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HAI Assay

1. Principle

The basis of the HAI assay is that antibodies of influenza virus will prevent attachment of the virus to red blood cells. Therefore hemagglutination is inhibited when antibodies are present. The highest dilution of serum that prevents hemagglutination is called the HAI titer of the serum. If the serum contains no antibodies that react with the new H1N1 strain, then hemagglutination will be observed in all wells. Likewise, if antibodies to the virus are present, hemagglutination will not be observed until the antibodies are sufficiently diluted.

2. Materials and Equipment

2.1. Working solution of chicken red blood cells (cRBC)
2.2. Receptor Destorying Enzyme II (RDE)
2.3. Influenza virus with known HA titer
2.4. Phosphate Buffer Saline (PBS)
2.5. 0.9% saline/NaCl solution
2.6. ≥50uL per serum sample
2.7. V-bottom 96 well plates
2.8. 15ml and 50ml conical tubes
2.9. 1.5ml eppendorf tubes
2.10. 50uL per serum sample

3. Procedure

3.1 Preparing 10% cRBC Stock Solution

3.1.1 Place 5mL of cRBCs in 50mL conical tube
3.1.2 Centrifuge at 1200rpm for 10 minutes and 4°C
3.1.3 Aspirate supernatant and resuspend with 45mL PBS
3.1.4 Centrifuge at 1200rpm for 10 minutes and 4°C
3.1.5 Repeat 3.3 and 3.4 twice more
3.1.6 Resuspend with 12mL PBS and transfer to 15mL conical tube
3.1.7 Centrifuge at 1200rpm for 10 minutes and 4°C
3.1.8 Aspirate supernatant and resuspend with ~7.2mL of PBS to make a 10% stock solution of cRBs. Packed cRBs should be roughly 800uL
3.1.9 Store at 4°C until use

3.2 Preparing Receptor Destroying Enzyme II

3.2.1 Resuspend the lyophilized RDE with 20mL of 0.9% saline solution
3.2.2 Incubate on ice for 5 minutes or until fully dissolved
3.2.3 Aliquot 1mL in 1.5mL eppendorf tubes

3.2.4 Store in -80°C freezer until use

Note: Cannot grow through another freeze-thaw

3.3 Sample Preparation Day 1

3.3.1 Thaw serum samples and RDE in 37°C water bath
3.3.2 Transfer 50uL of each sample into a labeled 1.5mL microcentrifuge tube
3.3.3 Add 150uL of RDE to each tube, mix well
3.3.4 Place into the CO_2 incubator at 37°C for overnight incubation

3.4 Sample Preparation Day 2

3.4.1 Place samples into 56°C water bath for a 45 minute incubation
3.4.2 Prepare 0.5% cRBC working stock solution from 10% stock solution and virus concentrations, see appendix 4.1 – keep on ice
3.4.3 Remove samples from water bath and chill on ice
3.4.4 Immediately add 300uL of 0.9% NaCl solution to each sample, the final volume should be 500uL per sample
3.4.5 Allow samples to rest at room temperature for the remainder of the assay

3.5 HAI Assay

3.5.1 Label v-bottom 96 well plates accordingly – only 1 virus strain per plate, 8 samples per plate, and duplicate plates per sample
3.5.2 Add 25uL of PBS to each well from columns 1-10, with row A empty
3.5.3 Add 50uL of PBS to all of column 12
3.5.4 Aliquot 50uL of serum as in figure below in row A and in column 11 respectively

		1		2		3		4					
Serum		V1	V3	V1	V3	V1	V3	V1	V3	CONS2	VC	SC	PC
Dilution		1	2	3	4	5	6	7	8	9	10	11	12
1/10	A										8HA	1V1	
1/20	B										4HA	1V3	
1/40	C										2HA	2V1	
1/80	D										1HA	2V3	
1/160	E										0.5HA	3V1	
1/320	F										0.13HA	3V3	
1/640	G										0.06HA	4V1	
1/1280	H										0.03HA	4V3	

3.5.5 Add 75uL of virus working dilution to well A10 and serially dilute 25uL from A10 to H10, discard the last 25uL
3.5.6 Add 25uL of virus working dilutions to every well of columns 1-9
3.5.7 Incubate for 15 minutes at room temperature
3.5.8 Add 50uL 0.5% cRBC solution to every well of columns 1-12
3.5.9 Incubate for 1 hour at room temperature then read plates

4. Appendix

4.1 Virus Concentration and Preparation Guide

Use $TCID_{50}$ information to prepare the working virus solutions ($8HA_{50}$) as follows:

a. A/California 1/32
b. A/Perth 1/128
c. A/South Dakota 1/256
d. A/Uruguay 1/32
e. B/Brisbane 1/16

Note: 5 ml of the working virus solution is enough for 4 donors per strain, 2 plates total.

	Titer (8HA50)	uL vir. stock	uL PBS
A/California	1/32	375	111625
A/Perth	1/128	94	11906
A/ So. Dakota	1/256	47	11953
A/Uruguay	1/32	375	11625
B/ Brisbane	1/16	750	11250

4.2 Virus Control Plate

4.2.1 Use v-bottom 96 well plate and add 25uL of PBS to columns 1-6, leaving row A empty
4.2.2 Add 75uL of the working virus solution into the wells of row A columns 1-6 in duplicates
4.2.3 Serial dilute 25uL from row A (columns 1-6) down to row H. Discard the last 25uL
4.2.4 Add 50uL of 0.5% working cRBS solution into each well
4.2.5 Incubate at room temperature for 1 hour
4.2.6 Read plates – the last well with hemagglutination should correspond to 1 HA unit

		A/CALI		A/PERTH		B/BRIS							
Conc.		1	2	3	4	5	6	7	8	9	10	11	12
8HA	A	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
4HA	B	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
2HA	C	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
1HA	D	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
1/2HA	E	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
1/4HA	F	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
1/8HA	G	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---
1/16HA	H	CA	CA	Per	Per	Bris	Bris	---	---	---	---	---	---

4.3 Reading Plates

- "+" – hemagglutination is present, the well is hazy with no cRBC button
- "-" – hemagglutination is absent, the well is relatively clear with cRBC button
- HAI titer is the reciprocal of dilution of last well that inhibits hemagglutination

4.4 Hemagglutinin Assay

Used to quantify HA titer of influenza antigen or virus

4.4.1 Use v-bottom 96 well plate and add 50uL of PBS in columns 2-12
4.4.2 Aliquot 100uL of virus samples in first column
4.4.3 Serially dilute 50uL across plate for 1:2 dilutions, discard last 50uL
4.4.4 Add 50uL of 0.5% cRBCs to all wells
4.4.5 Incubate at room temperature for 1 hour, then read plates

4.5 Troubleshooting

If non-specific hemagglutinins are present within the serum, they need to be absorbed. After step 3.4.1 mix the serum (10:1 ratio) with a stock solution of packed chicken red blood cells. Incubate for 1 hour at 4°C. Centrifuge at 1200rpm for 10 minutes and remove the supernatant and continue with the protocol.