

CENTRO DE INVESTIGACIÓN EN SANIDAD ANIMAL (CISA – INIA)	PROCEDURE FOR THE GENOTYPING OF AFRICAN SWINE FEVER VIRUS (ASFV) ISOLATES REV. 2018	SOP/CISA/ASF/GENOTYPING/1/ Page 1 of 8
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CENTRO DE INVESTIGACION EN SANIDAD ANIMAL (CISA-INIA)

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SOP/CISA/ASF/GENOTYPING/1

STANDARD OPERATING PROCEDURE FOR GENOTYPING OF AFRICAN SWINE FEVER VIRUS (ASFV) ISOLATES

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1. PURPOSE

The purpose of this procedure is to describe of molecular characterization of African swine fever virus (ASFV) isolates for genotyping purposes.

2. SCOPE

This procedure is applied to the ASFV DNA extracted following the procedure described in the **SOP/CISA/ASF/DNA EXTRACTION/1** (*“Standard operating procedure for the extraction of African Swine Fever Virus (ASFV) DNA”*) in any kind of porcine clinical sample such as EDTA-blood, serum and tissue homogenates and in cell culture supernatants.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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1. Arias, M., Sánchez-Vizcaino, J.M. (2012). African swine fever. In: Zimmerman, J., Karriker, L.A., Ramirez, A., Schwartz, K.J, Stevenson, G.W. (Eds), Diseases of swine, 10th Edition. John Wiley and Sons, United States of America, pp. 396-404.
2. Food and Agriculture Organization of the United Nations (FAO). AFRICAN SWINE FEVER: DETECTION AND DIAGNOSIS. A manual for veterinarians. FAO 2017
<http://www.fao.org/3/a-i7228e.pdf>
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3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure of samples processing for African swine fever (ASF) diagnosis (**SOP/CISA/SAMPLE/1**)
- Procedure for the extraction of African Swine Fever Virus (ASFV) DNA (**SOP/CISA/ASF/DNA EXTRACTION/1**)

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
Bp: base pairs
CVR: central variable region
DNA: Deoxyribonucleic acid
E+: ASFV positive extraction control
E-: ASFV negative extraction control
Kbp: kilobase pairs
PCR: polymerase chain reaction.
R+: ASFV DNA reaction positive control
R-: ASFV DNA reaction negative control
r.p.m.: revolutions per minute

4.2. BACKGROUND

Molecular epidemiology has proved useful in investigating epidemiological patterns of ASF as well as the likely origin of the disease when introduced into new regions. The ASFV genome consists in a double-stranded DNA molecule of 170 to 193 kilobase pairs encoding among 151 to 167 genes depending on ASFV strain. Restriction enzyme site mapping and sequence analysis of virus genomes have established that the central region is conserved but large length variations occur at the terminal ends, particularly within 40 kbp of the left end of the genome, but also within 15 kbp from the right end of the genome.

The current approach for ASFV genotyping is based on the analysis of four independent regions located at the conserved central area of the ASFV genome comprising; i) the **C- terminal end of VP72** coding protein gene, which differentiates up to **24 distinct genotypes** (Boshoff et al., 2007; Achenbach JE et al., 2016; Quembo et al., 2017), ii) the **intergenic region** located between the **I73R**

and **I329L** genes and characterized by the presence of tandem repeat sequences (TRS) which allow to **differentiate among genotype II ASFV strains circulating in Eastern Europe** (Gallardo et al., 2014), iii) the **central variable region (CVR)** within the *B602L*-gene (Gallardo et al., 2011) characterized by the presence of amino acid repeat sequences and the iv) the **full E183L-gene encoding the p54 protein** (Gallardo et al., 2009) as a valuable additional genotyping method for molecular epidemiological studies of p72 genotype I viruses, particularly in West Africa where this genotype predominates.

The CVR remains the genome target of choice when attempting to determine the origin and map the spread of closely related virus.

5. PROCEDURE DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Analytical Balance.
- Adsorbent paper.
- Conventional thermocycler with heated lid.
- Freezer <-10°C.
- Freezer ≤-70°C.
- Fridge 4±3°C
- Glass or plastic pipettes for volume of 1-10 ml.
- Heating block or water bath (72±2°C).
- Latex or nitrile gloves
- Microcentrifuge for eppendorf tubes.
- Microcentrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- Micropipette disposable tips with aerosol resistant filter of 1-20, 20-200 and 200-1000 µl, sterile
- Single channel pipette 1-10µl

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- Single channel pipette 10-100µl.
- Single channel pipette 10-200µl.
- Single channel pipette 200-1000µl
- Photograph camera and printer.
- Pipetboy acu or equivalent.
- Power supply.
- Tray for horizontal agarose gels, tank, combs, and powder leads.
- Tube racks.
- UV transilluminator.
- Vortex.

REAGENTS.

A) Reagents for DNA amplification step:

- AmpliTaq Gold® DNA polymerase with buffer II and Cl₂Mg [Ref.: N8080243 (Roche) or similar characteristics]. *Store at <-10°C.*
- **ASFV genotyping primers** *Store <-10°C in aliquots (expiry date: 1 year).* Three different set of primers are using to amplify three independent regions of the ASFV genome comprising:
 - ⇒ **p72- U** [5'- GGCACAAGTTCGGACATGT - 3']
 - ⇒ **p72-D** [5'- GTACTGTAACGCAGCACAG- 3']
 - ⇒ **ECO1A** [5'-CCATTTATCCCCGCTTTGG-3']
 - ⇒ **ECO1B** [5'-TCGTCATCCTGAGACAGCAG-3']
 - ⇒ **PPA89** [5'- TGTAATTTTCATTGCGCCACAAC - 3']
 - ⇒ **PPA722** [5'- CGAAGTGCATGTAATAAACGTC - 3'].
 - ⇒ **CVR1** [5'- ACTTTGAAACAGGAAAC (AT) AATGATG -3']
 - ⇒ **CVR2** [5'- ATATTTTGTAATATGTGGGCTGCTG- 3']
- Deoxyribonucleotide triphosphate (dNTP) mix containing 10 mM of each dNTP [Ref.: 11581295001 (Roche) or similar characteristics]. *Store at <-10°C.*
- Nuclease-free sterile H₂O, PCR grade.
- **Positive and negative controls:** the following controls must be included in each PCR run:

- ⇒ **E+→ ASFV positive sample target control for the extraction:** positive ASFV isolate. *Store <-10°C in aliquots (expiry date: 6 months).*
- ⇒ **E-→ negative sample control for the extraction:** distilled water which is included during the extraction process to exclude contaminations.
- ⇒ **R+→ASFV positive DNA target control for the reaction:** ASFV positive DNA. *Store <-10°C in aliquots (expiry date: 6 months).*
- ⇒ **R-→ negative DNA target control for the reaction:** distilled water which is included during the PCR process to exclude contaminations.

B) Reagents for amplified DNA detection step:

- Agarose MP 100 [Ref. 1 388 983001 (Roche) or similar characteristics]. *Store at room temperature.*
- Bromophenol blue [Ref.: 1.08122.0025 (Merck) or similar characteristics]. *Store at room temperature.*
- GelRed Nucleic Acid Gel Stain [ref 4003, Biotium or similar characteristics]. *Store at room temperature in aliquots of 100µl*
- Glycerol 87% [Ref. 1.4094.2500 (MERCK) or similar characteristics]. *Store at room temperature.*
- Molecular Weight Marker VI DNA [Ref.: 11062590001 (Roche) or similar characteristics]. *Store at <-10°C.*
- TAE buffer 50x (Tris base, acetic acid and EDTA) [Ref.: A16911000 (AppliChem) or similar characteristics]. *Store at room temperature.*
- Xylene cyanol [Ref.: X4126 (Sigma) or similar characteristics]. *Store at room temperature.*

5.2. PREPARATION

5.2.1. REAGENTS PREPARATION

- **Agarose 2% solution** → Dissolve 2gr (±0.1gr) of agarose MP in 100 ml of TAE 1x and heat in microoven until the agarose appears completely melted.

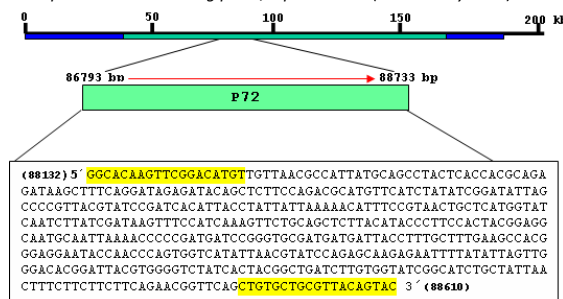
- **Loading sample buffer 6x** [bromophenol blue 0.25%, xylene cyanol 0.25%, glycerol 30%] → Dissolve 0.1gr (±0.01gr) bromophenol blue + 0.1 gr (± 0.01gr) xylene cyanol in 17.24 ml of glycerol. Adjust with distilled water to a final volume of 50ml. *Store at <-10°C in aliquots (expiry date: 1 year).*
- **Electrophoresis buffer 1x**→ Dilute 40 ml of TAE (50x) in 1,960 liter of distilled water. *Store at room temperature (expiry date: 2 months).*
- **Molecular Weight Marker VI DNA** → 200 µl of Marker VI + 200 µl of loading buffer 6x + 400 µl electrophoresis buffer 1x. *Store at 4°C (±3°C) (expiry date: 6 months).*

5.3. METHODS

5.3.1 DNA amplification procedures.

- A. **PCR amplification of the C-terminal region of p72 protein using primers p72- U and p72-D.** These primers amplify 478 bp from the protein p72 of the Ba71V ASFV isolate (*GenBank accession no. ASU18466*- Figure 1) and have been previously described by Bastos et al., 2003.

Fig. 1: Sequence obtained using p72U/D primers set (marked in yellow) inside P72 protein.



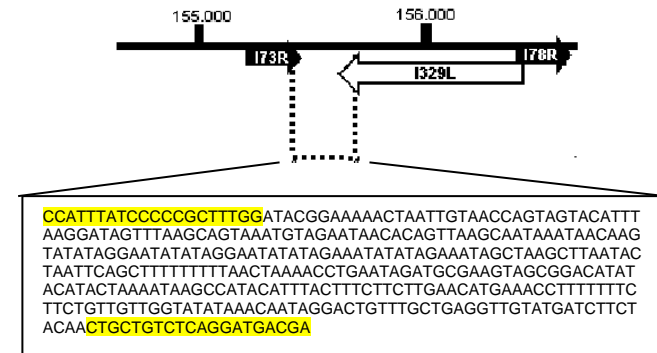
In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixtures described below for the number of samples to be assayed (including R+ and R- controls) allowing for at least two extra samples.

Pipetting step	MASTER MIX REAGENTS	1x VOLUME (reaction 25µl)	FINAL CONCENTRATION
1	H ₂ O	16.375µl	
2	PCR Buffer 10X	2.5µl	1X
3	Cl ₂ Mg 25 mM	2.5µl	2.5 mM
4	dNTPs 10 mM	0.5µl	0.2 mM
5	Primers p72U 20 µM	0.5µl	0.4 µM
6	Primer p72D 20 µM	0.5µl	0.4 µM
7	Taq Gold 5 U/µl	0.125µl	0.025 U/µl
Master mix volume		23 µl	

Add 2µl of DNA template to each PCR tube. Include R+ control and R- control

- B. **PCR amplification of the intergenic region located between the I73R and I329L genes.** These primers amplify 356 bp located between the I73R and I329L genes and characterized by the presence of TRS of the Gergia ASFV isolate (*GenBank accession no. FR682468.1*- Figure 2) and have been previously described by Gallardo et al., 2014).

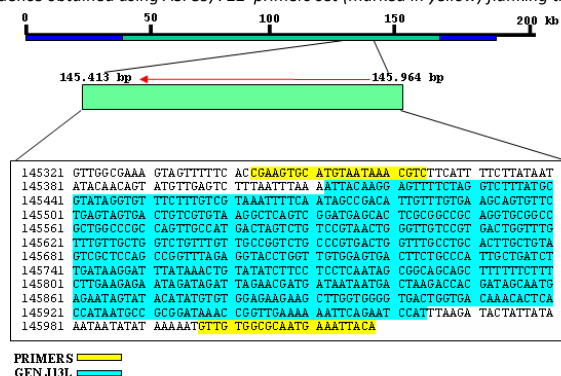
Fig. 2. Sequence obtained using Eco1A/B primers set (marked in yellow) in the Gergia ASFV strain.



In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixtures described below for the number of samples to be assayed (including R+ and R- controls) allowing for at least two extra samples.

- D. **PCR amplification of the full *E183L*-gene encoding the p54 protein using primers PPA89 and PPA722.** These primers amplify 676 bp flanking the complete VP54 sequence of the Ba71V ASFV isolate (*GenBank accession no. ASU18466*- Figure 4) and have been previously described by Gallardo *et al.*, 2009.

Fig. 4: Sequence obtained using ASF89/722 primers set (marked in yellow) flanking the P54 protein



In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixtures described below for the number of samples to be assayed (including R+ and R- controls) allowing for at least two extra samples.

Pipetting step	MASTER MIX REAGENTS	1x VOLUME (reaction 25µl)	FINAL CONCENTRATION
1	H ₂ O	16.375µ	
2	PCR Buffer 10X	2.5µl	1X
3	Cl ₂ Mg 25 mM	2.5µl	2.5 mM
4	dNTPs 10 mM	0.5µl	0.2 mM
5	Primers PPA89 20 µM	0.5µl	0.4 µM
6	Primer PPA722 20 µM	0.5µl	0.4 µM
7	Taq Gold 5 U/µl	0.125µl	0.025 U/µl
	Master mix volume	23 µl	

Add 2µl of DNA template to each PCR tube. Include R+ control and R- control

PCR CYCLE CONDITIONS.

PCR STEP	Temperature	Time	Nº cycles
Activation of TaqGold DNA pol	95°C	10 min	1x
DNA denaturation	95°C	30 sec	
Primer annealing	55°C	1 min	40 x
Elongation DNA	72°C	1 min	
Extra elongation step	72°C	10 min	1x
Hold at 4°C.			

Keep the amplified products at 4±3°C until proceed with the electrophoresis (maximun 18 hours).

5.3.2 Agarose gel electrophoresis

1. Make a **2% agarose solution in 1x TAE buffer**. Heat the solution in a microwave oven until the agarose appears completely melted and add the nucleic acid staining (Gel red) in 1/10,000 dilution. (Ex add 10 microlitres of Gel Red in 100ml of agarose). Shake carefully to homogenate.
2. Prepare the gel tray, sealing the ends and placing the adequate number of combs. Pour the melted agarose into the gel tray. Wait until the gel become solid (aprox 20 minutes).
3. Carefully remove the sealing of the tray and place it in the tank. Add the **electrophoresis buffer** until gel is covered. Remove carefully the combs.
4. Add **1 µl of 6x loading buffer** to 5 µl of the PCR amplified product.
5. **Load 6 µl of each sample** to one well of the gel.
6. Add **6µl of molecular weight marker DNA VI** to one well on each lane of the gel.
7. Connect to power supply (DNA samples will move towards positive electrode). Run the gel at a constant voltage of 150-200 volts for about 30 – 40 minutes.

Finally, the **sequence analysis of the intergenic region between the I73R and I329L genes (IGRI73R-I329L)** can allow to distinguish among ASFV strains circulating in the Eastern EU countries due to the insertion or deletion of the nucleotide tandem repeat (TRS) insertion (**GGAATATATA**) which is representative of all the ASFV isolates circulating within the EU countries Estonia, Latvia, Poland, Lithuania since the entrance of the disease in January 2014. This intergenic region

variant named **GII-IGRI73R-I329L-2** is also present in the Ukraine 2012 and 2015 isolates, in the Belarus 2013 viruses, in the Moldova 2016 and, since 2012, in Russian Federation where are co-circulating the two IGR variants (Gallardo et al., 2014; Goller et al., 2015).

Figure 7: Partial nucleotide sequence alignment of the intergenic region between 173R and I329L in African swine fever virus (ASFV) isolates from eastern and central Europe, including a virus isolated in 2007 in Georgia (Georgia2007; GenBank accession no. FR682468.1). The mutation that results in the insertion of a single nucleotide internal repeat sequence (GGAATATATA) is indicated by gray shading

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61                                     136                                     18
Georgia2007  TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Abx07        TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Arx07        TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Che07        TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Az08D        TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Az08B        TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Ing08        TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Oren08       TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
NO08/Av      TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
NO08/Ap      TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Dagestan09  TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
StPet09     TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Kalmykia09  TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Rostov09    TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Tver0511/Torjo TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Tver0312/Torjo TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Tver0312/Movo TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Ukr12/Zapo  TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Tver0712/Les TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Tver0812/Bolo TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Bel13/Grodno TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
LT14/1490   TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
LT14/1482   TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Pol14/Sz    TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
Pol14/Krus  TAGTTTAAAGCAGTAAATGTAGATAACACAGTTAAGCAATAAATACCAAGTATATAGGAATATATAGGAATATATA-----GAAATATATAGAAATAGCTAAGCTTAATACTAAT
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5.5. CRITICAL POINTS

Because of PCR is a highly sensitive technique, the most critical point along all the analysis procedure is the considerable risk of carry-over contamination, and the false positive results that could be obtained in this situation. The contamination could be due to the ASFV itself present in the positive analysed samples or in the positive controls included in the DNA extraction procedure; also, it could be due to ASFV DNA obtained after amplification and manipulated by agarose gel

electrophoresis during the amplicon analysis of a previous PCR. **It is mandatory that personnel working on PCR follow and carry out some strict work rules in order to minimize the contamination risk associated to PCR technique:**

- All steps of sample analysis by PCR should be performed in separate locations, using equipment and material specific for each one: sample preparation, DNA extraction, PCR mix preparation, and analysis of PCR products by agarose gel electrophoresis.
- Personnel must work always with clean nitrile or latex gloves in the PCR laboratory.
- Whenever personnel goes into a different PCR area, should be remove the gloves and take clean ones.
- The material will be of exclusive use for the PCR procedure step in which is located/labeled.
- Use a new pippete tip each time that a tube containing any sample or DNA is manipulated.
- Tubes containing amplified product should never be opened and manipulated in other laboratory distinct to that exclusively assigned to their analysis by electrophoresis.

5.6. SECURITY MEASURES

- Read and follow carefully the complete procedure.
- Keep reagents to the appropriate temperature before and after use.
- Do not pool reagents or instructions from different kits.
- Avoid any contamination of reagents.
- Do not use any reagent after it expiration date has passed.
- Do not eat, drink or smoke in the laboratory.
- Do not pipette by mouth.
- Wear always protective disposable nitrile or latex gloves.