STANDARD OPERATING PROCEDURE			
Title:	**Classical Swine Fever – Detection of CSFV RNA by Real-Time TaqMan PCR**		
Reference:	**European Union Diagnostic Manual, 2007 ; Hoffmann *et al*., (2005)**	*Author:*	**DR N N Barman, MS Elina Khatoon**

1. INTRODUCTION

Purpose/Scope of this SOP

1.1.1. This SOP outlines the method for the detection of CSFV RNA in blood and tissue samples. RNA is isolated from samples using the QIAGEN QIAamp® Viral RNA mini-kit or the conventional method using TRIZOL reagent, following which CSFV specific RNA sequences are detected using a commercial one-step quantitative RT-PCR assay.

2. MATERIALS

2.1. Documentation and software

2.1.1 7300 Real Time PCR system Software

7300 Real Time PCR system set up and users guide (APPLIED BIOSYSTEMS)

2.2. CHEMICALS AND REAGENTS

Reagent	**Supplier**
SuperScript™ III Platinum One-Step Quantative RT-PCR System	Invitrogen 100 reactions: 11732-020 500 reactions: 11732-088
Oligonucleotide primer CSF 100-F (20µM)	Sigma Genosys 5’ ATG CCC AYA GTA GGA CTA GCA 3’
Oligonucleotide primer CSF 192-R (20µM)	Sigma Genosys 5’ CTA CTG ACG ACT GTC CTG TAC 3’
CSF Hoffmann TaqMan dual labelled probe (5µM)	HPLC purified Sigma Genosys TGGCGAGCTCCCTGGGTGGTCTAAGT (MGB probe, Dye 6-FAM)

All PCR reagents are stored at -20ºC. Probes should be kept in the dark as much as possible.Primers purified either by desalting or HPLC purification are acceptable for this protocol.

2.3. EQUIPMENT

2.3.1	Consumables	Supplier
	Optical Tubes, 8/Strip	APPLIED BIOSYSTEMS
	Optical 96w plates barcoded	APPLIED BIOSYSTEMS
	Optical Flat Cap 8/Strip	APPLIED BIOSYSTEMS
2.3.2	**Equipment**	**Supplier, Model**
	Class 2 safety microbiological safety cabinet	LABCHEM & LABORTENIK INSTRUMENTS
	Micro-centrifuge	TARSONS
	7300 Real Time PCR system	APPLIED BIOSYSTEMS

3. PROCEDURE/METHOD

3.1.1. Extraction of total RNA by RNA-TRI reagent : (Approximately-2hours)

The procedure employed for total RNA extraction was as per manufacturer's recommendations.

The procedure is as follows:

- 200 µl of tissue homogenate (20% suspension in PBS, pH 7.2) was taken in 1.5 ml microfuge and 1 ml of RNA-TRI reagent (Ambion) was added, mixed vigorously by shaking and incubated at room temperature for 10 minutes for complete dissociation of nucleoproteins.
- Following incubation 200 µl of chloroform was added to the mixture and again incubated at room temperature for 10 minutes after mixing thoroughly for 15 seconds (Vortex).
- The mixture was then centrifuged at 12000xg at 4 ◌̊ C for 10 minutes and the colourless upper aqueous phase was transferred to a fresh micro centrifuge tube.
- Then 500 µl of isopropanol (Amresco) was added and incubated for 10 minutes. The samples were then centrifuged on 12000xg at 4 ◌̊ C for 10 minutes to pallet the total RNA.
- The RNA pallet was washed once with 1 ml 75% ethanol and thereafter, centrifuged at 7500xg at 4 ◌̊ C for 8 minutes.
- The supernatant was removed and the RNA pallet air dried at room temperature was resuspended in 20 µl of nuclease free water (Ambion)/DEPC treated water (Ambion).
- The RNA samples were properly labeled and stored at -20/-40 ◌̊ C, till further use.

3.1.2. Isolation of RNA from samples using kit

3.1.2.a Isolate RNA from samples using either the QIAamp® Viral RNA Mini spin protocol or the the conventional method using TRIZOL reagent.

3.1.2..b Once the RNA has been eluted, store at -18°C or below or keep on ice and proceed to CSFV RNA detection by RT-PCR.

3.2. Preparation of PCR master mix

3.2.1 Preparation of PCR master mix must be carried out in the PCR clean room.

3.2.2 Prepare a master mix containing the following reagents and preferably add the reagents in the order given.

Reagent	Volume per reaction
Water	4µl
2 x reaction buffer (Invitrogen)	12.5µl
50mM $MgSO_4$(Invitrogen)	1µl
ROX passive reference dye(Invitrogen)	0.5µl
CSF 100-F	1 µl
CSF 192-R	1 µl
CSF Hoffmann FAM probe	1 µl
SS III/Taq polymerase(Invitrogen)	0.5 µl
RNAase inhibitor (40U/µl)	0.5 µl

3.2.3 Thoroughly mix the master mix and aliquot 22µl into the appropriate number of Optical Flat Cap 8/Strip or plates.

3.3. Addition of templates

3.3.1. Add 3µl of the RNA samples to the 22µl master mix.

3.3.2 Once RNA is added, fit caps to all wells. It is important that the caps are fitted firmly and correctly onto the wells before being used on the real time machine.

3.4. Single Step TaqMan Real Time PCR: Reverse transcription and PCR

3.4.1 Incubate the reactions with the following thermocycling profile:

PCR step:		50ºC for 15 minutues
		95ºC for 2 minutes
	(x 50 cycles):	95ºC for 15 seconds
		60ºC for 1 minute

Collect fluorescence data during the 60ºC step.

3.4.2. The complete run takes approximately 1 hour and 20 minutes

4. RESULTS

4.1. Analysis and display of results

4.1.1 The fluorescence data can be viewed during, and after, the PCR reaction using the "Raw Data Plots" tab in the run section.

4.1.2 To analyse the data, select the "Analysis" button and select the wells to be examined in the "Analysis Section/Setup" window.

4.1.3 To view the results, click on the "Results" tab in the "Analysis" section and view the amplification plots.

4.2. Interpretation of Results

4.2.1 Analyse the data by comparing the results obtained for the samples with those obtained for the no template controls, positive controls and standard known quantities.

An increase in fluorescence will be observed at an early cycle for a positive sample (generally between 10 and 28). The no template controls and negative samples should not result in an increase in fluorescence above the baseline.

The cycle at which the increase in fluorescence occurs (the threshold cycle or Ct) is earlier (ie: lower) for samples with higher concentrations of CSFV RNA than for samples with lower concentrations of CSFV RNA.

B. Detection of CSFV RNA by Real-Time SybrGreen PCR

5. PROCEDURE/METHOD

5.1. Isolation of RNA from samples: Same as mentioned in 3.1.1 and 3.1.2.

5.2 Reverse transcription: cDNA preparation step:

Complementary DNA was synthesized using following reaction conditions:

Total RNA	6 µl
Random primer (50 ng/ µl)	1 µl
Nuclease free water to make	13 µl

The contents were mixed properly and spun briefly and placed in the thermal cycler for incubation on following condition:

70^0C	5 minutes
25^0C	10 minutes
4 ^{0}C	Hold

The contents were brought to the bottom of the tube by brief spinning. Thereafter, the following reagents were added to the mixture:

5X RT buffer	4 µl
RNAase inhibitor (1U)	1 µl
10mM dNTP mix	1 µl
M-MuLV RT (200U/ µl)	1 µl

The contents were mixed properly and spun briefly and placed in the thermal cycler for incubation on following condition:

25 ^{0}C	5 minutes (Primer annealing)
42^0C	1 hour (Extension)
70 ^{0}C	10 minutes (Inactivation of enzymes)
4 ^{0}C	Hold

The cDNA thus formed was stored at -20 ^{0}C after proper labeling till further use.

5.3. Preparation of PCR master mix:

Primer Identity	Sequence	Tm
*CSFL1	5'TGG GTG GTC TAA GTC CTG AGT-3'	59.8^0C
**CSFR1	5'- GTG TGA TTT CAC CCT AGC GA-3'	57.3^0C

5.3.1 Preparation of PCR master mix must be carried out in the PCR clean room.

5.3.2 Prepare a master mix containing the following reagents and preferably add the reagents in the order given.

Total reaction volume : 20µl	
Reagents/Ingredients	**Volume**
Nuclease free water	7µl
SybrGreen mastermix	10µl
Forward primer (5 pmol/µl)	0.5µl
Reverse primer (5 pmol/µl)	0.5µl
Thoroughly mix the master mix and aliquot 18µl into the appropriate number of Optical Flat Cap 8/Strip or plates. Add 2µl of the cDNA samples to the 18µl master mix. Once cDNA is added, fit caps to all wells. It is important that the caps are fitted firmly and correctly onto the wells before being used on the real time machine.	

5.4. SYBR GREEN BASED REAL TIME PCR

5.4.1. Place the reaction tubes or plate in to the 7300 Real Time PCR system (Applied Biosystems).

5.4.2. Incubate the reactions with the following thermo cycling profile:

PCR step:	Dissociation stage :
1. 94^0C for 5 min	1. 95^0C for 15 sec.
2. 40 cycles	2. 60^0C for 1 min.
94^0C for 30 sec	3. 95^0C for 15 sec.
60^0C for 1 min	4. 60^0C for 15 sec

Collect fluorescence data during the 60ºC step using ROX, FAM and HEX filters. The complete run takes approximately 2 hour and 10 minutes

NESTED TR-PCR (GEL BASED):

5.5. MATERIALS

5.6.1	**Consumables**	**Supplier**
	0.2ml PCR tubes	GENAXY
5.6.2	**Equipment**	**Supplier, Model**
	Class 2 safety microbiological safety cabinet	LABCHEM & LABORTENIK INSTRUMENTS
	Micro-centrifuge	TARSONS

VERITI 96-well Thermalcycler	APPLIED BIOSYSTEMS
Micropipette (0.5-10 µl, 10-100 µl, 20-200 µl, 100-1000 µl)	GENAXY (Nichipette)
Filter Tips	GENAXY

6. PROCEDURE/METHOD

6.1. Isolation of RNA from samples: Same as mentioned in 3.1.1 and 3.1.2.

6.2. Reverse transcription: Same as mentioned in 5.2.

TABLE 1: Primer sets (E2, 5' NTR and NS5B gene regions of CSFV) used:

Primer designation	Genomic region	Primer sequence	Nucleotide position	Reference
Prim I	E2-F	5'AGRCCAGACTGGTGGCCNTAYGA3'	2228-2250	Lowings *et.* al, 1996
Prim II	E2 -R	5 'TTYACCACTTCTGTTCTCA 3'	2898-2880	Lowings *et.* al, 1996
Prim III (Internal)	E2 -F	5' TCRWCAACCAAYGAGATAGGG 3'	2477-2497	Lowings *et.* al, 1996
Prim IV (Internal)	E2 -R	5'CACAGYCCRAAYCCRAAGTCATC3'	2748-2726	Lowings *et.* al, 1996
Prim V	5' NTR-F	5'CTAGCCATGCCCWYAGTAGG 3'	94-113	Greiser-Wilke *et.* al,1998.
Prim VI	5'NTR-R	5'CAGCTTCARYGTTGATTGT 3'	514-496	Greiser-Wilke *et.* al, 1998.
Prim VII (Internal)	5'NTR-F	5'AGCTCCCTGGGTGGTCTA 3'	146-163	Greiser-Wilke *et.* al, 1998.
Prim VIII (Internal)	5'NTR-R	5'TGTTTGCTTGTGTTGTATA 3'	417-399	Greiser-Wilke *et.* al, 1998.
Prim IX	NS5B-F	5'GACACTAGYGCAGGCAAYAG 3'	11138-11157	Bjorklund et al 1999
Prim X	NS5B-R	5'AGTGGGTTCCAGGARTACAT 3'	11586-11567	Bjorklund et al 1999

F= Forward Primer; **R**= Reverse Primer

6.3. Preparation of PCR master mix

6.3.1 Prepare a master mix containing the following reagents
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Reagent	Volume per reaction
Water, Nuclease-free	33.5µl
10x Buffer	5µl
25mM MgCl2	3µl
10 mM dNTPs	1µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
Taq polymerase	0.5µl

6.3.2. Thoroughly mix the master mix and aliquot 45µl into the appropriate number of 0.2ml PCR tubes.

6.3.3. Remove the tubes containing the PCR master mix and an appropriate number of tubes from the clean room

6.4. Addition of templates

6.4.1. Add 5µl of the cDNA samples to the 45µl master mix.

6.4.2 Once cDNA is added, fit caps to all wells, including empty wells if a PCR plate is used. It is important that the caps are fitted firmly and correctly onto the wells before being used on the real time machine.

6.5. Amplification of E2 Gene:

6.5.1. Place the reaction tubes or plate into the Thermal cycler (Veriti 96-well Applied Biosystems).

6.5.2 Incubate the reactions with the following thermo cycling profile:

PCR step:

1. 95^0C for 2 min
2. 34 cycles
   - 95^0C for 30 sec
   - 56^0C for 45 sec
   - 72^0C for 1 min
3. 72^0C for 1 min and hold at 4^0C

6.5.3. The complete run takes approximately 1 hour and 35 minutes.

6.5.4. For Nested PCR, the procedure was essentially the same except that the template cDNA was replaced by 5 µl of primary PCR amplicons and also the annealing temperature was kept at 58° C. The primers used in this case were internal primers (Prim III & IV) as described in the table.

6.6. **Amplification of partial 5' NTR:**

The procedure for the amplification of 5' NTR was also similar to that used for the amplification of E2 gene region, except for the annealing temperatures and the primer sets used.
For primary PCR, Prim V and VI (Table 1) were used at the annealing temperature of 58^0C and for nPCR the primers used were Prim VII and VIII (Table 1) at an annealing temperature of $56\ ^0$C.

The PCR amplicons thus obtained were stored at -20 ^{0}C, till further use.

6.7. **Amplification of NS5B gene :**

The procedure for the amplification of NS5B was also similar to that used for the amplification of E2 gene region, except for the annealing temperatures and the primer sets used.
For primary PCR, Prim IX and X (Table 1) were used at the annealing temperature of 56^0C .
The PCR amplicons thus obtained were stored at -20 ^{0}C, till further use.

7. Confirmation of PCR amplicons : Gel Electrophoresis

The confirmation of RT-PCR amplicons was carried out by their sizes in agarose gel. The PCR products were electrophorosed in 1.7% agarose gel containing ethidium bromide in 0.5X tris borate EDTA and visualized on a UV transilluminator as per standard procedures. For size comparision, a 100bp DNA ladder marker was run parallel to the PCR amplicons.
The Nested PCR amplicons of E2 and NTR show a product size of 271 bp while that of NS5B shows at 449 bp.

PREPARATION OF CHEMICALS:

1. 0.1% DEPC (diethyl poyrocarbonate):
DPEC……1ml
Distilled water to……..1000ml
All the plastic and glass wares to be used should be treated first with 0.1% DEPC overnight followed by autoclaving at 121^0C for 15 mins.

2. 0.5M EDTA:
Dissolve 18.61g of EDTA (disodium salt, dihydarte) in 80 ml of TDW by vigorous stirring, then adjust the pH to 8.0 with NaOH and autoclaved.

3. 10X Tris Borate EDTA (TBE) stock solution:
Tris base (hydroxyl methyl amino methane)…108g
Boric acid……..… 55g
0.5M EDTA……. 40ml
Dissolve in 800 ml of distilled water by stirring and adjust the volume to 1 litre by autoclaved distilled water.

Working concentration: 0.5X: 25ml of 10X TBE + 475 ml of TDW

4. Ethidium bromide (10mg/ml):
Ethidium bromide……..10mg
Distilled water to…….1 ml
Store at 4^0C

5. RANDOM PRIMER DILUTION (Fermentas)**:**
Concentration in stock = 200ng/µl
Working concentration = 50ng/µl
Dilution = 2 µl of Random Primer + 6 µl of nuclease free water

6. PREPARATION OF 10mM dNTPs (Fermentas)**:**
Concentration in stock = 100mM/ µl
Working concentration = 10mM/ µl
Dilution = 10 µl of each dNTPs + 60 µl of nuclease free water

7. RIBONUCLEASE INHIBITOR DILUTION (Fermentas)**:**
Concentration in stock = 40U/µl
Working concentration = 1U/µl
Dilution = 1 µl of ribonuclease inhibitor + 39 µl of nuclease free water

8. 75% ETHANOL:

Absolute Ethanol……....75ml
Triple distilled water……..25m