.
5) Transfer 3 ml from tube 2 into tube 3, and mix well.
6) Transfer 3 ml from tube 3 into tube 4, and mix well.
7) Transfer 3 ml from tube 4 into tube 5, and mix well.
8) Transfer 3 ml from tube 5 into tube 6, and mix well.
9) Transfer 3 ml from tube 6 into tube 7, and mix well.
10) Transfer 3 ml from tube 7 into tube 8, and mix well.

b. Working procedure:

- Coat the microtiter plate with **1**) 100 µl per well of serial dilutions of the standard reference IgG4 solution in PBS coating buffer in columns 1 and 2 on each plate and **2**) 100 µl per well of rGLURP-R0 in PBS coating buffer at 1.0 µg/ml in columns 3 to 12. Cover the plates with a plastic sealer and label each plate with antigen name, IgG subclass, series number, date and personnel initial. The plates can be stored for 21 days in the fridge at 2 to 8 ^{o}C.
- Dilute serum / plasma samples 1:50 in serum dilution buffer.The dilutions can be stored for 21 days in the fridge at 2 to 8 ^{o}C.
- Remove the plates from the fridge and wash them four times in washing buffer. For each washing step leave the plate filled with washing buffer for 1 minute before it is emptied. Pad the plates dry and add 150 µl of blocking buffer and keep at room temperature for 1 hour.
- Empty the plates, pad them dry, and wash the plates 4 times in washing buffer. Add 100 µl of dilution buffer to wells in columns 1 and 2. Add 100 µl of the diluted 4 positive and the 2 negative control samples and a PBS blank to duplicate wells in columns 3 and 4.
- Add 100 µl of the diluted test sample to duplicate wells in columns 5 to 12. Incubate for 2 hours with shaking on a rocker platform at room temperature.
- Wash the plates 4 times in washing buffer. For each washing step leave the plate filled with washing buffer for 1 minute before it is emptied.
- Add 100 µl of monoclonal mouse anti-human IgG4 diluted 1:3000 in dilution buffer, to all wells. Incubate for 1 hour with shaking on a rocker platform at room temperature.
- Wash the plate 4 times in washing buffer. For each washing step leave the plate filled with washing buffer for 1 minute before it is emptied.
- Add to each well 100 µl of peroxidase conjugated goat anti-mouse IgG diluted 1:3000 in dilution buffer. Incubate for 1 hour with shaking on a rocker platform at room temperature.
- Add to each well 100 µl of colour solution. Incubate at room temperature for 30 min in the dark.
- Add to each well 100 µl 0.2 M H_2SO_4 and read the absorbance at 450 nm, ref. 620 nm on a plate reader and save the results in a text file
- The conversion of absorbance values into A.U. is performed by using Microsoft Excel-based curve-fitting program.
- For each test fill out a work protocol

References:

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