
# A Comparative Examination of Influenza Haemagglutination-Inhibition Assay Protocols - Development of a Consensus HI Protocol

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# Plan for HI comparison

- HI assay protocols differ worldwide. It is not known how much these differences affect between-laboratory variability
- Differences occur in use of materials, determination and expression of HI assay endpoint
- At Hong Kong CONSISE meeting
  - Planned to share and compare laboratory protocols for HI assays within CONSISE laboratories.
  - Planned to develop a consensus HI protocol that could be evaluated in a collaborative study

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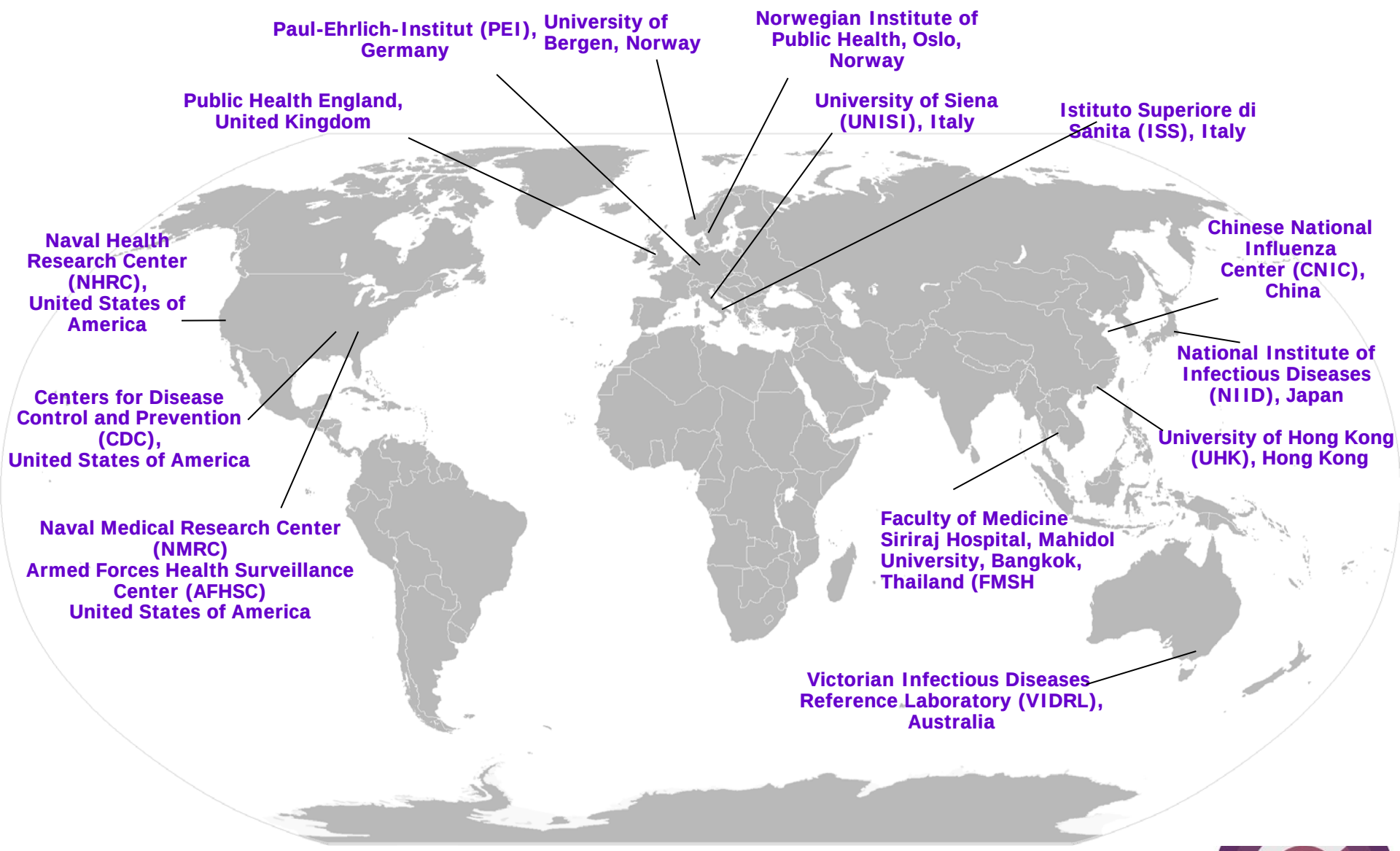
**Fourteen laboratories took part**

# Haemagglutination-Inhibition Assay Comparison – participating laboratories



# HI Assay Protocol Comparison

| Parameter or variable | | Most frequent variables used (# of laboratories) | Alternate variables (# of laboratories) |
|---|---|---|---|
| **Red blood cell preparation** | RBC species used | Turkey (H1N1, H1N1pdm09, B, some H3N2( (12),<br>Guinea pig (some H3N2, H1N1) (9) | Chicken (3), goose (1) |
| | RBC species available | Turkey (13), guinea pig (10) | Chicken (9), horse (6), human O (5), goose (2), pig (1), duck (1)<br>One lab cannot access turkey |
| | RBC washing | Resuspension in PBS + two washes in PBS (8) | Use of NaCl (3), only one wash (1), three washes (2), wash until no lysed cells (1) |
| | Resuspension buffer | PBS (10) | NaCl (2), PBS+BSA (1), Dulbecco PBS (1) |
| | Method for RBC cell estimation | Packed cells estimated by eye (6) | Use of haematocrit (4), haemocytometer (3), UV absorbance at 541 nm (1) |
| | Final RBC concentration in assay | Turkey 0.5% (6, chicken 1% (1), guinea pig 1% (3), guinea pig 0.75% (3) | Turkey 1% (3), turkey 0.7% (1), turkey 0.35%, turkey 0.25% (1) |
| | Final RBC concentration in assay well (adjusted for vols of all reagents in well) | Turkey 0.25% (5), chicken 0.25% (2), human O 0.23% (1), human O (0.375% (1), goose 0.25% (1), guinea pig 0.374% (4) | Turkey from 0.124-0.5% (7), chicken 0.5% (1), chicken 0.175% (1), guinea pig from 0.167-0,5% (4) |

# HI Assay Protocol Comparison
# - WHO manual in red*

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| | RBC species available | Turkey (13), guinea pig (10) | Chicken (9), horse (6), human O (5), goose (2), pig (1), duck (1) One lab cannot access turkey |
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*WHO Global Influenza Surveillance Network. Manual for the laboratory diagnosis and virological surveillance of influenza.WHO 2011.*
*http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf pages 43-59*

# HI Assay Protocol Comparison - continued

| Parameter or variable | | Most frequent variables used (# of laboratories) | Alternate variables (# of laboratories) |
|---|---|---|---|
| **Serum preparation** | *V. Cholerae* Receptor Destroying Enzyme | Supplied by Denka Seiken C Ltd (9) - No evaluation of activity by recipient lab | WHO flu reagent kit (1), Sigma Aldrich (4), local lab produced (1) |
| | RDE:serum ratio for serum treatment | 3 vols RDE to 1 vol serum (8) | 4 vols RDE (5), 5 vols RDE (1) |
| | Incubation conditions | 37°C for 16-20 h (14) | No variables |
| | RDE inactivation | 56°C for 30 min, allow to cool and add 6 vols PBS or 0.85% NaCl (4) | Use of sodium citrate (4), different time (2), different vols (5) |
| | Final serum dilution | 1:10 (13) | 1:4 (1) |
| | Detection of non-specific agglutinins in treated serum | Performed before assay (7) | Performed in parallel (2), Not done (5) |
| | Treatment of serum to remove non-specific agglutinins | 20 vols RDE-treated serum with 1 vol packed RBC for 1 h at 4ºC (3) | Different temp. (3),different vol RDE (3), different time (1), different vol RBC (1), not done (5) |

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# HI Assay Protocol Comparison - continued

| Parameter or variable | | Most frequent variables used (# of laboratories) | Alternate variables (# of laboratories) |
|---|---|---|---|
| **Haemagglutination conditions** | Chicken RBC – concentration | 0.5% (3) | 1% (2), 0.35% (1) |
| | Chicken RBC - type of microtitre well | V (5) | U (1) |
| | Chicken RBC - incubation time at RT after RBC addition | 30 min (3) | 45 min (1), 60 min (1), 30 min at 4ºC |
| | Turkey RBC - concentration | 0.5% (6) | 1% (3), 0.7% (1), 0.25% (1), 0.35% (1) |
| | Turkey RBC - type of microtitre well | V (9) | U (3) |
| | Turkey RBC - incubation time at RT after RBC addition | 30 min (9) | 45 min 91), 30-60 min (1) |
| | Guinea pig RBC - concentration | O.75% (4) | 1% (2), 0.5% (1) |
| | Guinea pig RBC - type of microtitre well | U (5) | V (2) |
| | Guinea pig RBC - incubation time at RT after RBC addition | 60 min (5) | 45 min (1), 60 min at 4ºC |
| | Human type O RBC - concentration | 0.7% (1), 0.75% (1), 1%( 1), 1% in 0.5%BSA/PBS (1) | |
| | Human type O RBC - type of microtitre well | V (2), U (2) | |
| | Human type O RBC - - incubation time at RT after RBC addition | 60 min (4) | No variables |

# HI Assay Protocol Comparison - continued

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| | Chicken RBC - type of microtitre well | V (5) | U (1) |
| | Chicken RBC - incubation time at RT after RBC addition | 30 min (3) | 45 min (1), 60 min (1), 30 min at 4ºC |
| | Turkey RBC - concentration | 0.5% (6) | 1% (3), 0.7% (1), 0.25% (1), 0.35% (1) |
| | Turkey RBC - type of microtitre well | V (9) | U (3) |
| | Turkey RBC - incubation time at RT after RBC addition | 30 min (9) | 45 min 91), 30-60 min (1) |
| | Guinea pig RBC - concentration | O.75% (4) | 1% (2), 0.5% (1) |
| | Guinea pig RBC - type of microtitre well | U (5) | V (2) |
| | Guinea pig RBC - incubation time at RT after RBC addition | 60 min (5) | 45 min (1), 60 min at 4ºC |
| | Human type O RBC - concentration | 0.7% (1), 0.75% (1), 1%( 1), 1% in 0.5%BSA/PBS (1) | |
| | Human type O RBC - type of microtitre well | V (2), U (2) | |
| | Human type O RBC - - incubation time at RT after RBC addition | 60 min (4) | No variables |

# HI Assay Protocol Comparison - continued

| Parameter or variable | | Most frequent variables used (# of laboratories) | Alternate variables (# of laboratories) |
|---|---|---|---|
| **Haemagglutination conditions** | Complete haemagglutination definition | RBC in suspension after RBC control has settled completely (14) | No variables |
| | Incomplete haemagglutination definition | Some RBC in suspension after RBC control has settled completely (14) | No variables |
| | No haemagglutination definition | RBC settled completely: turkey and chicken RBC 'run' or form 'tear drop' when tilted; guinea pig or human O RBC form 'halo' (14) | No variables |
| | Haemagglutination endpoint | Highest dilution of virus that causes complete haemagglutination (13) | Highest dilution of virus that causes 50% haemagglutination (1) |
| | Haemagglutination titre | Reciprocal of haemagglutination end-point (14) | No variables |

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| | Haemagglutination titre | Reciprocal of haemagglutination end-point (14) | No variables |

# HI Assay Protocol Comparison - continued

| Parameter or variable | | Most frequent variables used (# of laboratories) | Alternate variables (# of laboratories) |
|---|---|---|---|
| **HI assay preparation** | Vol RDE-treated serum added to each well | 25 µl (13) | 50 µl (1) |
| | Sera diluted down or across micotitre plate | Down (ie 1:10-1:1280) (10) | Across (ie 1:10-1:20480) (3), either dependent on study design (1) |
| | Diluent for serum dilution | PBS (12) | 0.9% NaCl (1), Dulbeccos phosphate buffered saline (10 |
| | Final antigen concentration added to wells | 4HAU per 25 µl (12) | 8 HAU per 25 µl (1), 8 HAU per 50 µl (1) |
| | Vol of antigen added to wells | 25 µl (13) | 50 µl (1) |
| | Amount of antigen per well | 4 HAU (12) | 8 HAU (2) |
| | Mixing protocol | Thorough manual agitation (10) | Lab shaker for 10 sec (1), lab shaker for 10 sec then manual agitation (1), None (2) |
| | Incubation for antigen/serum | Room temp for 60 min (7) | Room temp for 20-30 min (7) |
| | Vol RBC suspension added to each well | 50 µl (12) | 25 µl (2) |

# HI Assay Protocol Comparison - continued

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| | Incubation for antigen/serum | Room temp for 60 min (7) | Room temp for 20-30 min (7) |
| | Vol RBC suspension added to each well | 50 µl (12) | 25 µl (2) |

# HI Assay Protocol Comparison - continued

| Parameter or variable | | Most frequent variables used (# of laboratories) | Alternate variables (# of laboratories) |
|---|---|---|---|
| **HI assay preparation** | Incubation for antigen/serum/RBC | Turkey - RT for 30 min (8)<br>Goose - RT for 30 min (1)<br>Guinea pig – RT for 60 min (4)<br>Chicken – RT for 30 min (3)<br>Human O – RT for 60 min (1)<br>Horse - 4ºC for 60 min (1) | Turkey – 45 (1) or 60 min (1)<br>Guinea pig - 4ºC for 60 min (2)<br>Chicken – 45 min (1) |
| | Final vol in well | 100 µl (11) | 75 µl (2), 150 µl (1) |
| | Starting serum dilution | 1:10 excluding antigen and RBC vol (14) | 1:8 excluding antigen and RBC vol (1) |
| | Number of replicates | Duplicates (10) | Triplicates (1), Single (3) |
| **End-point HI estimation** | HI end-point definition | Highest dilution of serum that prevents complete haemagglutination (13) | Highest dilution of serum that prevents 50% haemagglutination (1) |
| | Hi titre definition | Reciprocal of HI end-point (14) | No variables |

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| | Number of replicates | Duplicates (10) | Triplicates (1), Single (3) |
| **End-point HI estimation** | HI end-point definition | Highest dilution of serum that prevents complete haemagglutination (13) | Highest dilution of serum that prevents 50% haemagglutination (1) |
| | Hi titre definition | Reciprocal of HI end-point (14) | No variables |

# Consensus HI Assay Protocol
# - WHO manual in red

| Parameter or variable | | Required Parameter | Recommended parameter |
|---|---|---|---|
| **RBC preparation** | RBC species used | Either turkey or guinea pig | |
| | RBC washing | | Resuspension in PBS plus two washes in PBS |
| | Method for RBC cell estimation | | Estimate vol of packed cells by eye, by haematocrit or by haemocytometer |
| | Final RBC concentration in assay | 0.5% turkey, 0.75% guinea pig | |
| **Serum preparation** | *V. Cholerae* Receptor Destroying Enzyme | Either Denka Seiken C Ltd, WHO reagent kit or Sigma-Aldrich | Evaluate RDE activity by recipient lab |
| | RDE:serum ratio for serum treatment | 3 vols RDE to 1 vol serum | |
| | Incubation conditions | 37 ºC in water bath or incubator for 16-20h | |
| | RDE inactivation | 56°C for 30 min, allow to cool and add 6 vols PBS or 0.85% NaCl (4) | |
| | Final serum dilution | 1:10 | |
| | Detection of non-specific agglutinins in treated sera | Performed before HI assay | |
| | Treatment of sera to remove non-specific agglutinins | 20 vols RDE-treated sera incubated with 1 vol packed RBC for 1 h at 4ºC | |
| | Control sera | | Run positive and negative sera in each assay |

# Consensus HI Assay Protocol continued
# - WHO manual in red

| Parameter or variable | | Required Parameter | Recommended parameter |
|---|---|---|---|
| **Haemagglutina -tion conditions** | Turkey RBC – concentration | 0.5% | |
| | Turkey RBC – type of microtitre well | V | |
| | Turkey RBC - incubation time at RT after RBC addition | 30 min | |
| | Guinea pig RBC – concentration | 0.75% | |
| | Turkey RBC – type of microtitre well | U | |
| | Turkey RBC - incubation time at RT after RBC addition | 60 min | |
| | Complete haemagglutination definition | RBC in suspension after RBC control has settled completely | |
| | Incomplete haemagglutinatiion definition | Some RBC in suspension after RBC control has settled completely | |
| | No haemagglutination definition | RBC settled completely: turkey RBC 'run' or form 'tear drop' when tilted; guinea pig RBC form 'halo' | |
| | Haemagglutination end-point | Highest dilution of virus that causes complete haemagglutination | |
| | Haemagglutination titre | Reciprocal of haemagglutination end-point | |

# Consensus HI Assay Protocol continued
# - WHO manual in red

| Parameter or variable | | Required Parameter | Recommended parameter |
|---|---|---|---|
| **HI assay preparation** | Vol RDE-treated serum added to each well | 25 µl | |
| | Serum dilution across or down microtitre plate | | Either down or across depending on assay design |
| | Diluent for serum dilution | | PBS |
| | Final antigen concentration | 4 HAU per 25 µl | |
| | Vol antigen added per well | 25 µl | |
| | Amount of antigen per well | 4 HAU | |
| | Mixing protocol | | Lab shaker for 10 mins or thorough agitation |
| | Incubation of antigen/serum | | Room temperature for 20-60 mins |
| | Vol RBC added per well | 50 µl | |
| | Incubation of antigen/serum/RBC | Turkey RBC - room temp for 30 min<br>Guinea pig RBC – room temp for 60 min | |
| | Final vol per well | 100 µl | |

# Consensus HI Assay Protocol continued
# - WHO manual in red

| Parameter or variable | | Required Parameter | Recommended parameter |
|---|---|---|---|
| **HI assay preparation continued** | Starting serum dilution | 1:10 excluding antigen and RBC vols | |
| | Number of replicates | Duplicate | |
| | Control of virus titre | | Back-titration to check 4HAU |
| **HI end-point estimation** | HI end-point defintion | Highest dilution of serum that prevents complete haemagglutination | |
| | HI titre definition | Reciprocal of HI end-point | |

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