

NASBA for the detection of avian influenza virus H1-H16 subtypes  
in veterinary and environmental samples

### MICROPLATE DETECTION METHOD

Includes main components for 50 reactions



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PLEASE READ THROUGH THE ENTIRE PROTOCOL BEFORE  
STARTING.

## 1. KIT COMPONENTS

### Storage Conditions

Components should be stored at -20°C with the exception of the small inner box, which should be stored at 4°C. Expiry dates shown on the box indicate the date beyond which reagents should no longer be used.

### Nucleic Acid Amplification

- 5 x 6.5 mg Enzyme spheres (red cap; contained in a foil pack with silica gel desiccant)
- 1 x 0.5 ml Enzyme sphere diluent (red cap)
- 5 x 10 mg Reagent spheres (blue cap; contained in a foil pack with silica gel desiccant)
- 1 x 0.6 ml Reagent sphere diluent (blue cap)
- 1 x 0.5 ml KCl stock solution (yellow cap)
- 1 x 60 µl AIV primer mix
- 1 x 25 µl AIV positive control
- 2 x 1.5 ml NASBA water (white cap)

### Nucleic Acid Detection

- 2 x 55 µl AIV Probe solution
- 5 x 1.1 ml Substrate solution (protect from light)
- 1 x 10 ml 10x TBS Washing buffer, pH 7.4 (store at room temperature)
- 12 x 8 well Microtitre plate strips and support frame (store at room temperature in the dark)

### Small Inner Box Contents (store at 4°C):

- 3 x 800 µl Hybridisation buffer
- 5 x 1.1 ml Detection solution
- 1 x 11 µl Detection concentrate
- 5 x 1.1 ml Stop solution

## 2. PREPARATION OF REAGENTS

### A. Nucleic Acid Amplification

- Re-use of reagents: The reconstituted reagent sphere/enzyme sphere can be re-used once within two weeks of opening provided they have been stored at -70°C. Re-use of all other amplification reagents is possible if the remainder of the opened reagents has been stored at -20°C.*
- Bring all reagents to room temperature before use.

### Preparation of Amplification Solution

- Add 80 µl **reagent sphere diluent** to a lyophilised **reagent sphere** and immediately vortex well.
- Add 14 µl **NASBA water** and 16 µl **KCl stock solution** and vortex.
- Add 10 µl **AIV primer mix** and vortex.
- DO NOT** centrifuge.
- Use within 30 min.

### Preparation of the Enzyme Solution

- Add 55 µl **enzyme diluent** to a lyophilised **enzyme sphere**. Leave this enzyme solution to stand for at least 20 min at room temperature.
- Mix gently by flicking the microfuge tube with your finger.
- DO NOT** vortex any solution containing enzymes.
- Centrifuge briefly before use.
- Use within one hour.

### B. Nucleic Acid Detection

- Re-use of detection reagents is possible if the remainder of opened reagents has been stored at 2 - 8°C.*
- Bring all reagents to room temperature before use.
- Spin down all microfuge tubes to bring liquid to the bottom of the tube.

#### 1. **AIV Probe solution**

Thaw to room temperature and mix thoroughly before use.

#### 2. **Hybridisation buffer**

Mix thoroughly before use.

#### 3. **Detection solution**

Mix thoroughly before use.

#### 4. **Detection concentrate**

Dilute 1:500 in Detection solution and mix thoroughly before use.

#### 5. **Substrate solution**

Thaw to room temperature in the dark and mix thoroughly before use.

#### 6. **Stop solution**

May be used without further treatment.

#### 7. **10x TBS washing buffer**

Dilute 1:10 with DEPC-treated water and mix thoroughly before use.

**Note:** Frequent thawing and freezing of the frozen reagents may reduce the sensitivity of the kit. Components frozen without being thawed are stable for 12 months.

#### 8. **Microtitre plate strips**

Remove sufficient microtitre strips as are required for the number of samples to be tested.

#### 9. **Microtitre plate support frame**

May be used without further treatment.

## 3. PROCEDURES

### *Remarks:*

- Wear gloves throughout the procedure to protect your RNA samples from nucleases.*
- All procedures should be carried out at room temperature unless otherwise stated.*
- DO NOT vortex any solutions containing enzymes.*

### Nucleic Acid Amplification

- For each amplification reaction, pipette 5 µl of the nucleic acid extract into a fresh microfuge tube.
- Add 10 µl of **amplification solution** (see section 2).
- Incubate microfuge tubes for 5 min at 65°C in a heat block.
- Cool microfuge tubes for 5 min at 41°C in a heat block.
- Add 5 µl of **enzyme solution** (see section 2) and mix well by flicking the microfuge tube with your finger.
- Immediately return microfuge tubes to 41°C for 5 min.
- Briefly centrifuge microfuge tubes and incubate for 90 min at 41°C in a water bath.

- Perform the detection or store the RNA amplicons at  $-20^{\circ}\text{C}$  for up to 1 month.

#### Nucleic Acid Detection

- Set-up sufficient microtitre strips on the support frame as are required for the assay.
- Add 2  $\mu\text{l}$  probe solution, 5  $\mu\text{l}$  NASBA product and 43  $\mu\text{l}$  hybridisation buffer to a 0.5 ml or 1.5 ml microfuge tube and mix thoroughly.
- Repeat for each NASBA assay product.
- Add hybridisation mixture to separate wells of the microtitre strip. Avoid introducing bubble into the wells. Cover firmly with plastic wrap to prevent evaporation.
- Incubate at  $41^{\circ}\text{C}$  for 1 hr.
- Wash the wells three times with 250  $\mu\text{l}$  1x TBS, pH 7.4. Flick dry the plate each time after discarding solution.
- Add 100  $\mu\text{l}$  detection solution to each well. Cover firmly with plastic wrap to prevent evaporation.
- Incubate at room temperature for 30 min.
- Wash the wells three times with 250  $\mu\text{l}$  1x TBS, pH 7.4. Flick dry the plate each time after discarding solution.
- Add 100  $\mu\text{l}$  substrate solution to each well (take care to not introduce bubbles into the wells).
- Incubate at room temperature for 5 min in the dark.
- Add 100  $\mu\text{l}$  stop solution and gently shake to stop the colour development.
- Load the microtitre frame with microtitre strips into a standard 96-well microtitre plate spectrophotometer and read the absorbance at 405 nm. Use 100  $\mu\text{l}$  substrate solution with 100  $\mu\text{l}$  stop solution (without hybridisation and detection reagents) as background control.

#### 4. DATA COLLECTION

See your spectrophotometer instruction manual for detailed guidelines on sample loading and analysis.

Table 1. Typical absorbance readings

Sample	Qualitative result	Signal (Abs <sub>405</sub> $\pm$ S.D)
Assay negative (all reagents/water instead of RNA)	Negative	0.080 $\pm$ 0.016
Negative control (non-AIV RNA)	Negative	0.119 $\pm$ 0.047
Positive control (AIV RNA)	Positive	0.959 $\pm$ 0.053

#### 5. DETERMINING THE CUT-OFF LIMIT

The calculation of the most appropriate cut-off limit to adequately discriminate between genuine positive and negative samples is a relatively straightforward procedure. However, care must be taken to calculate the cut-off limit for each set of experiments that are conducted.

- Test 10 known negative samples and 10 known "weak positive" samples (i.e. dilutions of known positives). Define a cut-off limit that can correctly differentiate the negative and weak positive samples tested above.
- The cut-off limit will be greater than the highest Abs<sub>405</sub> signal obtained for a known negative sample and lower than the lowest Abs<sub>405</sub> signal obtained for a known weak positive.
- Further known negative and weak positive samples can then be tested to further qualify the appropriateness of the selected cut-off value.
- The chosen cut-off value will be valid for the current set of experiments only and must be defined again when the assay is run in the future or if other parameters are altered (e.g. buffer composition, primer sequence, length of incubation, etc).

A suggested cut-off limit is the mean negative control value plus 10 S.D (i.e.  $0.12 + (10 \times 0.012) = 0.24$ ).

Our laboratory usually obtains a S.D. in the range of  $0.15 \pm 0.3$  for our negative control samples. The safe-harbour for our cut-off value is, therefore, approximately 0.45.

**Note:** If you obtain a cut-off value outside this range we suggest that you re-evaluate your microplate system or contact Hai Kang Life Corporation Limited for technical advice.

#### 6. TROUBLESHOOTING GUIDE

Symptoms	Possible Causes	Problem solving
Low absorbance signal	RNase contamination	The reagents provided are all RNase-free. The other solutions and apparatus used (pipettes, tips and microfuge tubes, etc) should be treated to ensure they are also RNase-free.
	Poor amplification efficiency	Add enzyme solution to the microfuge tubes by taking the microfuge tubes out of the heating block one at a time and returning them immediately after addition of enzyme. Maintain the amplification temperature at $41^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .
	Enzymes denatured	<b>DO NOT</b> vortex the enzyme solution. Enzymes are quite sensitive to physical influences and lose their activity when vortex mixed. <b>DO NOT</b> incubate the enzymes above $42^{\circ}\text{C}$ . It will also denature enzyme activity.
	Poor hybridisation between target RNA and probe	Make sure the incubation temperature ( $45^{\circ}\text{C}$ ) is correct. Mix the probe solution, NASBA product and hybridisation buffer well before adding the mixture into the well. Avoid making bubbles when adding the mixture into the well.

#### 7. TECHNICAL ASSISTANCE

Our technical staff will provide technical assistance you may need in using this kit. Simply call +(852) 2111 2123 during office hours:

**Monday – Friday: 9:00am to 5:30pm**  
**Saturday: 9:00am to 1:00pm**

A recorded message (in English, Cantonese or Putonghua) may be left outside office hours. Alternatively, you may contact our technical staff by fax or email.

**Fax:** +(852) 2111 9762  
**Email:** technical@haikanglife.com

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