Montreal Neurological Institute and Hospital
McGill University

CBIG-02-009

PREPARATION AND TESTING OF HUMAN AB SERUM FOR SUBSEQUENT STORAGE AND USE

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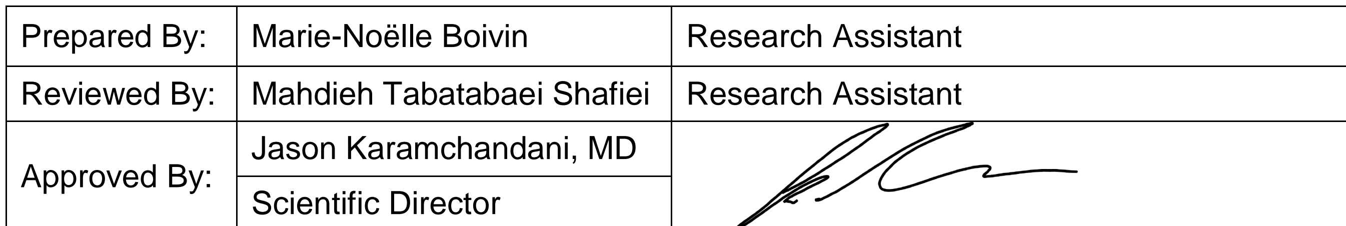

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1. REVISION HISTORY

Version	Summary of revisions	Effective Date
1.0	Initial	18-Jun-2020

2. SCOPE AND APPLICATION

This protocol is to be used for the preparation and testing of Human AB serum used to freeze PBMC.

3. REFERENCE TO OTHER SOP OR DOCUMENTS

When adopting this SOP for local use, please reference *C-BIG Repository: CBIG-02-009 Preparation and Testing of Human AB Serum for Subsequent Storage and Use*.

3.1 Reference to Other C-BIG SOPs or Documents

1. C-BIG Repository: CBIG-02-001 PBMC Isolation from Whole Blood (Conventional Method)
2. C-BIG Repository: CBIG-02-002 PBMC Isolation from Whole Blood (Leucosep Method)
3. C-BIG Repository: CBIG-02-010 Cell Thawing
4. C-BIG Repository: CBIG-02-015 Antibody Staining for Flow Cytometry
5. C-BIG Repository: CBIG-03-001 Different Methods to Count Cells
6. C-BIG Repository: CBIG-03-002 RPM Conversion

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3.2 Reference to External SOPs or Documents

1. Experimental Therapeutic Program: ETP-P-0022 Preparation and Testing of Human AB Serum for Subsequent Storage and Use

4. PERSONNEL QUALIFICATION AND RESPONSIBILITIES

To be read by all personnel who prepare and test Human AB serum. All personnel who read this SOP should sign the form found in the reading log binder.

5. ABBREVIATIONS AND DEFINITIONS

Abbreviation	Definition
C-BIG	Clinical Biological Imaging and Genetic Repository
CFSE	Carboxyfluorescein Succinimidyl Ester
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
HI	Heat Inactivated
HuAB serum	Human AB Serum
mg	Milligram
Min	Minutes
mL	Milliliters
mM	Millimolar
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
QA	Quality Assurance

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QC	Quality Control
RT	Room Temperature
SOP	Standard Operating Procedure
µg	Microgram
µL	Microliters
°C	Degree Celsius
%	Percent

6. MATERIALS AND EQUIPMENT

The materials and equipment listed below are recommendations only and may be substituted by alternative/equivalent products more suitable for site-specific task or procedure.

NOTE: All disposable items are sterile (gamma-irradiated) unless otherwise specified. All equipment, disposables or reagents can be substitutes with equivalent materials following evaluations and approval, unless specified otherwise.

NOTE: All sample contact materials must be suitable for RNA work (i.e., RNAse-free). Use clean gloves at all times to prevent inadvertent RNAse contamination during processing. All equipment, disposables or reagents can be substitutes with equivalent materials following evaluation and approval, unless specified otherwise.

Material/Equipment	Material/Equipment

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	(site specific)
Disposable serological pipets, 5 mL	
Disposable serological pipets, 10 mL	
Disposable serological pipets, 25 mL	
Disposable transfer pipets	
Polypropylene centrifuge tube	Globe Scientific; Cat # 6321
Refrigerated ultracentrifuge	Beckman Coulter; Avanti J-E series; Cat # 369001
50 mL polypropylene conical tube	
15 mL polypropylene conical tube	
Parafilm	
Water-bath	
Filtration System	Millipore Sigma Stericup; Cat # S2GVU05RE
Human Serum	Human Serum from human male AB plasma, Sigma; Cat # H4522
Pipet tips, 20 μL	
Pipet tips, 200	
Pipet tips, 1000 μL	
Pipet-aid	
Class II biological safety cabinet	
Micropipets, range 100 to 1000 μL	
Centrifuge with swinging bucket	Eppendorf centrifuge 5810, Cat # 022625004; with rotor S-4-104, Cat # 5820759003
Phosphate buffered saline (1X), pH 7.4, CA^{2+} and Mg^{2+} free	Wisent; Cat # 311-010-CL
DMSO	Fisher Scientific; Cat # D1391
X-Vivo 15 with gentamicin, phenol red from	Lonza; Cat # 04-418Q

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24 well plate	
CFSE	Thermofisher Scientific; Cat # C34570
RPMI1640 medium	Thermofisher Scientific; Cat # 11875101
Fetal Bovine Serum	Thermofisher Scientific; Cat # 16000036
CD3 Monoclonal antibody (OKT3)	Thermofisher Scientific; Cat # 16-0037-85
Incubator	
96 well v-shape plate	Corning; Cat # 3897
Live/Dead fixable aqua dead cell stain kit	Thermofisher Scientific; Cat # L34965
APC mouse anti-human CD3	BD Pharmingen; Cat # 561810
PE mouse anti-human CD4	BD Pharmingen; Cat # 561843
BV421 mouse anti-human CD8	BD Pharmingen; Cat # 562429
CompBead anti-mouse Ig	BD Pharmingen; Cat # 552843
Arc Amine reactive compensation bead kit	Thermofisher Scientific; Cat # A10628
FACS tube	
Flow cytometer	
-20°C Freezer	

7. PROCEDURES

PRECAUTIONS: All biological samples derived from human source are considered to be biohazardous. Use appropriate precautions when working with such samples (i.e. personal protection equipment such as gloves, lab coat and safety glasses). All waste

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(samples and related contact materials) must be placed in marked biohazardous waste containers and disposed of under hospital guidelines.

NOTE: All subsequent manipulations to be performed under aseptic conditions.

7.1 Heat inactivation and preparation of human AB serum

1. Thaw HuAB serum at 4°C overnight for next day heat inactivation or at RT for same day heat inactivation.
2. Aseptically, transfer HuAB serum into several 35 mL polypropylene centrifuge tubes.
3. Centrifuge HuAB serum at 30,000Xg for 30 min at 4°C in the ultracentrifuge with no break.
4. Transfer tubes on ice.
5. Carefully transfer HuAB serum in 50 ml conical tubes with transfer pipette while trying to avoid aspirating fat layer.
6. Seal caps with parafilm.
7. Heat inactivate HuAB serum at 56°C for at least 30 min in a water bath, excluding the time required for the HuAB serum to reach the desired temperature (approximately 5 min).
8. Allow HI HuAB serum to cool down and return to RT.
9. Filter-sterilize HI HuAB serum with filter units.

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10. Aliquot the sterilized HI HuAB serum in 15 mL polypropylene conical tubes making sure the tubes are not filled up to the top to leave room for expansion of serum upon freezing.
11. Store at -20°C freezer for a maximum of one (1) year.

NOTE: Once a HI HuAB serum aliquot is thawed, it can be kept at 4°C for a maximum of one (1) week. Once thawed, an aliquot should not be frozen again.

7.2 Testing of human AB serum

1. Isolate PBMC from whole blood. Refer to SOP CBIG-02-001 *PBMC Isolation from Whole Blood (Conventional Method)* or to SOP CBIG-02-002 *PBMC Isolation from Whole Blood (Leucosep Method).*
2. After the cell count, split the PBMC into two (2) 50 mL conical tubes and prepare to freeze down.
3. The cells from one (1) 50 mL conical tube will be frozen in the control HI HuAB serum and the others will be frozen in the HI HuAB lot to be tested.

NOTE: Leave the PBMC in liquid nitrogen for at least one (1) week before continuing the protocol.

4. Thaw the PBMC according to SOP CBIG-02-010 *Cell Thawing*.
5. In a 50 mL conical tube, re-suspend the PBMC into 9 mL of X-Vivo medium and centrifuge at 310Xg for 12 minutes.

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6. Discard the supernatant and re-suspend the cells into 10 mL of X-Vivo medium. Count cells according to the SOP CBIG-03-001 *Different Methods to Count Cells*.
7. Centrifuge at 310Xg for 12 minutes and discard supernatant.
8. Make the cell suspension at $5X10^6$ cells/mL with pre-warmed X-Vivo medium.

<u>NOTE:</u> Keep at least one (1) million cells on the side for control purposes i.e. cells without CFSE staining. At least X number on the side.

9. Prepare the CSFE working solution, see below:

Add 18 µL of DMSO to the CSFE stock solution to make a 5 mM stock solution. Dilute five (5) times the stock solution with PBS to make a working solution of 1 mM.

10. Add 1 µL of CFSE working solution to 1 mL of cell suspension, 2 µL of CFSE working solution to 2 mL of cell suspension and so on.
11. Incubate the cell suspension and CFSE at 37°C for 10 min in the dark and keep shaking gently intermittently.
12. Add RPMI1640 with 5% FCS up to 50 mL, centrifuge at 310Xg for 12 minutes and discard the supernatant.
13. Re-suspend the cells with 10 mL of X-Vivo medium and count according the SOP CBIG-03-001 *Different Methods to Count Cells*.
14. Centrifuge at 310Xg for 12 min.

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15. Discard the supernatant and re-suspend the cells with X-Vivo medium to make a cell suspension at $1X10^6$ cells/mL.
16. Plate cells into 24-well plate, add 1 mL of cell suspension per well, plus 0.5 mL of X-Vivo medium containing anti-CD3, for a final volume of 1.5 mL per well, as described below:

Old lot HuAB serum	New lot HuAB serum	Control
Nil	Nil	No CSFE and no anti-CD3
Low anti-CD3 concentration	Low anti-CD3 concentration	CFSE and no anti-CD3
High anti-CD3 concentration	High anti-CD3 concentration	CSFE and low anti-CD3 concentration
-	-	CFSE and high anti-CD3 concentration

NOTE: The usual final concentration of anti-CD3 used is 1 µg/mL, for the low concentration, use anti-CD3 at 0.3 µg/mL and for the high concentration use anti-CD3 at 0.8 µg/mL. For the preparation of anti-CD3 see below:

For high concentration of 0.8 µg/mL:

Stock at 1 mg/mL=1000 µg/mL: dilute 1:100 (10 µl of anti-CD3 + 990 µl X-Vivo) = concentration 10 µg/mL

$C_1V_1=C_2V_2$

10 µg/mL*V_1=0.8 µg/mL *1.5mL

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V_1=0.12 mL= 120 µL Add 1 mL of cell suspension + 120 µL of anti-CD3 (1:100) + 380 µL of X-Vivo medium
For low concentration of 0.3 µg/mL: Stock at 1 mg/mL=1000 µg/mL: dilute 1:1000 (1 µl of anti-CD3 + 999 µl X-Vivo) = concentration 1 µg/mL $C_1V_1=C_2V_2$ 1 µg/mL*V_1=0.3 µg/mL *1.5mL V_1=0.45 mL= 450 µL Add 1 mL of cell suspension + 450 µL of anti-CD3 (1:1000) + 50 µL of X-Vivo medium

17. Incubate the plate at 37°C with 5% CO_2 for three (3) to five (5) days or until clumps are formed.

NOTE: If a longer incubation than 3 days is needed, change the medium by replacing 0.5 mL of the in culture medium with 0.5 mL of new X-Vivo medium.

18. Stain the cells with Live/dead, CD3, CD4 and CD8. See section 7.3 for more details.

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7.3 Flow cytometry staining

1. Transfer cells into a 15 ml conical tube (1 well per conical tube).
2. Centrifuge at 650Xg for 5 min.
3. Discard the supernatant and add 300 µl of PBS.
4. Transfer cells with a pipette into a 96 well v-shaped plate.
5. Centrifuge the plate at 650Xg for 5 min.
6. Discard supernatant by inverting it over a container and re-suspend in 300 µL of PBS by pipetting up and slowly and gently.
7. Centrifuge at 650Xg for 5 min.
8. Discard supernatant and re-suspend in 100 µL of PBS and add 1 µL of live dead aqua stain and mix (if too many samples, do a master mix; for example, for 10 wells, prepare a mix for 12 wells – 12 µL of live dead stain with 1200 µL of PBS and distribute 100 µL in all the wells). To reconstitute the live dead stain, see below:

1. Thaw the DMSO (component b) that came with the kit. 2. In the BSC, in the dark, add 50 µL of DMSO to the vial containing the stain (component a). 3. Mix well and vortex. 4. Store in the same container that it came in at -20˚C in the dark and away from moisture. The live dead stain is good up to two (2) weeks when it is reconstituted.

9. Incubate for 30 min in the dark at RT.
10. Wash with 150 µL of PBS and centrifuge at 650Xg for 5 minutes.

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11. Discard supernatant and perform the surface stain in PBS with the following antibodies CD3 APC, CD4 PE and CD8 BV421.For further information see SOP CBIG-02-015 *Antibody Staining for Flow Cytometry*.
12. Incubate for 20 to 30 min at RT in the dark.
13. Wash with 150 µL of PBS and centrifuge at 650Xg for 5 min.
14. Discard supernatant and re-suspend in 200 µL of FACS buffer (500 mL of PBS + 5 mL of FBS).
15. Transfer the samples into FACS tubes.
16. Pass the sample on the cytometer.

NOTE: All the fluorochrome for the stain used here are light sensitive and can be changed for another fluorochrome.

NOTE: Don't forget to do the compensation beads for surface stain and Live Dead stain.

NOTE: To select a new lot number for HuAB serum, the results after flow cytometry should be similar to the current lot number results.

8. QUALITY CONTROL / QUALITY ASSURANCE

All the equipment used should be monitored, cleaned and calibrated per their specific SOPs.

Reagents with an expiry date should be monitored and used before this date. If used after expiry date, it should be recorded.

9. APPENDICES/FORMS

NA