

CLART[®] FluAVir

**DETECTION AND CHARACTERIZATION OF VIRUSES CAUSING
NEW FLUA H1N1**

CLART® FluAVir
Extraction-Purification

48 determinations Ref: AS-0509-48
96 determinations Ref: AS-0509-96

CLART® FluAVir
Amplification

48 determinations Ref: AT-0609-48-MT
96 determinations Ref: AT-0609-96-MT

CLART® FluAVir
Visualization

48 determinations Ref: AS-0709-48
96 determinations Ref: AS-0709-96

Version 2
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Check, handling instructions



Expiration date



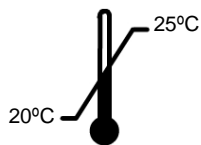
Medical Device *for In Vitro* Diagnostics



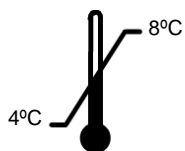
Only for the evaluation of the *In vitro* diagnostic device performance



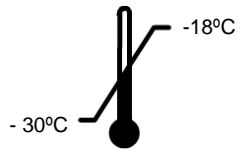
Lot



Store at room temperature



Store between 4°C to 8°C



Store between -30°C to -18°C

2. INTRODUCTION

Influenza viruses are RNA virus of the *Orthomyxoviridae* family, very diverse and with a high rate of evolution. These viruses affect respiratory epithelium tissues by destroying the epithelial and mucus forming cells, via cytolysis and apoptosis. This damage of the body barriers might produce secondary bacterial infections, (e.g. pneumonia) that use to be the most frequent cause of patient's death.

The 2009 FluA outbreak has been originated by a new variant of the influenza A (subtype H1N1) of swine origin. This new variant of the virus contains genetic material from an avian, two swine and one human stump. It's thought to be a mutation, the responsible of the "jump" from a swine to a human host⁴. First patient registered in the world was a 10 years old boy who felt ill on March 30th in San Diego (USA)⁵ without being in touch with pigs. According to the World Health Organization (WHO), first cases of Influenza were detected in Mexico on April 11th in the Mexican state of Veracruz. Afterwards, FluA outbreak, started to spread out to multiple Mexican states (Distrito Federal, Estado de Mexico and San Luis de Potosi) and United States (Texas and California). From then on, the virus has infected patients from countries all over the world. Initially, just cases from people who have visited Mexico were reported, but later on, indirect infections were reported in many other countries like Spain, Germany, South Korea or United Kingdom⁶. On April 29th of 2009, the WHO classified the H1N1 outbreak as a level 5 alert, which means, imminent pandemic level⁷. These alerts levels do not refer to the illness seriousness caused by the virus, but to its geographical spreading. On June 11th of 2009, the WHO increased the alert level due to New Influenza A (H1N1) from level 5 to level 6, the highest level, which means pandemic level.

This new viral stump is known as *SwineFlu*, *North American Flu*⁸ and *New Flu*, although these names have been the object of diverse controversies. On April 30th 2009, the WHO gave it the official name of Flu A virus (H1N1)¹⁰.

Among the Influenza viruses, Influenza A viruses are those causing the most severe and extended outbreaks. When antigenic variations are produced in those viruses, pandemic or global epidemics occur. Since 1918, pandemics have been happening periodically every 10 to 15 years. The minor antigenic variations within Influenza A, Influenza B and, to a lesser extent, Influenza C viruses, lead to the appearance of yearly seasonal Flues. Generally, seasonal Flues are not really serious and have variable extension. Morbidity rates in the FluA affected areas varies a lot, but in general it ranges from 10% to 20% of the global population.

Human H1N1 circulating stumps during the last few years, are considered less virulent, causing milder symptoms, even in subjects without immunity against the virus¹¹. In fact, the last Flu A pandemic, was caused by the subtype H3N2 and took place between 1968 and 1969 (so called Hong Kong Flu) when socio-sanitary conditions were very different than those of nowadays.

Up to date, although the symptoms of Virus Flu A (H1N1) are being mild, little is known about the evolution of the virus, as Margaret Chan, the General Manager of the WHO states: "The evolution of the actual pandemic is unpredictable"¹².

The kit for the detection of the New FluA (H1N1), developed by GENOMICA, is based on the amplification of specific fragments of the viral genome of the New FluA (H1N1) and the subsequent detection via hybridization with type-specific binding probes, placed onto an array. This procedure entails a series of advantages:

- Sensitivity: since it allows detection from minimal quantities of viral genomic material.
- Easy to standardize in a clinical laboratory.
- Rapid: analysis results in 8 h.

3. PROTOCOL DESCRIPTION

The CLART[®] FluAVir kit is able to detect and characterize the presence of new H1N1 subtype and differentiate it from the seasonal H1N1 and H3N2 subtypes, causing, all of them, respiratory infections in humans.

Virus detection is performed via RT-PCR (reverse transcriptase PCR) amplification of a specific fragment of the viral genome. For detecting New FluA, a 229 pb sized fragment is used, while for the seasonal FluA the fragment size is 261 pb. Detection of the amplified product is possible thanks to a new technological platform: **CLART[®]** (Clinical Array Technology). The platform is based on a low-density microarray fixed at the well's bottom of a classical microtiter plate. The product is offered in an 8 wells strips format (Array Strip)(Fig. 1). Thus, this system considerably simplifies the processes of hybridisation and visualisation when compared with classic microarray systems.

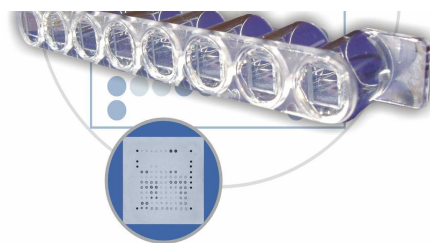


Figure 1: CLART[®] Platform.

The **CLART® FluAVir** kit is based on the precipitation of an insoluble product on those sites of the array where hybridization has been produced. During RT-PCR, amplified products are labeled with biotin. Afterwards, they hybridize with their respective specific probes on the arrays and incubated with streptavidin-peroxidase conjugate. The conjugate binds, via streptavidin, with the biotin present in the amplified products, while, in the presence of o-dianisidine, the peroxidase activity of the conjugate induces the appearance of an insoluble product which precipitates at the specific hybridization site (Fig. 2).

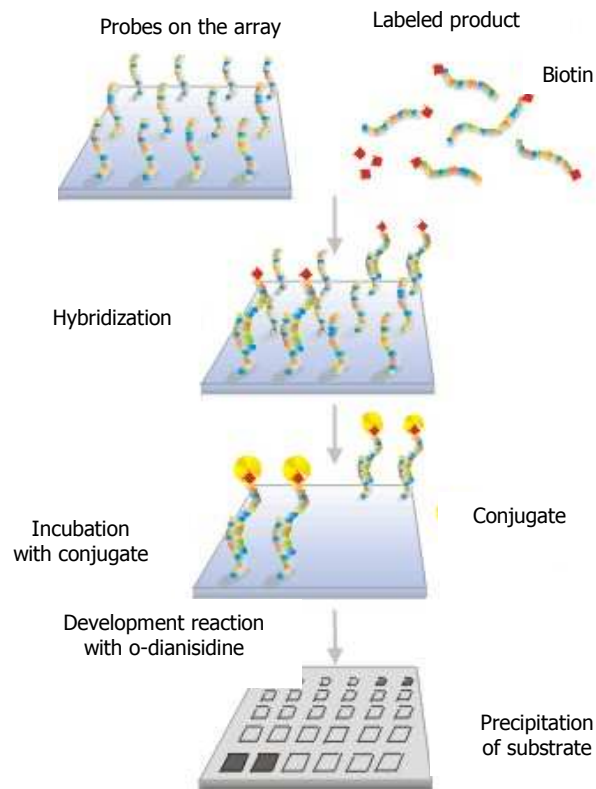


Figure 3: Diagram of the visualization method. Probes, immobilized on the surface, capture their complementary biotin-labeled amplified products. With the help of the biotin, the conjugate binds, in this case streptavidin-HRP (*HorseRadish Peroxidase*). Thanks to the HRP action, the o-dianisidine substrate produces a precipitation on the hybridization site.

The sensitivity obtained with the **CLART® FluAVir** kit, combining the amplification reaction with the visualization in the array, is so high that it is not necessary to perform double amplifications (nested-PCR), thus avoiding any contamination risk involved.

4. KIT COMPONENTS AND STORAGE

CLART® FluAVir kit contains enough reagents for the extraction and the analysis of 48 to 96 clinical samples. Reagents included in the kit have been grouped in various packages, depending on the shipment and storage temperatures. When storage recommendations are observed, all reagents remain stable until the kit's expiration date.

4.1. Extraction-purification reagents

The extraction-purification kit is shipped at -20°C and it should be stored at this temperature until its use. Reagents included in this kit are:

- **SEML** (extraction solution for liquid samples). **Once thawed, it should be stored at 4°C and used within 8 days.**
- **SD** (Dilution solution). Store at -20° or 4°C.
- **IP** (Isopropanol) Store at -20°C.
- **DE** (Ethanol 70%) Store at -20°C.

4.2. Amplification reagents

They are shipped and stored at -20°C.

- **Amplification tubes:** Ready-to-use for the amplification of FluA subtype H1N1(Swine origin), as well as the seasonal FluA H1N1 and H3N2. It contains 43 µL of reaction mixture. Thaw on ice just the exact number of amplification tubes to be used and store the rest at -20°C.

WARNING! The enzyme mixture should be added before the introduction of the extracted genetic material.

- **Enzyme mixture** (this is a mixture of the **RT** (retrotranscriptase) enzymes and **DNA polymerase**). Ready to use. Store at -20°C.

Note: **The kit package includes a self-adhesive and irreversible temperature indicator; the appearance of a reddish color on the visualization window indicates that, at a certain moment, products have exceeded storage temperature of -20°C and they should not be used.**

4.3. Visualization reagents

The visualization kit is shipped at 4°C and should be stored at same temperature until its use.

WARNING! Upon receipt the kit, ArrayStrips should be stored at room temperature.

- **Array Strips** (specific probes included). They are provided in a sealed thermal envelope. **Always store closed, at room temperature, away from direct sunlight.**
- **SH** (Hybridization Solution). **Store at 4°C.**
- **DC** (Conjugate Diluent). **Store at 4°C.**
- **CJ** (Conjugate). **Store at 4°C.** Centrifuge briefly before use.
- **RE** (Development Solution). **Store at 4°C.**
- **TL** (Wash Pad). **Store at 4°C.**

4.4. Other components

The technique requires a device to capture and process the image obtained from the micro-array, rendering one report per sample in a totally automatic way:

- CAR (Clinical Array Reader): allows the automatic analysis and interpretation up to 12 ASs (96 samples). CAR has been design for its exclusively used with GENOMICA's diagnostic kits (Fig. 3).
- Software: **Specific CLART® FluAvir software**, designed and validated by GENOMICA.

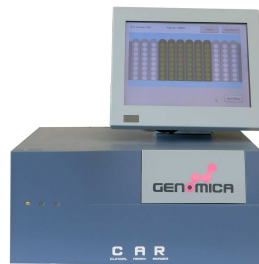


Fig.3: CAR (Clinical Arrays Reader)

5. MATERIALS REQUIRED NOT PROVIDED

Below you can find a list of all materials required but not provided with the kit.

5.1. Reagents and materials

- Distilled water.
- Saline solution.
- Disposable gloves.
- Filter tips or positive displacement pipettes.
- Crushed ice container.
- 1.5 ml autoclaved Eppendorf tubes.
- 1.5 ml tube grids.
- 0.5 ml/0.2 ml tube holder.

5.2. Equipment

- Microcentrifuge.
- Thermal cycler.
- Laminar flow chamber for the extraction laboratory.
- Three adjustable micropipettes ranging from 1-20 μ l, 20-200 μ l, and 200-1000 μ l for the extraction laboratory.
- One adjustable micropipette ranging from 1-20 μ l, to add the enzyme mixture to the amplification tubes.
- One adjustable micropipette ranging from 1-20 μ l, to add the genetic material to the amplification tubes.
- Three adjustable micropipettes ranging from 1-20 μ l, 20-200 μ l, and 200-1000 μ l for the visualization laboratory.
- Heating block with agitation, adjustable at 25°C, 30°C and 50°C. Compatible with Eppendorf microtubes.
- Vortex.
- Vacuum system (optional)

6. RECOMMENDATIONS AND HANDLING PROCEDURES

Very important in order to avoid contamination! Read carefully before starting with the assay.

6.1. General recommendations

1. This assay should be performed in FOUR physically separated AREAS, in order to avoid sample contamination with the previously amplified product. Separate working materials should be available in each area (pipettes, tips, tubes, grids, gloves, etc.) which should never be used outside these areas.

- **Pre-PCR extraction area:** for extracting DNA/RNA. A laminar flow hood must be used.
- **Pre-PCR area for the preparation of the amplification tubes.** In this area, the enzyme mixture is added to the amplification tubes. It is recommended to use a laminar flow hood.
- **Pre-PCR area for the addition of the extracted material.** In this area, the extracted DNA/RNA is added to the amplification tubes where the enzyme mixture has been previously introduced. A laminar flow hood should be used.
- **Post-PCR area:** for the amplification and visualization of the amplified products

2. Always use gloves. It is recommended to change gloves frequently, while it is mandatory to change them prior to start working in each of the above-mentioned areas. New gloves should be used for the preparation of the amplification tubes and every time DNA/RNA is added to them.

3. Clean working areas (laboratory benches, hoods, grids, pipettes) thoroughly with diluted bleach (preferably) **following every sample batch processing**; it is mandatory to disinfect all working areas in case of contamination.

4. Always use filter tips and positive displacement pipettes to avoid contamination due to micropipettes. A different set of pipettes should be used in each area.

5. Use disposable and autoclaved laboratory material.

6. Do never mix reagents from two different tubes, even if they belong to the same lot.

7. Close reagent tubes immediately after use in order to avoid contamination.

8. Discard the micropipette tip after pipetting.

9. Always keep tubes separate during handling, paying special attention **during extraction procedure.**

10. GENOMICA is not responsible for results obtained using this kit, in case of use of samples other than those indicated or DNA/RNA extracted with a protocol other than the one indicated herein.

6.2 Precautions for visualization

1. Avoid the pipette tip or the vacuum system touching the bottom of the tube, since this could damage the microarray.
2. It is recommended to add all solutions to the side wall of the AT or AS; never directly on the bottom.
3. It is advisable not to add the SH solution until the addition of the denaturalized PCR products.
4. Following incubation with the CJ solution, it is very important to wash the AT/AS and the tube cap thoroughly to avoid any residues that could react with the RE solution, resulting in a non-specific precipitation that could lead to false interpretations of the result.
5. Avoid bubbles on the surface of the microarray when adding any solution.
6. When visualizing the image in the reader, confirm that position markers appear and that there are no bubbles or spots interfering with the reading. You may clean the bottom of the tube with cellulose paper or gently tap the tube with your finger.

7. SAMPLING

7.1. Nasopharyngeal lavage.

Introduce 3 to 7 ml of sterile saline solution into the nasal fossa, maintaining the patient's head leant backwards and then collect the solution in a sterile container placed below nasal fossae, inclining the patient's head forwards. Store the sample at 4°C if it is going to be processed within the day or at -80°C if it is going to be processed later.

7.2. Pharyngeal exudate.

For the collection of pharyngeal exudate (which is the second most usual practice for the detection of respiratory viruses after nasopharyngeal lavages), use a tongue depressor in order to avoid contamination with saliva, and collect a sample from the posterior pharynx, from the inflamed or erythematous areas or there where visible lesions exist. Rotate the swab trying to detach some epithelial cells from the lesion. In case there are exudates or mucous residues adhered to the lesion, they should be withdrawn with another swab before proceeding to the sampling. Introduce the swab in its tube with the transport media. Store at 4°C if the sample is going to be processed within the day or at -80°C if it is going to be processed later.

7.3. Nasopharyngeal exudate.

For the collection of a nasopharyngeal exudate sample, insert a flexible wire swab into the nose and the pharynx and rotate it gently several times. Introduce the swab in its tube with the transport media. Store at 4°C if the sample is going to be processed within the day or at -80°C if it is going to be processed later.

8. WORKING PROTOCOL

8.1. Extraction of the genetic material of a clinical sample

In order to optimize results, extraction performance should be at least [...], with independence of the extraction method used (manual or automatic device).

Specific recommendations before initiating extraction:

- Work in the **pre-PCR extraction area**, always using a laminar flow hood and following the recommendations mentioned in section 6.1.
- Keep samples at 4 °C.
- Add reagents in the indicated order.
- Do not use saline solution for swabs.

8.1.1 Manual Extraction Method:

1. Process a negative control with each sample batch, consisting of 200 µl of SD (dilution Solution), and process it like the rest of the samples.
2. Pipette 200 µl of clinical sample. In case of swabs with transport media, vortex for 30 seconds and then pipet 200 µl.
3. Add 600 µl of SEML (liquid sample extraction solution). Wait until the solution thaws and turns clear before using it. Mix by inverting the tubes several times and allow 15 min. at room temperature.
4. Add 600 µl of isopropanol (stored at -20°C); mix by inverting the tubes several times and centrifuge, preferably at 4°C, at 13.000 rpm for 20 min.
5. Remove supernatant using a micropipette. A 1000 µl micropipette can be used to remove the supernatant, as long as a smaller micropipette is used at the end, for example a 20 µl one, for removing the residues at the bottom of the tube without removing the precipitate.
6. Add 1000 µl of 70% ethanol (stored at -20°C). Agitate gently to clean the precipitate at the bottom.
7. Centrifuge preferably at 4°C, at 13,000 rpm for 15 min.

8. Remove the supernatant carefully as indicated at step 4. Let them in the hood to dry for 15 or 20 min. until there is no ethanol residues left. Prior to resuspending the sample, confirm that there are no ethanol residues.

9. Resuspend in 20 µl of Dilution Solution.

8.1.2 Automatic Extraction Method:

Please proceed following manufacturer specifications.

8.2. RT-PCR amplification

Amplification-specific recommendations:

- Work in the **pre-PCR area for the preparation of the amplification tubes**, always using a hood and following the recommendations mentioned in section 6.1.
- Be very careful when adding the enzyme mixture, since it contains a high percentage of glycerol. This way, if you introduce the pipette tip too deep, the mixture adheres on the walls causing the addition of a larger amount of mixture than the necessary this could result in an insufficient quantity of product for the rest of the amplification tubes of the kit.
- Add the DNA/RNA in the **pre-PCR area**, always using a hood and following the recommendations mentioned in section 6.1. During the process, keep tubes well separate and at 4 °C.

Amplification reaction protocol:

1. For each sample to be processed, thaw and keep 1 amplification tube on ice.
2. Centrifuge the reaction tubes in the microcentrifuge for a few seconds, so that all liquid can get to the bottom of the tube. In case there are no microcentrifuge adaptors available for reaction tubes, larger tubes can be used instead, after having cut their cap off.
3. **Add 2 µl of the enzyme mixture** to each amplification tube.
4. Add 5 µl of the extracted RNA/DNA to each one of the reaction tubes and resuspend several times with the micropipette. Keep the tubes on ice.
5. Program the following temperature cycles on the thermal cycler:

| | |
|---|---|
| 1 cycle | 45°C 45 min. 95°C 15 min. |
| 45 cycles | 95°C 0.5 min. 50°C 1.5 min. 68°C 1.0 min. |
| 1 cycle | 68°C 10 min. |
| 4°C continuously until tube collection (optional) | |

The amplification lasts around 4 hours, although this could slightly vary depending on the thermocycler.

8.3. Visualization of the amplified product on Array Strips (AS)

Specific recommendations before starting the visualisation process:

THE PROTOCOL DESCRIBED BELOW SHOULD BE FOLLOWED IN THE POST-PCR AREA. NEVER TAKE THE AMPLIFIED PRODUCT TO THE PRE-PCR AREA.

At the beginning of the assay:

1. PREPARE THE **WASHING SOLUTION BEFORE EACH ASSAY**. DO NOT USE PREVIOUS SOLUTIONS OR ANY REMAINING FROM PREVIOUS ASSAYS.
2. Before starting the denaturing program, wash the thermocycler with 10% bleach solution. During the denaturing process, place the amplification tubes separated into the thermocycler. Do not go beyond 10 minutes.
3. It is not necessary to use filter tips during the visualisation process. However, a different tip for each sample and for every reagent must be used. This precaution must also be undertaken for the SL buffer.
4. The 8-tip combs used with the aspiration pumps must be cleaned after use or decontaminated with 10% bleach solution after each assay.
5. Warm up the SH solution at RT until total disappearance of crystals.
6. One hour before starting the hybridisation process, switch on the thermomixer at 53°C.
7. Switch on the CAR (Clinical Arrays Reader) in order to allow auto-calibration of the device.

8. Input the sample IDs. It is important to have the equipment ready to read the samples at the end of the assay so that they are not exposed to an excess of developing time.
9. Once the assay has been initiated, it is important not to leave the AS to dry in any step of the protocol until they are ready to be read. **The reading is performed with dry AS.**

Visualization protocol (AS):

1. Denaturalization: use the thermal cycler to denaturalize the amplified products. For this step, place the tubes in the thermal cycler and incubate at 95°C from 8 to 10 min. Program 15 minutes in the thermal cycler so that after 10 minutes have elapsed amplified products can continue at 95°C. Remove the tubes from the 95°C incubation and place them immediately in a container with ice.

2. Diluted TL Solution preparation: Dilute 15 mL/strip of fresh TL solution by diluting 1500µL TL into 13,5 mL distilled water

3.- AS preliminary washing: Add 200 µl of diluted TL Solution to every array and resuspend 10 to 15 times. Discard the diluted TL Solution using a pipette or preferably a vacuum system.

This step is necessary in order to wash already packaged strips, before adding the sample. The tube should not contain any washing solution residues.

4.- Hybridization: Once PCR products have been denaturalized, add 100 µl of tempered SH solution in each Array, avoiding foaming. Add 4 µl of the amplified product of the amplification tube. Resuspend several times in order to mix with the SH, without touching the glass. Incubate in the heating block for **1 hour** at 53°C, agitating at 550 rpm.

Following incubation, remove the Strips and discard the SH Solution using a pipette or a vacuum system. Program the heating block at 30°C and leave it running so that it can be used later on at step 6. You can remove the lid off the heating block so that it can cool down quicker.

5.- Washing: Wash **twice** the AS with 200 µl of diluted TL Solution to every Array and resuspend 10 to 15 times. Discard the diluted TL Solution using a pipette or a vacuum system, leaving a small volume. In case that the heating block has not reached a temperature of 30°C when you get to this step, leave the ASs filled with diluted TL Solution until the heating block reaches the necessary temperature.

6.- Blocking and conjugate: It is recommended to centrifuge the CJ solution for 10 seconds before its use. Then, prepare the diluted CJ solution. To this end, mix in a tube **1000 µl of DC** solution and **15 µl of CJ** solution for each Strip (for every strip, prepare enough mixture allowing for one extra sample, to compensate any pipetting errors). When stored at 4 °C, the diluted CJ Solution

remains stable until 4 hours after its preparation. Do not use once this time has elapsed.

Add 100 µl of diluted CJ Solution to the Array. Incubate for exactly 15 minutes at 30°C, agitating at 550 rpm. Following this incubation, **rapidly** discard the solution of the Array using a pipette or vacuum system.

Reduce the temperature of the heating block to 25°C for its use in step 9.

7.- Washing: Wash twice with 200 µl of diluted TL Solution to every array, resuspending 10 to 15 times, and then, discard the solution using a pipette or vacuum system. **If such washing is not performed rapidly, it can cause an illegible signal during reading.**

8.- Washing: Wash one more time. **This is the most important washing.** Add 200 µl of diluted TL Solution to every Array and discard the TL Solution using a pipette or a vacuum system. It is important to **avoid any residues of the CJ Solution**, since they could react with the RE Solution producing an unspecified signal. It is not necessary to change tip for every tube, although it is important not to touch the glass.

9.- Development with RE Solution: Remove the TL solution, add **100 µl** of RE solution to each array of the AS and incubate for 10 minutes at 25°C in the heating block **without agitating**.

WARNING! It is very important to use the heating block without agitation and read the samples immediately after incubation.

10.- Remove the RE Solution using a pipette or vacuum system. The microarray should be dry.

9. RESULTS READING

Processing of data obtained from each analysis is carried out automatically. The reading and analysis system will show a report indicating the final results.

The reader monitor displays a three-column table; the left column shows the virus species and the subtypes characterized in the micro-array. The central column shows a positive or negative result for each virus species, while the right one shows the validity determined by the amplification control.

10. RESULTS INTERPRETATION

One of the main drawbacks of genomic amplification is the utilisation of poor quality DNA samples (too little DNA, degradation of the DNA, or lost of DNA during extraction) or the presence of DNA polymerase inhibitors (e.g., haemoglobin, remains of paraffin wax, salts etc.) in the samples to be analysed, thus interfering with the genomic amplification and resulting in false negatives. However, the **CLART® FluAVir Kit** eliminates false negatives via the use of internal control within the same AS.

When the viruses are present in the sample, there is always a prevalence to amplify the genotypes instead of amplifying the controls. Hence, under certain conditions (i.e. high number copies of one particular virus genotype or when several genotypes are present in the samples) **internal controls may not appear (NO SIGNAL)**.

Positive samples

| Virus | Result | Control |
|--------------|---------------|----------------|
| Type | Positive | Valid |

Negative samples

| Virus | Result | Control |
|--------------|---------------|----------------|
| Type | Negative | Valid |

Inadequate, inhibited samples or samples with very low viral load

| Virus | Result | Control |
|--------------|---------------|----------------|
| Type | Uncertain | Invalid |

11. TECHNICAL AND OPERATIONAL SPECIFICATIONS

Control of known interferences:

There are substances that might interfere in the detection when using the **CLART® FluAVir** kit. They are mainly substances inhibiting enzymes and, thus, they could inhibit the amplification reaction. The most common factors are shown in this list:

- **Use of unsuitable samples.** The analysis of any kind of clinical samples other than those specified in the manual of the **CLART® FluAVir** kit, as well as incorrect sampling, can produce an invalid analysis result due to the lack of amplification as a consequence of sample shortage or inhibited reaction.
- **Inadequate storage of samples** can influence the result of the analysis. If samples are subject to conditions that can result in the degradation of their DNA/RNA, then the result of the analysis will be false.

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