RIDA®GENE Flu LC2.0

real-time RT-PCR

Art. Nr.: PG0525

100 Reactions

For *in vitro* diagnostic use.

-20 °C

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1. Intended use

For *in vitro* diagnostic use. RIDA®GENE Flu LC2.0 is a real-time RT-PCR for the direct, qualitative detection and differentiation of influenza viruses (Influenza A and Influenza B) from human nasal and throat swabs. RIDA®GENE Flu LC2.0 real-time RT-PCR is intended for use as an aid in diagnosis of influenza infection.

2. Explanation of the test

The influenza, also called flu, is the most common respiratory infectious disease which is caused by influenza viruses.

Worldwide, 3 - 5 million people are infected with influenza and approximately 250,000 - 500,000 die from this disease each year. The annual influenza epidemics cause serious public health and economic problems.[1]

In the U.S., influenza causes more than 200,000 hospitalizations and more than 36,000 deaths annually.[2] In the season 2010/2011 more than 41,000 cases of influenza were reported in Germany.[3]

Influenza viruses are RNA viruses belonging to the family of Orthomyxoviridae. They are divided into the subtypes A, B and C. Characteristic for influenza viruses is their high mutational variation (antigenic drift) of the surface antigens, hemagglutinin (HA) and neuraminidase (NA). Influenza types A and B cause the annually occuring influenza epidemics whereas infection with influenza C viruses cause milder disease.

Epidemiologically, influenza A play the major role due to diversity: they are responsible for three major pandemics in the 20th Century and for the majority of influenza epidemics. The majority of influenza A infections in humans are caused by the H1N1 and H3N2 subtypes. In addition to the antigenic drift induced by mutation, new influenza A subtypes can be generated by reassortment of a human and nonhuman influenza A strain (antigenic shift), which may trigger a pandemic. The influenza A subtype H1N1, is associated with the past and potentially new influenza pandemics (e.g. the Spanish Flu in 1918/19; Swine Flu 2009). Today, this influenza A subtype is known as H1N1v. The transmission of influenza viruses occurs by droplets and aerosols. The incubation period is 1 - 4 days. Clinical symptoms are mainly severe diseases of the respiratory tract with high fever and cough. The onset of symptoms is characteristically abrupt (sudden onset). During severe courses of disease pneumonia and bacterial super-infection can occur, which can be fatal, especially for the elderly and children. [4]

3. Test principle

The RIDA®GENE Flu LC2.0 is a real-time RT-PCR for the direct, qualitative detection and differentiation of influenza viruses (Influenza A and Influenza B) from human nasal and throat swabs. The detection is done in a one step real-time RT-PCR format where the reverse transcription is followed by the PCR in the same reaction tube. The isolated RNA is transcribed into cDNA by a reverse transcriptase. Gene fragments specific for influenza virus (Influenza A and Influenza B) are subsequently amplified by real-time PCR. The amplified targets (M-Gene and NP1-Gene) are detected with hydrolysis probes, which are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophore). In the presence of a target the probes hybridize to the amplicons. During the extension step the Taq-polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal which is detected by the optical unit of a real-time PCR instrument. The fluorescence signal increases with the amount of formed amplicons. The RIDA®GENE Flu LC2.0 assay contains an Internal Control RNA (ICR) as an internal control of sample preparation procedure and to determine possible PCR-inhibition.

4. Reagents provided

Tab.1: Reagents provided (Reagents provided in the kit are sufficient for 100 determinations)

Kit Code	Reagent	Amount	Lid Color
1	Reaction Mix	2x 700 µl	yellow
2	PP-Mix	1x 770 µl	green
3	Enzyme Mix	1x 80 µl	red
R	Internal Control RNA	2x 1800 µl	brown
N	PCR Water	1x 500 µl	white
P	Positive Control	1x 100 µl	blue

5. Storage instructions

- Protect all reagents from light and store at -20 °C. All reagents can be used until the expiration date. After expiry the quality guarantee is no longer valid.
- Carefully thaw reagents before using (e.g. in a refrigerator at 2 - 8 °C).
- Reagents can sustain up to 5 freeze/thaw cycles without influencing the assay performance. (e.g. after the first thawing separate it in aliquots and freeze immediately).
- During PCR preparation all the reagents should be stored cold in an appropriate way (2 - 8 °C).

6. Additional equipment and materials required

- Sterile, media-free Rayon or Nylon flocked swabs (e.g. Copan Diagnostic Inc., catalogue no. 155C or 552C)
- RNA-Extraction kit (e.g. RIDA® Xtract)
  or
  RNA-Extraction system (e.g. MagNA Pure (Roche), m2000sp (Abbott), m24sp (Abbott), NucliSENS easy®MAG™ (bioMérieux))
- Real-time PCR instrument: LightCycler® 2.0 (Roche)
- RIDA®GENE Color Compensation Kit II (PG0002)
- Real-time PCR consumables (reaction vials)
- Centrifuge with a rotor for the reaction vials
- Vortexer
- Pipettes (0.5 – 20 µl, 20 – 200 µl, 100 – 1000 µl)
- Filter tips
- Powder-free disposal gloves

7. Precautions for users

- For *in vitro* diagnostic use only.
- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- This test must only be performed by laboratory personnel trained in molecular biology methods.
- Strictly follow the working instructions.
- When handling samples, wear disposable gloves. After finishing the test, wash your hands.
- Do not smoke, eat or drink in areas where samples or test reagents are being used.
- Samples must be treated as potentially infectious as well as all reagents and materials being exposed to the samples and have to be handled according to the national safety regulations.
- Do not use the kit after the expiration date.
- Calcium alginate swabs and swabs with wooden or aluminum shafts and/or cotton tips may inhibit PCR and can lead to false negative results. Use the recommended swabs for specimen collection (refer to section 6. Additional equipment and materials required).

8. Test procedure

8.1 Specimen collection

Moisten the swab with sterile saline or use dry swabs. Nasal/throat specimen should be collected with the recommended swab (refer to section 6. Additional equipment and materials required) according to manufacturer's instruction.

8.2 RNA-Preparation

For RNA isolation of nasal and throat swabs the following procedure is recommended: Add 200 µl water (RNase-free) into a preparation tube. Insert the swab into the water and cut or break the swab stem. Cap the preparation tube tightly, vortex shortly and continue according to manufacturer's instruction of the RNA-extraction kit or RNA-extraction system.

The RIDA®GENE Flu LC2.0 assay contains an Internal Control RNA (ICR), which can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as a PCR inhibition control.

If the ICR is used only as a PCR inhibition control, 1 µl of the ICR should be added to the Master-Mix (see Tab.3).

If the ICR is used as a extraction control for the sample preparation procedure and as PCR inhibition control, 20 µl of the ICR has to be added during extraction procedure. The ICR should always be added to the specimen-lysis buffer mixture and must **not** be added directly to the specimen. We also recommend to add 1 µl of the ICR to the negative and positive control RT-PCR Mix.

8.3 Master-Mix preparation

Calculate the total number of PCR reactions (sample and control reactions) needed. One positive control and negative control must be included in each assay run.

We recommend to calculate an additional volume of 10 % to compensate imprecise pipetting (see Tab.2, Tab.3). Thaw, mix gently and centrifuge briefly the Reaction Mix, the PP-Mix, the Enzyme Mix, the Positive Control, the PCR Water and the ICR before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab.2: Calculation and pipetting example for 10 reactions of the Master-Mix (ICR as extraction and PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	12.5 µl	137.5 µl
2	PP-Mix (Primer-Probe-Mix)	6.9 µl	75.9 µl
3	Enzyme Mix	0.7 µl	7.7 µl
	Total	**20.1 µl**	**221.1 µl**

Mix the components of the Master Mix gently and briefly spin down.

Tab.3: Calculation and pipetting example for 10 reactions of the Master-Mix (ICR only as PCR inhibition control)

Kit Code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	12.5 µl	137.5 µl
2	PP-Mix (Primer-Probe-Mix)	6.9 µl	75.9 µl
3	Enzyme Mix	0.7 µl	7.7 µl
R	Internal Control RNA	1.0 µl	11 µl
	Total	**21.1 µl**	**232.1 µl**

Mix the components of the Master Mix gently and briefly spin down.

8.4 Preparation of the RT-PCR Mix

Pipette 20 µl of the Master-Mix in each reaction vial (tube or plate).

Negative control: Add 5 µl PCR Water as negative control to the pre-pipetted Master-Mix.

Note: *If the ICR is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the ICR to the negative control RT-PCR Mix.*

Sample: Add 5 µl RNA-Extract to the pre-pipetted Master-Mix.

Positive control: Add 5 µl Positive Control to the pre-pipetted Master-Mix.

Note: *If the ICR is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the ICR to the positive control RT-PCR Mix.*

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The RT-PCR reaction should be started according to the PCR instrument Set-up (see Tab.4).

8.5 PCR Instrument Set-up

Tab.4: Real-time PCR profile for the LightCyler® 2.0

Reverse Transcription Initial Denaturation Cycles PCR Denaturation Annealing/Extension	10 min, 58 °C 1 min, 95 °C 45 Cycles 15 sec, 95 °C 30 sec, 55 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: *Annealing and Extension occur in the same step.*

8.6 Detection Channel Set-up

Tab.5: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection Channel	Note
Roche LightCycler® 2.0	Influenza A	530	**RIDA®GENE Color Compensation Kit II (PG0002) is required**
	ICR	560	
	Influenza B	705	

9. Result interpretation

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer`s instructions. Positive and negative controls have to show correct results (see Fig.1, Fig. 2).

The positive control has a concentration of 10^3 copies / µl. In each PCR run it is used in a total amount of 5 x 10^3 copies.

Fig.1: Correct run of the positive and negative control (Influenza A) on the LightCycler® 2.0

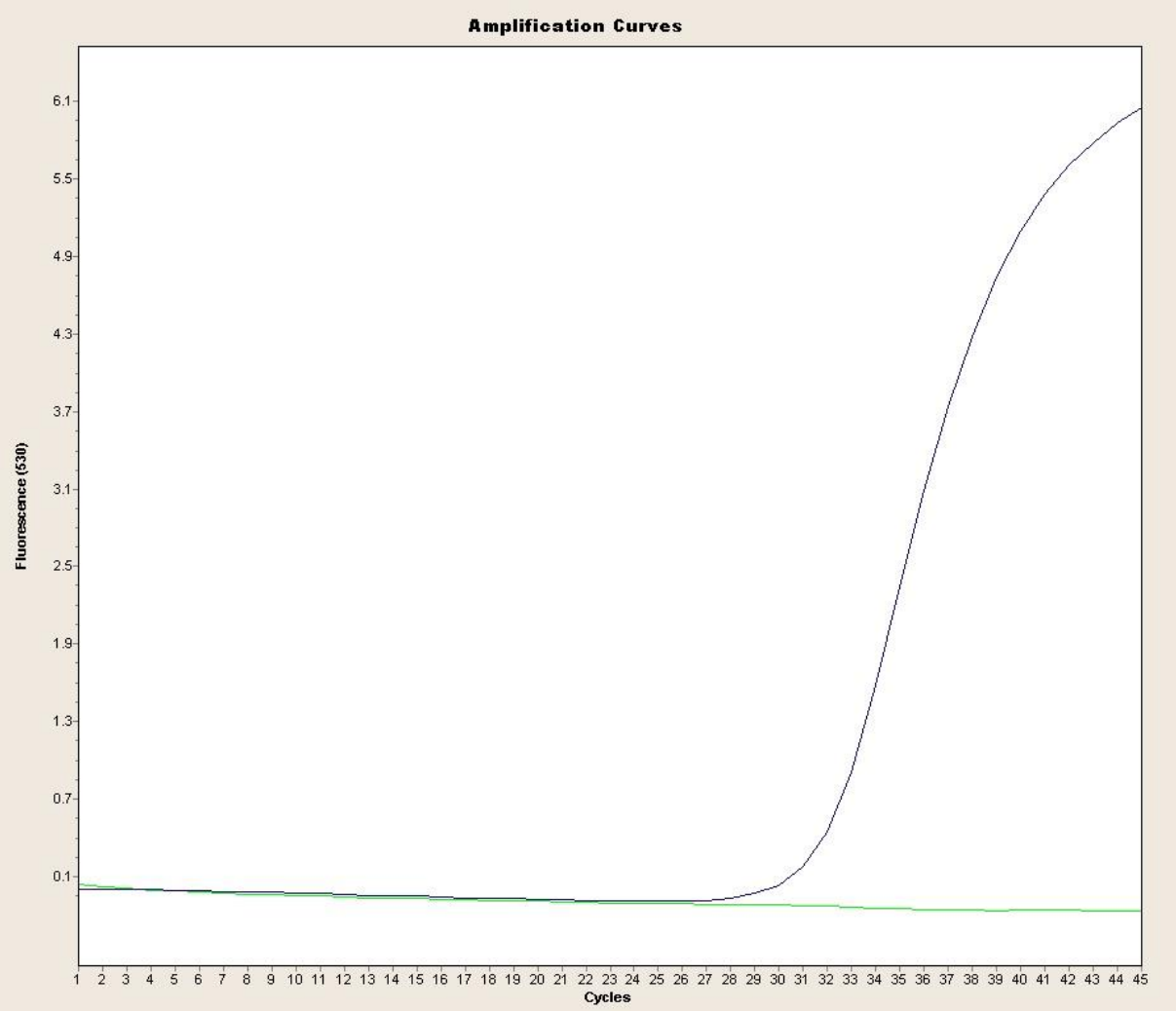
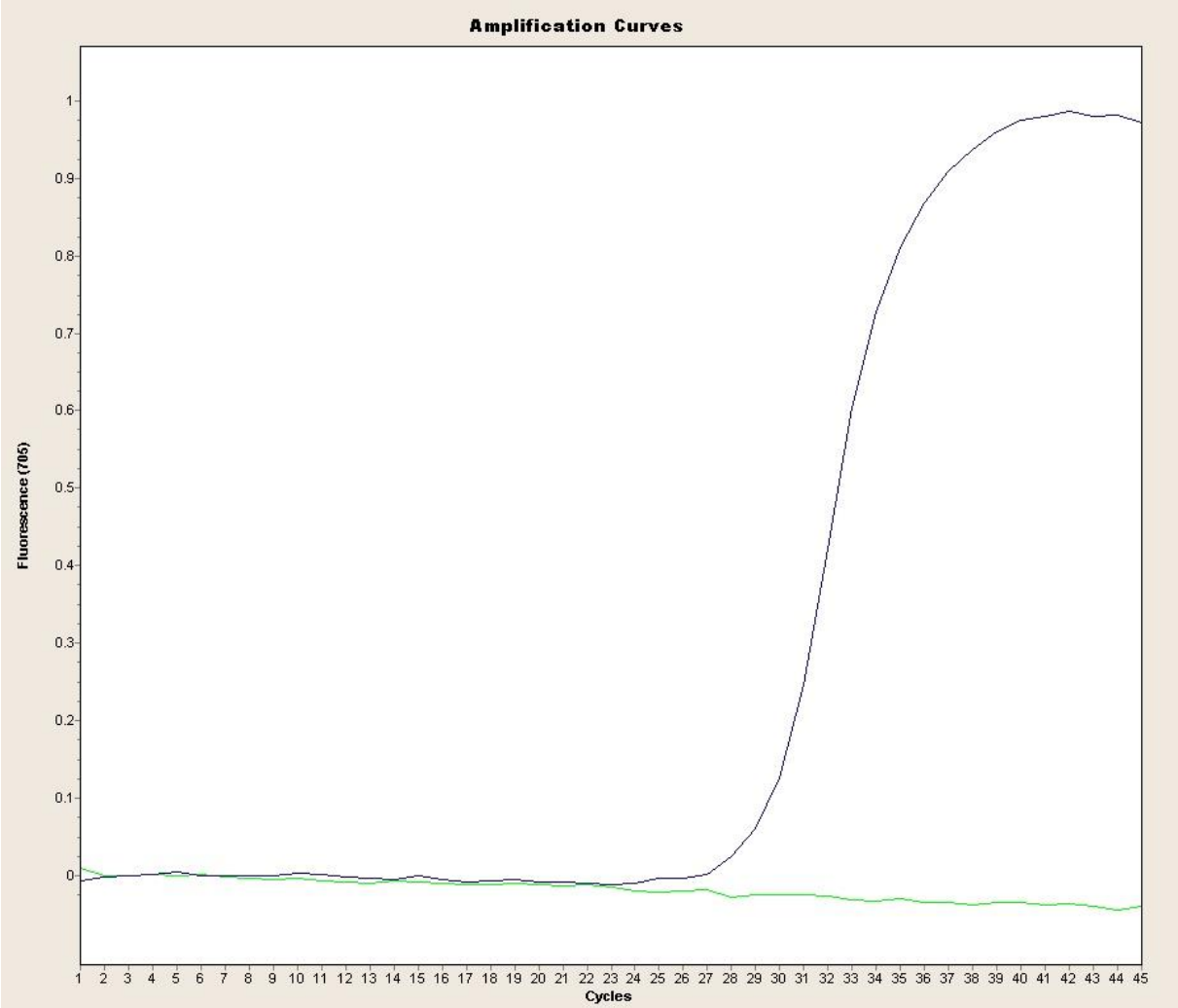


Fig.2: Correct run of the positive and negative control (Influenza B) on the LightCycler® 2.0



The result interpretation is done according to Table 6.

Tab.6: Sample interpretation

Target gene			
M-Gene (Influenza A)	**NP1-Gene (Influenza B)**	**ICR**	**Result**
positive	negative	**positive/negative**	**Influenza A**
negative	**positive**	**positive/negative**	**Influenza B**
negative	negative	**positive**	**Negative** (Target genes not detectable)
negative	negative	negative	**Not evaluable**

A sample is evaluated negative, if the sample shows no amplification signal in the detection system, but the Internal Control RNA (ICR) is positive. An inhibition of the PCR reaction or a failure in the extraction procedure can be excluded by the detection of the Internal Control RNA (ICR).

A sample is evaluated positive, if both, the sample and the Internal Control RNA (ICR), show an amplification signal in the detection system.

A sample is evaluated positive, if the sample RNA shows an amplification signal in the detection system, but the Internal Control RNA (ICR) is negative. The detection of the internal amplification control is not necessary, because high concentrations of the amplicon can cause a weak or absent signal of the internal amplification control.

A sample is evaluated invalid, if both, the sample and the Internal Control RNA (ICR) show no amplification signal in the detection system. The sample contained a RT-PCR inhibitor or a failure occurred in the extraction procedure. The extracted sample needs to be further diluted with PCR water (1:10) and re-amplified, or the isolation and purification of the sample has to be improved.

10. Performance characteristics

10.1 Analytical sensitivity

The RIDA®GENE Flu LC2.0 real-time RT-PCR has a detection limit of ≥ 50 RNA copies per reaction for Influenza A and Influenza B, respectively (see Fig.3, Fig.4).

Fig.3: Dilution series Influenza A (10^5 - 10^1 RNA copies / µl) on the LightCycler® 2.0

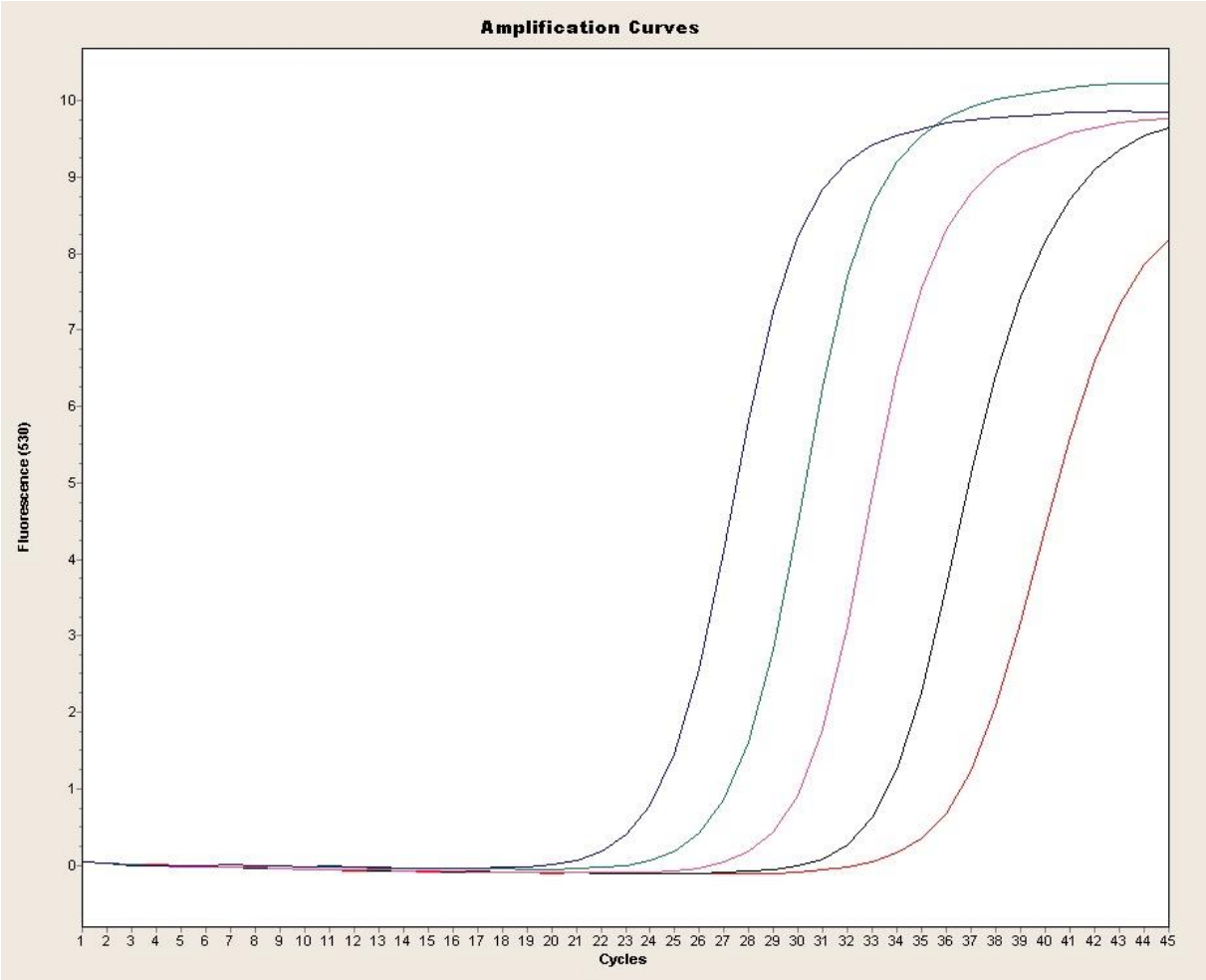
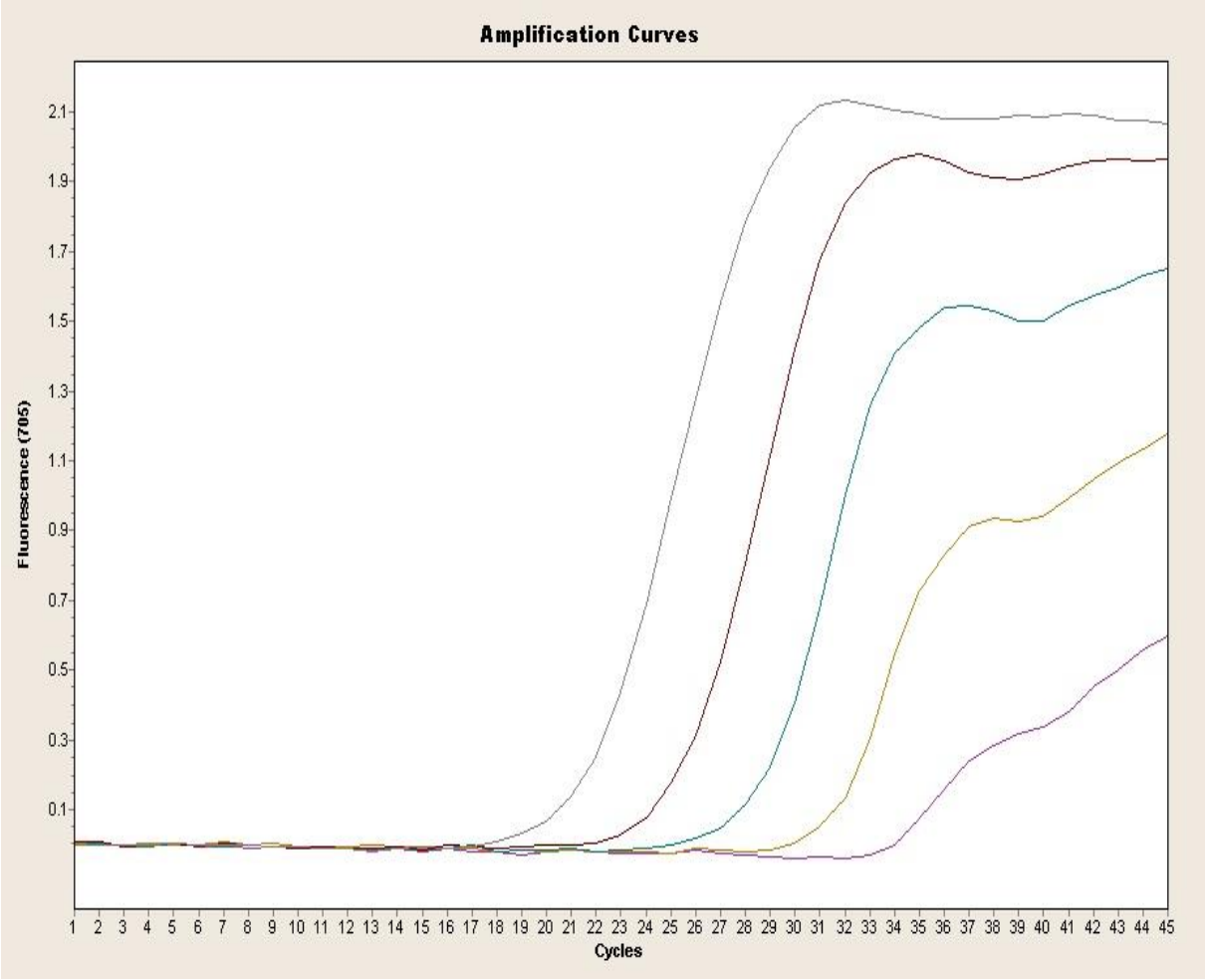


Fig.4: Dilution series Influenza B (10^5 - 10^1 RNA copies / µl) on the LightCycler® 2.0



The detection limit of the whole procedure depends on the sample matrix, RNA-extraction and RNA-concentration.

10.2 Analytical specificity

The RIDA®GENE Flu LC2.0 real-time RT-PCR is specific for influenza viruses (Influenza A and Influenza B). No cross-reaction could be detected for the following species (see Tab.7):

Tab.7: Cross-reactivity testing

Arcobacter butzleri	-	*Clostridium perfringens*	-	*Salmonella enteritidis*	-
Aeromonas hydrophila	-	*Clostridium sordellii*	-	*Salmonella typhimurium*	-
Adenovirus (Type 1, 4, 34)	-	Enteropathogenic *E.coli*	-	*Serratia liquefaciens*	-
Bacillus cereus	-	Enterotoxigenic *E. coli*	-	*Staphylococcus aureus*	-
Bacteroides fragilis	-	Shiga toxin producing *E.coli*	-	Methicillin-resistant *Staphylococcus aureus*	-
Campylobacter coli	-	*Enterobacter cloacae*	-	*Staphylococcus epidermidis*	-
Campylobacter jejuni	-	*Enterococcus faecalis*	-	*Shigella flexneri*	-
Candida albicans	-	*Klebsiella oxytoca*	-	*Vibrio parahaemolyticus*	-
Citrobacter freundii	-	*Proteus vulgaris*	-	*Yersinia enterocolitica*	-
Clostridium difficile	-	*Pseudomonas aeruginosa*	-		

10.2 Analytical reactivity

The reactivity of the RIDA®GENE Flu LC2.0 real-time RT-PCR was evaluated against multiple strains of influenza A and influenza B viruses (see Tab.8). All influenza A and influenza B strains of the panel were detected by the RIDA®GENE Flu LC2.0 real-time RT-PCR.

Tab.8: Analytical reactivity testing

Subtype	Strain	Influenza A	Influenza B
H1N1	Influenza A/Brisbane/59/2007	**positive**	negative
H1N1v	Influenza A/Bayern/63/2009	**positive**	negative
H1N1v	Influenza A/California/7/2009	**positive**	negative
H5N1	Influenza A/Chicken/Germany/R3294/2007	**positive**	negative
H3N2	Influenza A/Perth/16/2009	**positive**	negative
	Influenza B/ Brisbane/60/2008	negative	**positive**

11. Limitations of the method

1. The result of molecular analysis should not lead to the diagnosis, but always be considered in the context of medical history and symptoms of the patient.
2. This test is only validated for nasal and throat swabs.
3. This test is not intended to detect influenza C viruses.
4. Inappropriate specimen collection, transport, storage and processing or a viral load in the specimen below the analytical sensitivity can result in false negative results.
5. The presence of PCR inhibitors may cause invalid results.
6. Mutations or polymorphisms in primer or probe binding regions may affect detection of new influenza subtypes resulting in a false negative result with the RIDA®GENE Flu LC2.0 assay.
7. As with all PCR based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection (LoD) may be detected, but results may not be reproducible.

12. Literature

1. World Health Organisation 2009, Fact Sheet N°211, Influenza (Saisonal) www.who.int/mediacentre/factsheets/fs211/en/index.html. Accessed: 30.05.2012.
2. Center for Disease Control and Prevention 2008. Prevention and Control of Influenza: Recommendations of the Advisory Committee on Immunization Practices (ACIP) 2008.MMWR 57(RR07):1-60.
3. Robert Koch Institut. Arbeitsgemeinschaft Influenza. Bericht zur Epidemiologie der Influenza in Deutschland Saison 2010/2011.
4. World Health Organisation 2011, Manual for the laboratory diagnosis and virological surveillance of influenza.