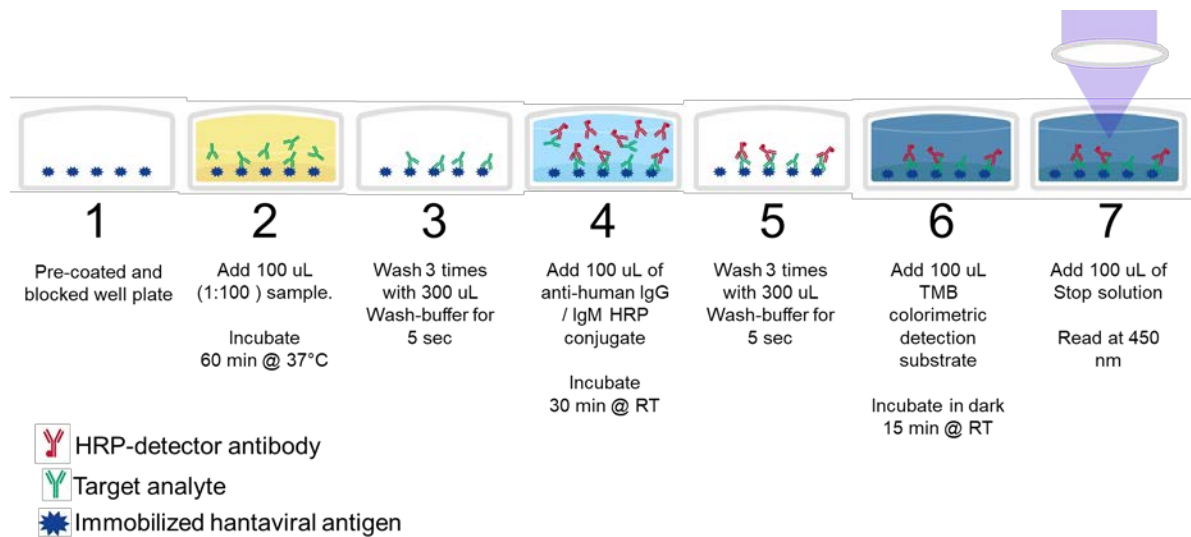# Human anti-hantavirus antibody detection through ELISA

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This protocol describes the technique for detecting human anti-hantavirus antibodies from serum or plasma samples using the Aviva Systems Biology® Hantavirus IgM/IgG ELISA kit (OKNX00139 & OKNX00138, respectively). This kit employs a reverse capture sandwich enzyme-linked immunosorbent assay (ELISA) in which the hantaviral antigen has been fixed to each well. This antigen is recognized by IgG or IgM antibodies if present in the sample and these antibodies are in turn recognized by an antibody conjugated to horseradish peroxidase (HRP) specific for human IgG or IgM, generating a color change whose absorbance at 450 nm is qualitatively proportional to the amount of anti-Hantavirus antibody present in the sample.

1	2	3	4	5	6	7
Pre-coated and blocked well plate	Add 100 uL (1:100) sample. Incubate 60 min @ 37°C	Wash 3 times with 300 uL Wash-buffer for 5 sec	Add 100 uL of anti-human IgG / IgM HRP conjugate Incubate 30 min @ RT	Wash 3 times with 300 uL Wash-buffer for 5 sec	Add 100 uL TMB colorimetric detection substrate Incubate in dark 15 min @ RT	Add 100 uL of Stop solution Read at 450 nm

Y HRP-detector antibody
Y Target analyte
✳ Immobilized hantaviral antigen

Adapted from Aviva Systems Biology® hantavirus ELISA kit manual

The detection of viral particles, antibodies against hantavirus or vRNA is essential to confirm hantaviral infection. The diagnostic gold standard for hantaviral infections is the Plaque Reduction Neutralization Test (PRNT), which requires BSL-3 facilities as it involves live viruses. Nucleic acid based molecular techniques provide sensitivity and specificity comparable to or superior to PRNT (2,3). For clinical and epidemiological applications, the anti-hantaviral IgG or IgM detection assay by ELISA is preferred (1). Direct capture ELISAs are more specific than indirect ELISA assays, than traditional direct immunofluorescence (IFD) and indirect immunofluorescence assays (IFA), or Western Blots (4). IgM levels in hantaviral infections rise

from day 5 post-infection, reach their maximum around day 10 and continue to be detectable until the 5th month. IgG levels begin to rise from day 5, reach their maximum on day 40 and usually remain elevated for over 10 years (4). The immune response to hantaviral infections is not virus-specific, this leads inevitably to cross-reactions. This kit has a sensitivity of $> 95.9\%$ and a specificity of 98.3%.

Required materials and instruments

1. Microplate reader with the capacity t oread absorbance at 450 nm.
2. P2, P10, P100 y P1000 micropipettes.
3. P2, P10, P100 y P1000 micropipette tips.
4. 0.6 ml microtubes.
5. One 1.5 ml microtube per sample.
6. A 50 ml conical tube (if < 20 samples) or 100 ml flask (if ≥ 21 samples) for 1x Wash Buffer.
7. Deionized, injection or ultrapure water.
8. Bacteriological incubator with humidifier set at 37°C.
9. Self-adhesive film included in the Aviva® kit.
10. Plastic tray used for Gram staining with 0.1% NaOCl.
11. Paper towels.

Required reagents

1. Aviva® Yellow IgG Sample Diluent. Be careful, don't confuse with that of IgM!
2. Aviva® Green IgM Sample Diluent. Be careful, don't confuse with that of IgG!
3. Aviva Stop Solution Aviva®.
4. Aviva® Blue Anti-Human IgG HRP Conjugate. Be careful, don't confuse with that of IgM!
5. Aviva® Pink Anti-Human IgM HRP Conjugate. Be careful, don't confuse with that of IgG!
6. Aviva® TMB (Tetramethylbenzidine) Substrate.
7. Aviva® IgG Cut-off Control (CC).
8. Aviva® IgG Positive Control (PC).
9. Aviva® IgG Negative Control (NC).
10. Aviva® IgM Cut-off Control (CC).
11. Aviva® IgM Positive Control (PC).

12. Aviva® IgM Negative Control (NC).

13. Aviva® Wash Buffer 20X.

Preparations

1. Turn the bacteriological incubator on and set it to 37°C and place a pipette tip box-top with water (humidity tray) and benzalkonium chloride inside (to prevent proliferation of microorganisms in water).

2. Label and add 990 µL of the corresponding sample diluent to a 1.5 mL microtube for each sample to be processed.

   NOTE: The sample diluent is specific for the IgG or IgM kit.

3. Prepare the required amount of 1X Wash Buffer from the 20X stock (see table below).

Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Number of PC wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of NC wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of dH$_2$0 wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of CC wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of sample wells	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44
Final 1x buffer needed (ml)	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52
Required 20x buffer (ml)	0.5	0.6	0.7	0.8	0.9	1	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2	2.1	2.2	2.3	2.4	2.5	2.6
top up with dH$_2$0 (ml)	9.5	11	13	15	17	19	21	23	25	27	29	30	32	34	36	38	40	42	44	46	48	49

Sample number	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
Number of PC wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of NC wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of dH$_2$0 wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of CC wells	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Number of sample wells	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	86	88
Final 1x buffer needed (ml)	54	56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	86	88	90	92	94	96
Required 20x buffer (ml)	2.7	2.8	2.9	3	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	4	4.1	4.2	4.3	4.4	4.5	4.6	4.7	4.8
top up with dH$_2$0 (ml)	51	53	55	57	59	61	63	65	67	68	70	72	74	76	78	80	82	84	86	87	89	91

4. Collect venous blood into two 6 ml vacutainer® tubes with red top (without anticoagulant to recover serum) or purple top (anticoagulated with EDTA or heparin to recover plasma) from the subjects of interest (must be humans, not other animals).

5. For red cap tubes, stand the sample vertically for 2 hours at room temperature or 6 hours at 4°C. Centrifuge at 1000 g for 15 minutes.

6. For violet cap tubes, centrifuge at 1000 g for 15 minutes at 2-8°C within the first 30 minutes of

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sample collection.

7. Working within the confines of the biological safety cabinet located in the Cell Biology area, recover the serum or plasma and distribute 500 µL aliquots into 0.6 ml microtubes.

8. Prepare a 1:100 dilution of each sample to be processed by adding 10 µL of serum or plasma to the 1.5 mL microtube previously labelled and loaded with 990 µL of sample diluent, mix using vortex for 1 second.

NOTE: IgG and IgM conjugates should only be used with the diluents corresponding to each kit, do not confuse them.

9. Process the ELISA immediately. If the ELISA will be performed within the next 24 hours, refrigerate the sample at 4°C, store at -20°C for a few weeks or at -80°C for months or years.

10. Avoid multiple freeze-thaw cycles.

ELISA procedure

BIOSAFETY: These steps must be carried out within the biological safety cabinet located in the cell biology area (BSL-2) of the Viral and Human Genomics Laboratory.

11. Incubate reagents and materials (except standards) at 37°C for 5 minutes before starting.

12. Determine the number of wells required, cut the number of tubes needed from the plate, and store the remaining plate in the metalic bag and keep refrigerated at 4°C.

    Number of wells = (8 wells for controls + 2 for each sample) see previous table

13. Add 100 µL of NC, CC, 1:100 dilution of each sample, and PC to duplicate wells of the plate in this exact order.

14. Cover the wells of the plate with Aviva® adhesive film and incubate at 37°C for 60 minutes in the bacteriological incubator equipped with a humidity tray.

15. Remove the adhesive film and pour the contents of the wells into a plastic tray with 0.1% NaOCl.

16. Remove excess liquid from the plate by inverting and blotting it onto clean paper towels. Do not allow the plate wells to dry completely.

17. Rinse the plate by adding 300 µL of 1x wash buffer to each well. Incubate for 5 seconds and then pour the contents onto a plastic tray with 0.1% NaOCl. Remove excess liquid from the plate by inverting and blotting it onto clean paper towels. Do not allow the plate wells to dry completely.

18. Repeat the rinse two more times for a total of 3 rinses.

19. Add 100 µL of Aviva® anti-human IgG HRP conjugate (blue color) or Aviva® anti-human IgM HRP conjugate (pink color), as appropriate for the assay to be performed.

NOTE: IgG and IgM conjugates should only be used with the diluents corresponding to each kit, do not confuse them.

20. Cover the plate wells with Aviva® adhesive film and incubate at room temperature for 30 minutes.

21. Remove the adhesive film and pour the contents of the wells into a plastic tray with 0.1% NaOCl.

22. Remove excess liquid from the plate by inverting and blotting it onto clean paper towels. Do not allow the plate wells to dry completely.

23. Rinse the plate by adding 300 µL of 1x wash buffer to each well. Incubate for 5 seconds and then pour the contents onto the plastic tray with 0.1% NaOCl. Remove excess liquid from the plate by inverting and blotting it onto clean paper towels. Do not allow the plate wells to dry completely.

24. Repeat the rinse two more times for a total of 3 rinses.

25. Darken the work area and add 100 µL of TMB substrate to each well and incubate at room temperature for 15 minutes inside the metal Zip-Lok® bag.

26. Add 100 µL of the stop solution to each well in the same order as the previous step.

27. Read the absorbance of the wells at 450nm with a standard microplate reader within 30 minutes after adding the stop solution. If possible, use wavelength correction at 620 nm.

Assay validation

1. For the assay to be considered valid, the following criteria must be met:

   a. The average absorbance of the water blank (Substrate blank) should be < 0.100
   b. The average absorbance of the NC must be < 0.200 and < CC.
   c. The average absorbance of the CC should be between 0.150 and 1.30.
   d. The average absorbance of PC should be > CC.
   e. If ALL these criteria are not met, repeat the test.

2. Average the absorbances (Abs) of the replicates of each sample.

3. Transform the average absorbance into nephelometric turbidity units (NTUs) using simple rule of three where $NTU^{Sample} = (Abs^{Sample} \times 10) / Abs^{CC}$.

4. Based on the NTU calculated for each sample, assign the test result according to the ranges described in the following table.

Result interpretation

CC	10 NTU	
Positive	> 11 NTU	Antibodies against the pathogen present. The individual has had contact with the pathogen or its vaccine.
Equivocal	9 a 11 NTU	Repeat test. If it remains inconclusive, take sample 2 to 4 weeks after the original and reassess.
Negative	< 9 NTU	There are no antibodies against the pathogen. The individual has not had contact with the pathogen or its vaccine.

Notes

1. If working with frozen samples (stored at -20ºC), they should be thawed gradually by moving them to a refrigerator between 4 and 8ºC for 24 hours before performing the ELISA test.

2. Store all reagents at 4°C for no more than 6 months.

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5. Hantavirus IgM / IgG ELISA Kit (Human) (OKNX00138 & OKNX00139) Aviva Systems Biology

Revision history

1.0 Original document.

2.0 Protocol modified.

3.0 Protocol layout modified.

4.0 Protocol modified to better comply with manufacturer's instructions.

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