Australian Government
Department of Health
Therapeutic Goods Administration

Laboratories Branch

Biochemistry – Cell Culture Manual	
Procedure	CC – 28 – Anti-D Activity – SOP
Written	s22 [redacted]
Authorised	s22 [redacted]
Date issued	27 August 2018
Revision #	4

Anti - D Activity – SOP

Principle of Assay:

The potency of human anti-D immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-D immunoglobulin and D-positive red blood cells. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

Purpose and Scope:

What is anti-D?
Anti-D is a plasma product produced from the blood of selected Rh (D) negative donors. The product is given to pregnant women who are Rh (D) negative to prevent their babies from developing Haemolytic Disease of the Newborn, or HDN.

Who needs anti-D?
On average, approximately 17% of mothers in Australia will need anti-D injections during each of their pregnancies and after the birth of an Rh (D) positive baby.

How is anti-D made?
Anti-D can only be produced from the blood of a select group of donors. These donors all have the Rh (D) negative blood type but they also have an antibody called anti-D. Very few people, and even fewer donors, have anti-D, so we rely heavily on these donors for this important product.

The Anti-D program
To maximise the supply of anti-D to meet demand, we have established a special program called the Anti-D program. In this program, we boost the anti-D levels of donors who already have anti-D. In addition, we can stimulate development of anti-D in specially selected donors who initially do not have it. This means that if you are a male or a female past child-bearing years, you may be eligible to join our Anti-D program.

Anti-D Supply

From 1 July 2011, Rhophylac (CSL) will replace WinRho (Baxter) as the product available to the Australian market under the National Blood Authority free of charge. Imported anti-RhD is used in Australia to complement domestic supply and where intravenous treatment is required. The previously imported product, WinRho is no longer available and the tender has selected a new product, Rhophylac. Rhophylac is supplied as a 1500IU and is presented in a ready to use prefilled syringe.

Australian Red Cross Blood Service website, June 2011

Responsibility:

The biennial review of this SOP is the responsibility of the Principal Biochemist or their delegate. Reviews will be recorded on the Document Amendment Record in the in the Cell Culture Manual on the Quality System (Biochemistry). The amended SOP is then circulated to staff with the Document Amendment Awareness Record.

Training for each operator should be recorded on a Training Sheet and stored in their individual training records folder located in TRIM. PH14/26. It is the responsibility of the staff member to maintain individual proficiency in the techniques detailed in this SOP.

WARNINGS AND PRECAUTIONS

Although plasma derived reagents, standards and samples are usually screened for viral contamination, no guarantee of safety can be given. Therefore plasma and plasma-derived substances are to be treated as potentially hazardous. Precautions to be taken:

- Personal Protective Equipment (PPE) is to be worn
- Spills are to be decontaminated with 0.5% bleach (allow 10 minutes contact time)
- Plastic tips, tubes and clean-up materials are to be disposed of via biohazard bins
- Re-usable glassware is to be decontaminated by autoclaving as per MPSU procedures

For detailed safety information refer to:

- TGA Laboratory Safety Guidelines are located on the intranet
- Risk Assessments on haematology assays located with unique identifier 12162532004:

@ http://tgalqs/riskassess/index.htm

References:

1. The method is based on Method C from 2.7.13 – Assay of Human Anti-D Immunoglobulin version 7.0 of the European Pharmacopeia. 01/2008:20713.
2. CombiStats is a computer program for the statistical analysis of data from biological dilution assays or potency assays. It can perform calculations according to Chapter 5.3 of the European Pharmacopoeia (5th Edition 01/2005, 6th Edition 01/2008 and 7th Edition 01/2011) including Parallel Line Analysis.

Method:

Materials/Equipment

- BDFACS Canto II, LIMS# 30004
- Thermo Scientific Heraues Megafuge 1.0, LIMS # 101406, rotor #2704

Reagents

- PBS (e.g. SIGMA tablets Cat#P4417-50 Tab – 1 tablet dissolved in 200ml of ddH_2O yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.)
- PBS / 1 % BSA (Bovine Serum Albumin) – 1g BSA in 100ml of PBS
- CSL: Abtectcell III 3% – O R_1R_1 and O rr Red Blood Cells
- Invitrogen: Goat F(ab)'2 anti-human IgG (gamma) – Alexa Fluor 488.

- 96 well plates, flat bottomed
- Spherotech - 6 peak Sphero™ Rainbow calibration particles, 3.0µm, Cat# QRCP-30-5

Standards

2nd International Standard: Anti-D

NIBSC – Cat# 01/572

- Assigned potency = 285 IU/vial.
- Stored at -20^{0}C.
- Bring the lyophilised standard to room temperature before reconstitution.
- Reconstitute the contents of each vial with 1.0 mL of ddH_2O. Swirl gently.
- IS can be aliquoted (≥ 100µl) and stored at -80°C for 18 months. Aliquot can be used once only.

Procedure:

Reference Solutions:

Using the Anti-D worksheet, prepare 3 independent replicates of the 2nd IS (285 IU/ml) of at least 4 serial two-fold dilutions starting with a concentration of 0.5 IU/ml, using PBS-BSA as a diluent.

Test solutions:

Using the Anti-D worksheet, prepare 3 independent replicates of the samples of at least 4 serial two-fold dilutions starting with a concentration of 0.5 IU/ml, using PBS-BSA as a diluent.

Red Blood Cells (RBC)

- Prepare an appropriate volume of CSL- Abtectcell III 'R1R1' and 'rr' cells at 1-5 x 10^7 cells/ mL (1:10 dilution of 3% cells)
- Distribute 50 µl of cells into a flat bottomed microtitre plate

Assay

- Add 50 µl of each 'sample' or 'standard' to the RBC as described in the pre-designed grid (for example fill in plate layout - Trim record- R13/672455)

Negative controls

- Use 50 µL of PBS-BSA as negative control on R1R1 cells
- Distribute 50 µl of rr (D negative) cells into 2 wells, add 50 µl of the 0.5 IU/ml sample

Incubations and washes

- Add 50 µl of RBC + 50 µl standard/sample into 96 well plate
- Seal plate with plastic and incubate at 37°C for 40 mins

Primary Incubation

- Spin at 300 (rcf) x 3 mins, flick gently into container lined with absorbent towel, take care of bubbles in wells
- Wash with 200 µl PBS-BSA (mix to resuspend pellets)

1st wash

- Spin at 300 (rcf) x 3 mins, flick into container
- Wash with 200 µl PBS-BSA (mix to resuspend pellets)
- Spin at 300 (rcf) x 3 mins, flick into container

2nd wash

- Add 50 µl of secondary antibody, diluted 1:100 in PBS-BSA (Goat F(ab) anti-human IgG Alexa Fluor 488)
- Seal plate with plastic and incubate at room temp., 30 mins, dark

Secondary Incubation

- Spin at 300 (rcf) x 3 mins, flick into container
- Wash with 200 µl PBS-BSA (mix to resuspend pellets)

1st wash

- Spin at 300 (rcf) x 3 mins, flick into container
- Wash with 200 µl PBS-BSA (mix to resuspend pellets)
- Spin at 300 (rcf) x 3 mins, flick into container

2nd wash

- Resuspend with 200µl of **PBS**

Analysis on Flow cytometer

- Aliquot 800 µl **PBS** into 5ml tubes, and add the 200 µl of resuspended cells from the microtitre plate
- Prepare 2x 5ml tubes of 6 peak particles – one for the start of the assay and one for the end (add 3 drops of particles to 1ml of **PBS**)
- Open the Anti-D folder in the cytometer. Set up assay by 'duplicating without data' then rename as necessary. Note: if more than 1 carousel is required ensure the second carousel is stored in the dark until loaded onto the cytometer.
- Acquire data from one of the 6 peak tubes to adjust the gates so that the main populations fall within each of the gates. Do not adjust again once the assay is started.
- Measure 10000 gated cells on flow cytometer within 1-2 hours; ensure mixing prior to reading and every 3rd tube.
- Measure median fluorescence of gated cells for FITC-A

Statistical Analysis

Statistical Package:	*Combistats Version 5.0 (EDQM, Council of Europe)*
Model:	*Parallel lines*
Design:	*Completely randomised*
Transformation:	*y' = log(y)*
Variance:	*Observed residuals*
Dose ratio:	*2.0*

- Perform batch analysis on the 'specimen' and export the CMV file-:
- Right Click on relevant 'specimen' select "Batch Analysis"
- In "Batch Analysis Window" – Auto – check "statistics" and "free bioexponential scales" and select "Start"
- In "Specify Export File Name" window –CSV to removable disk (USB) and type appropriate filename eg Rhophylac 1409003621 30Oct2014 then select 'Save'
- "Batch Analysis Complete"
- "OK"
- safely remove USB
- On a networked computer open the CMV file in Excel, and organise the data into dose and replicate tables
- Save as an 'excel workbook' file into the relevant TRIM testing file
- Record values for P3 to P8 of the 6 peak particles onto the worksheet and calculate system suitability
- Open a "supercopy" of the Anti-D CombiStats template (trim R11/432567)
    - Right click on the document in TRIM and open a "supercopy"
    - Re-name and Save to the I-drive whist you are working on the statistics
    - Open the re-named "supercopy" template from within the Combistats Program. Transfer into TRIM once you have finished the statistical analysis
- Enter or copy/paste median FITC-A values from Excel into appropriate tables in Combistats
- Check ANOVA is valid for each individual test
    - See worksheet for more details on statistical analysis
- Where necessary, you may exclude the highest or lowest dose if the data range does not fall within the linear part of the curve
- Record Anti-D potency in LIMS
- File electronic and paper data in the appropriate files