CYTOKINE EXPRESSION IN FOOT-AND-MOUTH DISEASE VIRUS INFECTION AND VACCINATION

PH. D. THESIS SUBMITTED

IN

VETERINARY VIROLOGY

By

AUDARYA SACHIN DIGAMBAR

Roll No - 1082

To

DEEMED UNIVERSITY

INDIAN VETERINARY RESEARCH INSTITUTE

IZATNAGAR-MUKTESHWAR

2010

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Thesis

Submitted in partial fulfillment of the requirement for the degree

OF

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PROJECT DIRECTORATE ON FOOT-AND-MOUTH DISEASE
Indian Council of Agricultural Research
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Dated: 26/7/2010

Dr. B. Pattnaik
Project Director

Certificate

Certified that the research work in the thesis entitled **"Cytokine expression in Foot-and-Mouth disease virus infection and vaccination"** submitted by **Dr. Audarya Sachin Digambar** (Roll No. 1082) for the award of the degree of Doctor of Philosophy of Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that **Dr. Audarya Sachin Digambar** has worked for more than 30 months in the institute and has put in more than 500 days attendance under me, from the date of registration for the degree of **Doctor of Philosophy** of the Deemed University as required under the relevant ordinance.

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Certificate

Certified that the thesis entitled **"Cytokine expression in Foot-and-Mouth disease virus infection and vaccination"** submitted by **Dr. Audarya Sachin Digambar** (Roll No. 1082) in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Indian Veterinary Research Institute, embodies the original work carried out by the candidate. The candidate has carried out the work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of the degree of **Doctor of Philosophy** of **Indian Veterinary Research Institute**.

It is further certified that the candidate has completed all the prescribed requirements governing the award of the degree of Doctor of Philosophy of the Deemed University.

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With the blessings of my parents and morale support from family members; Er. Rahul (brother - he sent few copies of printed figures) and Mr. Anil Joshi (uncle) and others it is possible for me to tread on the path of glory.

Audarya Sachin Digambar

INDEX

ABBREVIATIONS

A	Adenine
A	Allemagne
BAD	Bcl-2 associated death promoter
BAX	Bcl-2 associated X protein
Bo	Bovine
bp	Base pair
BRSV	Bovine respiratory syncytial virus
C	Cytosine
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CMI	Cell mediated immunity
CN	Catalogue number
Con A	Conconavalin A
CSF	Colony-stimulating factor
CT-1	Cardiotrophin-1
DIVA	Differentiation between infected and vaccinated animals test
DNA	Deoxyribonucleic acid
dNTP	2’ deoxyribonucleoside-5’ triphosphate
DPC	Days post FMDV challenge
DPI	Days post FMDV infection
DPV	Days post FMDV vaccination
ds	double stranded
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
FBS	Foetal bovine serum
FMD	Foot-and-Mouth disease
FMDV	Foot-and-Mouth disease virus
G	Guanine

GAPDH	Glyceraldehydes-3-phosphate-dehydrogenase
GDP	Gross domestic product
GMCSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodefeciency virus
HRPO	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN(s)	Interferon(s)
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IIL	Indian Immunologicals Limited
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl- β-D-thiogalactopyranoside
IRF	Interferon regulatory factor
IVD	Isovaleryl dehydrogenase
IVRI	Indian Veterinary Research Institute
LB	Luria-Bertani
LIF	Leukemia inhibitory factor
Log	Logarithm
LPBE	Liquid phase blocking enzyme linked immuno/immunosorbent assay
L^{Pro}	Leader proteinase
m	Messenger
MCP	Macrophage chemotactic protein
MDA	Malondialdehyde
ME	β-Mercaptoethanol
MHC	Major Histocompatibility complex
MMLV-RT	Moloney Murine Leukemia virus
MMR	Macrophage mannose receptor
mRNA	Messenger ribonucleic acid
MSR	Macrophage scavenger receptor

NCBI	National center for Biotechnology information
NF*K*B	Nuclear factor kappa B
NFW	Nuclease free water
NK	Natural Killer
NO	Nitric oxide
NSP	Non structural proteins
O	Oise
OD	Optical density
OIE	Office international des epizooties
OPD	Orthophenylenediamine dihydrochloride
OSM	Oncostatin M
PBL	Peripheral blood leucocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PDFMD	Project Directorate on Foot-and-Mouth disease
PN	Part number
r	Recombinant
RBC/RBCs	Red blood cells
RNA	Ribonucleic acid
RPMI-1640	Rosewell Parker Memorial Institute medium
RQ	Relative quantitation
RT	Reverse transcriptase
rt	Room temperature
RT-PCR	Reverse transcriptase – Polymerase chain reaction
SF	Superfamily
SOC	Solution of competence
SOD	Superoxide dismutase
T	Thymine
TAC	Total antioxidant capacity
TAE	Tris-acetate EDTA

TBE	Tris-borate EDTA
TGF	Tumour growth factor
Th	T helper
TLR(s)	Toll-like receptor(s)
TNF	Tumour necrosis factor
TSS	Transformation and storage solution
UK	United Kingdom
USA	United States of America
USP	United States Pharmacopeia
UV	Ultraviolet
VCAM	Vascular cellular adhesion molecule
WBC/WBCs	White blood cells
X-Gal	5-bromo-4chloro-3indolyl-β-galactopyranoside

Units

%	Percentage(s)
µg	Microgram(s)
µl	Microlitre(s)
^{0}C	Degree celcius
cm	Centimeter(s)
g	Gram(s)
hr(s)	Hour(s)
IU	International unit(s)
km	Kilometer
M	Molar
mA	Milliampere
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar
ng	Nanogram(s)
nM	Nanomole

rpm	Revolution per minute
s	Seconds
sq	Square
U	Unit(s)
V	Volts
v/v	Volume/Volume
w/v	Weight/Volume
ρM	Picomole(s)

Chemical formula

$C_{21}H_{20}BrN_3$	Ethidium bromide
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
KCl	Potassium chloride
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$Na_2HPO_4.2H_2O$	Disodium hydrogen phosphate
NaCl	Sodium chloride
$NaH_2PO_4.2H_2O$	Sodium dihydrogen phosphate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide

INTRODUCTION

“Well Begun is half done”

…Aristotle

Chapter-1

INTRODUCTION

Massive livestock population in India is a boon to the countrymen. Care of these animals is a responsibility, ably prosecuted by ancestors, which were evidenced from activities of '*Vedic era*'. India, mainly an agricultural nation, comprises of 28 states and 7 union territories elongated over 3.2,87,263 million sq km, which is inhabited by more than 1027.02 million human population. Broad 'Agriculture Sector' carves up over 25 % in total gross domestic product (GDP). Of the total GDP, the livestock sector contributes around 6.8 % which accounts about 33 % in the GDP contributed by 'Agriculture Sector'. A total of 8 % work force is involved in varied activities pertaining to livestock sector (Chilonda, 2005).

Contribution of cattle to the Indian economy lead to recognize the economy popularly, "*Cow economy or Dung economy.*" India enjoys number one position in dairy milk production in the globe with annual milk production of 106 million tons though average per head per year production of milk is far below compared to other developing and developed nations (Kaur, 2010). Of these 106 million tons of milk, share of cow, buffalo and goat is about 40 %, 55 % and 4 % in total milk production, respectively (Balaraman, 2006). Export in livestock sector fetched about Rs 34,776 million (1 % of the capital obtained from total export) of which value of exported bovine meat (70.5 %) and dairy products (0.13 %) was 70.63 % of the total export (Kumar, 2009). Average fiscal deficit from four major scourges of cattle was evaluated by Singh and Prasad (2008) and found to be highest due to Foot-and-Mouth disease (FMD), which was approximately three times more than the total of averages of fiscal deficits from other three bacterial diseases namely *Haemorrhagic Septicaemia*, *Black Quarter* and *Anthrax*. A total loss of about Rs 50,000 million is incurred to the Indian economy due to direct and indirect means by FMD (James and Rushton, 2002). Loss of Rs 240 to Rs 8720 per FMD outbreak in selected livestock population of Haryana was reported (Ahuja *et al.*, 1993). The tag of "***F**ast **M**oving **D**isease = **FMD**"* (Murphy *et al.*, 1999) which has negative impact on the economy of livestock owners attracts most of the attention of scientific communities

towards understanding the disease. Combating against FMD become more intense and critical as the virus is regaining its once lost territories; which was proved by an outbreak of FMD in cattle, goats and pigs of Southern Andaman after disastrous *Tsunami* on 26th December 2004 (Hemadri *et al.*, 2006; Sunder *et al.*, 2008). Close relation of bovine respiratory virus 2 and Foot-and-Mouth disease virus (FMDV) add new dimension in the control (Hollister *et al.*, 2008).

FMD is one of the most important list 'A' viral diseases of bovines in India. FMD is caused by an Apthovirus (ribonucleic acid (RNA) as genomic material) classified in the family *Picornaviridae* (OIE, 2009). The morbidity rate in an outbreak of FMD in susceptible animals can rapidly approach 100 % but few strains are limited in infectivity to particular species. Case fatality rate is very low in adult (2 %) unless in exceptional cases (Singh *et al.*, 2008) but in young animals it may reach upto 26 %. Severe outbreaks of more violent form may occur in exotic dairy calves where sometimes case fatality may reach upto 23 % (Radostits *et al.*, 2003). There are three Types ('O', 'A' and 'Asia 1') of FMDV present among livestock population in India (Annual Report, 2009). FMDV is known to spread by direct contact between infected and susceptible animals, by animal products such as meat and milk, by the airborne route, and mechanical transfer on people, wild animals, birds and by vehicles (Schijven *et al.*, 2008). Contaminated milk and animal products attract ban on export. People trespassing from infected premises passively transmit the virus to susceptible animals. *Outstandingly, person in contact with FMD infected animals can harbour the virus in their respiratory tract for at least a day* (Radostits *et al.*, 2003). FMD is extremely contagious in cloven-footed animals although African wildlife can experience an outbreak. Live infectious viral particles can survive upto 12 years in soil adhered to gumboot. At 4 ^{0}C virus may still be alive for a year in cell culture medium. Virus in aerosol form can travel beyond 250 km and cause infection. In asymptomatic carriers, persistence of FMDV in oro-pharynx leads to multiplication and excretion of virus and setting up of initial foci of infection. Even in vaccinated animals few may become carrier (Zhang *et al.*, 2009).

Technology is vital in combating dangers posed by existing and newer form of diseases (Kalam and Rajan, 1998). Various methods based on serology as well as molecular biology, have been pioneered in the abroad and also in the country to diagnose FMD (Ferris and Dawson, 1988; Archetti *et al.*, 1995; Pattnaik *et al.*, 1997;

Mackay *et al.*, 1998; Moss and Haas, 1999; Bergmann *et al.,* 2000; Mackay *et al.*, 2001; Reid *et al.*, 2002; Sanyal *et al.*, 2003; Giridharan *et al.*, 2005; Mohapatra *et al.*, 2006; Parida *et al.*, 2006a; Paton *et al.*, 2006; Mohapatra *et al.*, 2007; Muthukrishnan *et al.*, 2008). Availability of wide range of diagnostics lead to upsurge in the number of FMD cases identified which in turn benefit in the implementation of chimerical task of FMD control by collage of means and measures including vaccination in the country. One of the extension study indicated that 60 % respondents were known of vaccination against FMD and among total animal population only 8 % were vaccinated against FMD (Singh and Chander, 2005). More impetus is needed to spread awareness among livestock owners about FMDV vaccination and its benefits. Although few tests (Marquardt *et al.*, 1995; Parida *et al.*, 2005) have been reported to diagnose asymptomatic/persistent animals, early diagnosis in such cases is still difficult. In vaccinated animals virus localize in the oro-pharynx because of circulating neutralizing antibodies (Salt, 1993). Infection with FMD confers protection by inducing good humoural antibody response. Although antibodies produced in serum are serotype specific, T cells cross react with other serotypes in FMDV vaccinated or infected bovines (Mahy, 2005). Serum samples obtained from animals in first few days of clinical infection proved inconclusive in differentiation between infected and vaccinated animals (DIVA) test. In this scenario, it has been felt that cytokines might have been playing certain role either by modulating immune responses or by unknown means. Few reports are available on detection of FMDV induced cytokines in India (Yadav *et al.*, 2004; Alluri, 2009).

Cytokines are secreted in response to discrete stimuli and secretion is short lived, ranging from a few hours to a few days. Cytokines exhibit attributes of pleiotropy, redundancy, antagonism, synergy and cascade induction which permit them to regulate cellular activity in a co-ordinated, interactive way (Kindt *et al.*, 2007). A protein secreted from a cell in order to induce specific responses in other cells having specific receptors for it is termed a cytokine. Cytokines play a pivotal role in regulating immune responses of infected or vaccinated animals. Fate of the infectious process is also governed by antiviral cytokines induced after infection. Three primary classes of cytokines were divided into pro-inflammatory, anti-inflammatory and chemokines (Flint *et al*., 2004). Cytokine interleukin-1 (IL-1), tumour necrosis factor (TNF), IL-6 and IL-12 are examples of pro-inflammatory which promote leukocyte activation, while others IL-10, IL-4 and

tumour growth factor (TGF) β are anti-inflammatory and suppress activity of pro-inflammatory cytokines and return system to basal “circulate and wait state”. IL-8 is a chemokine.

Species specific interferons (IFNs) are produced immediately by virus infected cells so that body will get some time to take proper account of pathogen and build up specific immune responses. Suppression of viral replication in primed cells as well as their growth; macrophage, natural killer (NK) cells and T lymphocyte activation; stimulation of major histocompatibility complex (MHC) I and MHC II antigens and Fc receptors and production of fever are some of the functions of IFNs. IFNs are stable at acidic pH and in high dilutions they can be biologically active as well. There are two kinds of IFNs; Type I and Type II. Type I IFNs (α secreted by leukocytes and β by fibroblasts) are encoded by separate cellular genes, having same size and similar effects generally. Type II IFN (γ secreted by T and possibly by B lymphocytes) is known to modulate immune responses along with many other cytokines including antiviral TNF-α. Slowly replicating viruses are responsible in greater production of IFN than the rapidly replicating. RNA acts as inducer of IFN. It has been reported that the single molecule of RNA can be responsible for production of IFN in a cell (Wagner and Hewlett, 2004).

IL-8, a chemokine, recruits immune cells during early stages of immune response which acts on two different chemokine receptors; CXCR1 and CXCR2 to initiate neutrophil migration. In some tissues different sets of chemokines cause local accumulation of different groups of leucocytes. Chemokines and its receptors in disease conditions are being explored. Adhesion molecules (intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1) are induced at site of inflammation by TNF-α, IL-1 and IFN-γ. Lymphocytes lose some of their surface molecules while they leave the blood vessel (L-Selectin). Granules of cytotoxic T cells contain perforin and granzymes which are involved in destruction of pathogen infected cells. Self or non-self recognition of nucleic acids in viral infection is relied on several PRRs including toll-like receptors (TLRs). Double stranded RNA with the help of TLR7 activates immune cells to secrete cytokines. Investigations on TLRs involved in viral infections and vaccinations are increasing. TLR2, TLR4, TLR7, TLR8 and TLR9 are important in viral infections (Koyama *et al.*, 2008). Research is undergoing to understand novel cells involved in innate immune response and production of cytokines (Moro *et al*.,

2010; Strober, 2010).

Considering the above-mentioned facts the study is planned with following objective - To understand and analyze expression of various cytokines involved in the Foot-and-Mouth disease virus (FMDV) infection vis-à-vis vaccination so that will be of help in differentiating pathogenesis of the disease from immunization.

REVIEW OF LITERATURE

"I keep six honest serving men;
Their names are What and Why and
When and How and Where and Who"

…Rudyard Kipling

Chapter-2

REVIEW OF LITERATURE

Since, first notice of interferons by Isaacs and Lindenmann in 1957 (Flint *et al*., 2004), workers are engaged unrelentedly to unearth new cytokines, its receptors, cells involved in its secretions, mechanism of its actions and use in therapeutics. Marc Feldmann and Ravinder Maini awarded with Dr. Paul Janssen 2008 award in biomedical research for their role in discovery of tumour necrosis factor (TNF)-α as an effective target for treatment of autoimmune diseases.

Information is available on cytokines, chemokines, toll-like receptors and various other genes involved in viral infections to defend host body. Interferon (IFN)-α/β promotes expression of class I major histocompatibility complex (MHC) molecules which are important for presentation of cytoplasmic antigens to cluster of differentiation (CD)8 T cells. Few viruses produce large quantities of IFN-α/β (Type I) downregulating interleukin-12 (IL-12) productions leading to downregulation of the disease. In contrast some viruses are responsible for production of IL-12 and activating natural killer (NK) cells which produce IFN-γ. IFN-γ together with IL-12 aggravates disease. Viral replication in infected cells yields double stranded ribonucleic acid (dsRNA) which is a potent inducer of IFN-α/β. IFN-α/β levels in serum are increased in clinically severe Ebola and chronic human immunodeficiency virus (HIV) infections. Early IFN-γ secretion is detected in mouse and human viral infections except acute simian immunodeficiency virus infection. IFN-γ levels after vaccination against Yellow fever virus and in asymptomatic Ebola virus infections were undetectable. IFN-γ induce expression of both class II and class I molecules and induce inducible nitric oxide synthase (iNOS) enzyme which in activated macrophages catalyzes production of nitric oxide (NO) which react with molecules mediating antiviral activity by modifying proteins essential to viral functions. IFN-γ enhances TNF response by upregulating TNF receptors. IFN-γ induces chemokines in infected tissue which may recruit activated cells to bring antiviral effector mechanism. TNF-α mediate cytotoxic and necrotic effects and enhance inflammation. TNF-α enhance production of IFN-γ responses, upregulation of class I MHC expression and activation of antiviral pathways. High systemic levels of TNF-α promote fatal toxic shock syndrome.

T helper (Th) 1 and Th2 differentiating responses in mice can be used to determine the direction of immune response but in cattle and humans it fails to explain many immune cell reactions to pathogenic challenges (Mosmann *et al.*, 1986; Brown *et al.*, 1997). Mice lacking in particular cytokine gene show deficient antibody response. Cytokines not only determine the types of antibodies produced but also provide amplification mechanisms. Antigen recognition of B cells enhances the expression of receptors for cytokines. Cytokines act on each stage of B cell activation (proliferation, antibody secretion and isotype switching). IL-2, IL-4 and IL-5 contribute in B cell proliferation and act synergistically. IL-6 is a growth factor for already differentiated, antibody secreted B cells. IL-1, IL-10 and TNF promote some B cell proliferation. IL-4 and IL-5 are major inducers of antibody secretion by B cells in mice. In contrast to mice, humans B cells secrete high level of antibody in response to IL-2, IL-6 and IL-10. Controlling of the pattern of heavy chain isotype is most selective and only obligatory function of different cytokines in humoural immune responses. IL-4 is the principal switch factor for immunoglobulin (Ig) E. IFN-γ inhibits IL-4 induced B cell switching. IL-4 reduces IgG2a production which is regulated by IFN-γ. Tumour growth factor (TGF)-β and IL-5 stimulate IgA production in mucosal lymphoid tissues. Increased levels of TNF-α, IL-1 and IL-6 are linked to the immunopathogenicity. Though, IL-6 with the ability to cause secretion of glucocorticoids might limit the inflammatory reactions. EBV LMP1 acts to induce IL-12p40 and IL-12p35 gene expression. Nef protein of HIV stimulates IL-15 along with TNF-α, IL-6 and IFN-γ. Expression of IL-12 receptor is promoted by IFNs. But at high concentrations it also inhibits IL-12. Interferon regulatory factors (IRFs) are needed for induction of IFNs. Constitutive IL-15 expression is dependent on IRF-1 which can be activated by stimulation with IFN-α, IFN-β or IFN-γ. Cytokine production (IL-1, IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ) is inhibited after administration of drug steroid (Abbas *et al*., 1997; Fujinami, 2001; Kauffmann *et al.*, 2002; Roitt *et al.*, 2002; Tizard, 2004; Willey, 2008).

Mitogens are used in stimulation and proliferation of cells by researchers (Tomar *et al.*, 1995; Benveniste *et al.*, 1996; Stabel, 2000; Rajak, 2007). Reporters have found increase in certain cytokines after *in-vitro* stimulation of cells with live or heat-inactivated virus when tested by real-time PCR. Viral proteins of BRSV, G and F are known to increase production of IL-10 response in macrophages and others (Konig *et al.*,

1996; Byrnes *et al.*, 1999). Degradation of nuclear factor kappa β during Foot-and-Mouth disease virus (FMDV) infection was also reported (Santos *et al.*, 2007). IL-1β production in peripheral tissue was associated with hyperalgesia associated with fever (Morgan *et al.*, 2004). Cellular genes which are affecting the infectivity of FMDV were studied (Piccone *et al.*, 2009). IL-2 and IFN-γ kinetics were studied in bovine tuberculosis (Rhodes *et al.*, 2000). Mammalian toll-like receptors (TLRs - TLR3, TLR7, TLR8 and TLR9) initiate immune responses to infection by recognizing microbial nucleic acids (Akira *et al.*, 2001; Iwasaki and Medzhitov, 2004). TLR7 and antiviral immunity is also described (O'Neill, 2002). TLRs 1-10 have been identified in bovine and ovine tissues (Menzies and Ingham, 2005).

IL-12, IL-23 and IFN-γ form a loop and play important role against several viruses in mice than in humans (Novelli and Casanova, 2004). Both, IFN-α and IFN-β are powerful first line defense against pathogens and have potent immunomodulatory activities (Valarcher *et al.*, 2003). IFN-γ and IL-12 are required for the activation of $CD4^+$ T cells (Fujinami, 2001). Interferons (IFN-α, IFN-β, IFN-γ) may be involved in the host defense against FMDV infection (Grubman and Baxt, 2004). Plaque formation by FMDV in cell culture was linked to suppression of interferons; IFN-α and IFN-β (Chinsangaram *et al.*, 1999). Porcine interferons have implicated in anti FMDV activity and protection (Moraes *et al.*, 2007; Yao *et al.*, 2007). Bogdan and others (1992) opined that IL-10 was responsible for degrading cytokine transcripts by virtue of induction of endoribonucleases.

Reports on dynamics of white blood cells (WBCs) in the blood after FMDV infection and also vaccination against Foot-and-Mouth disease (FMD) in cattle are scanty. Selective lymphocyte depletion in the early stages of the immune responses to FMDV in swine was reported. FMDV in swine causes transient immunosuppression, weaken the immune system and spreads (Segundo *et al.*, 2006). In Indian cattle and buffalo there was transient but marked decrease in the circulating leukocyte and monocyte levels on 1 day post FMDV infection (Mohan *et al.*, 2008).

Vitamin D3 is linked to suppression of pro-inflammatory cytokines in humans. Yeotikar and others (2003) gave metabolic profile of healthy and FMDV affected cattle. IL-6 secretion changes during the day or night (Vgontzas *et al*., 2005). Elitok and others (2005) reported significant difference in the iron, copper and zinc values between the

healthy and FMDV affected sheep. Disturbed oxidative status and sub-functional ovarian activity in female buffaloes after FMDV infection were observed. Concentration of Malondialdehyde (MDA) increased while Superoxide dismutase (SOD) and Total antioxidant capacity (TAC) decreased significantly in FMDV infected buffaloes (Zaher and Ahmed, 2008). Oxidative stress can affect signal transcription and gene expression (Sen and Packer, 1996). Involvement of nutritional, metabolic and other miscellaneous factors influencing on expression of cytokines in FMDV infection and vaccination is rarely studied.

Liquid phase blocking enzyme linked immunoassay (LPBE) was used for assessment of protective antibody in the serum after vaccination. LPBE is the prescribed test (OIE, 2009) for FMD serology and adopted worldwide for routine serum testing. Differentiation from infected and vaccinated animals (DIVA) test is widely used around the globe and it was recently developed in the laboratory. Purity of vaccines is also a factor which may influence immune response and ultimately expression of cytokines. Removal of non structural proteins (NSPs) enables the immune responses of vaccinated animals to be distinguished from infected animals (Barteling, 2002). NSPs (not serotype specific - 2C, 3A, 3D, polyprotein 3 ABC of FMDV) were used in the development of DIVA which is simple, rapid and reproducible. Antibody to 3ABC is a most reliable indicator of FMDV infection in both bovine and porcine serum samples. Though, absence of antibodies to any or all NSPs does not provide definitive evidence that the animal has never been infected with FMDV. Repeated vaccinations may produce detectable antibodies against NSPs (Mackay *et al.*, 1998). Vaccines against FMD consist of semi-purified, chemically inactivated FMDV eliciting antibodies to structural proteins. Depending on the purification and concentration procedure there will be presence of variable amount of NSPs in the vaccines. Adjuvant (Aluminium hydroxide, saponin or mineral oil) may enhance the immune responses to NSPs. Vaccines concentrated by ultracentrifugation do not increase the immune response to NSPs 2C and 3 ABC (Lubroth *et al.*, 1998).

With the advent of recombinant molecular biology techniques and sequencing methods (Murphy *et al.*, 1999) nucleotide sequence data in the form of full sequences or shorter expressed sequence tags is readily available.

2.1 Cytokines, chemokines, toll-like receptors and others in FMDV infection and vaccination

Lal and others (1983) concluded that the chicken interferon did not inhibit the uptake of FMDV or intracellular degradation but able to block synthesis of viral progeny in BHK-21 (Razi) cells. Repeated doses of chicken interferon administered *in-vivo* conferred protection in guinea-pigs from primary as well as secondary lesions after infection with virulent strain of Type 'O' FMDV.

McCullough and others (1992) showed that when recombinant and pure "natural" IL-1 and IL-2 used together with the FMDV antigen there was increased immune response against the antigen.

Valcarcel and others (1996) demonstrated that the vaccination with single serotype of FMDV induced a serotype cross reactive proliferation of T cells and opined that the initial response to infection and vaccination may be directed towards activation of Th 2 cells.

Barnett and others (2002) stated that the cytokines IL-6, IL-8 and IL-12 play a critical role in innate immune response in early protection against FMD after FMDV emergency vaccination. IL-6, IL-8 and IL-12 were consistently detected in challenged animals. Not detection of IL-1, IL-2, TNF, TGF and IFNs was indicative of failure to produce systemic inflammatory response or increase in T lymphocyte activity after vaccination. Monocytic cell activity indicated by the production of IL-6, IL-8 and IL-12, appears to play a critical role in FMDV emergency vaccine induction of the innate immune defences.

Murphy and others (2002) studied expression of IFN (α, β and γ), IL-1α and TNF-α in experimentally FMDV infected pigs by real time revese transcriptase-polymerase chain reaction (RT-PCR) keeping glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) as housekeeping gene.

Wong and others (2002) observed increased immunogenicity in swine vaccinated with both the plasmid deoxyribonucleic acid (DNA) vaccine candidate and plasmid encoding the swine IL-2 complementary (c) DNA togetherly.

Zhang and others (2002) demonstrated that FMDV replication was affected by IFN-γ and continuous treatment with IFN-γ restricted FMDV replication or cured

persistently infected bovine epithelial cells and concluded that IFN-γ mediated pathway may be involved in the *in-vivo* clearance of FMDV.

Cox and others (2003) reported increased levels in vaccinated pigs of IL-6, IL-8, and in some pigs IL-12. High potency vaccines may promote longer lasting immunity than the conventional lower potency vaccines in ruminants hence for preventive purposes these vaccines may be cost-effective.

Rigden and others (2003) showed that after FMDV vaccination there was no specific antibodies produced or increase uptake and destruction of opsonised virus by macrophages at 3 to 6 days post vaccination (DPV). No change was observed in the peripheral blood leucocyte (PBL) subpopulations and vaccine-induced production of acute phase proteins. Chemotaxis assays identified vaccine related increase in PBL migratory activity indicating elevated chemotactic potential in serum samples from 3 DPV.

Yadav and others (2004) concluded that the vaccination along with recombinant human IL-2 enhanced specific immune response against FMDV (Types - 'O', 'A_{22}', 'Asia 1') in mice.

Barnard and others (2005) reported messenger (m) RNA level of five cytokines by real time RT-PCR in peripheral blood mononuclear cells (PBMCs) of pigs after emergency vaccination against FMD. Virus specifically induced mRNA of IFN-γ, IL-2, IL-4 and IL-6 while IL-10 was induced by virus as well as mock antigen. Emergency FMDV vaccine activates both Th1-like and Th2-like responses without lymphopenia and inflammatory responses.

Bautista and others (2005) analyzed the innate immune response of freshly isolated swine skin dendritic cells to FMDV. Speedy induction of IFN-β mRNA was noticed compared to IFN-α. These cells secreted IFN-α and IFN-β proteins in response to the live virus. Dendritic cells constitutively expressed intracellular IFN-α protein without stimulation. These cells express and store IFN-α in uninfected animals and may play a role in the innate response of swine to viral infection.

Borrego and others (2006) observed that DNA vaccine based on single FMDV B and T cell epitopes can protect mice, in the absence of specific antibodies at the time of challenge.

Botton and others (2006) examined adjuvant effect of porcine interferon alpha in swine after vaccination with a replication-defective adenovirus containing FMDV. Addition of IFN resulted in a delayed onset of lesions except in one animal which had detectable viremia. Four of five pigs vaccinated with high-dose replication-defective adenovirus containing FMDV plus IFN were completely protected from disease and only one animal had a lesion which was restricted to the site of challenge. Porcine interferon alpha may be a potential adjuvant in FMD vaccination strategies.

Li and others (2006) reported that P1 DNA plasmids and p granulocyte macrophage colony stimulating factor (GMCSF). GMCSF adjuvant plasmids induced stronger FMDV specific and neutralising antibody responses and promoted IL-8 and IFN-γ secretion in immunised pigs.

Parida and others (2006b) estimated IFN-γ produced from whole blood samples of FMDV vaccinated or control cattle before and after challenge by enzyme linked immunoassay (ELISA). Blood samples were stimulated by inactivated FMDV antigen. In vaccinated, infected and challenged cattle specific stimulation of IFN-γ was noticed.

Zhang and others (2006) measured expression of mRNA of six different cytokines (IFN-α/β and γ, IL-2, IL-1α and TNF-α) as well as two toll-like receptors (TLR3, TLR4) by real-time RT-PCR. In acute stage of infection, there was increased mRNA expression of IFN-α, IFN- γ and IL-1α, out of which mRNA expression of IFN-α was significantly higher. Expression of mRNA of TNF-α was recorded higher in carrier cases than non-carrier. Increased expression of TLR 4 was observed in acute cases than healthy cattle.

Cheng and others (2007) studied on adjuvanticity of IFN-α for recombinant FMD protein vaccine. Strong FMDV specific neutralizing antibody and significant T cell mediated immune response was observed after vaccination with recombinant protein FMD vaccine.

Du and others (2007) used two recombinant adenoviruses expressing VP1 epitopes fused with porcine GMCSF. Increased levels of FMDV-specific T cell proliferation, IFN-γ and IL-4 was observed. There was protection from viral challenge though neutralizing antibody titers were lower.

Shi and others (2007) observed enhanced immune response after inoculation of the fusion product recombinant (r) VP1/bovine (Bo) IL-18 and suggested that the rBoIL-18 has a potential to enhance the efficacy of vaccination against FMDV. Expressed

recombinant VP1 (rVP1) proteins produced humoural and marginal cell-mediated immune responses in mice. When expressed VP1 proteins inoculated with rBoIL-18 there was increased humoural and cell mediated immunity (CMI).

Greenwood and others (2008) used nano-beads to target FMDV-specific synthetic peptides to dendritic cells to induce immune responses in sheep. Single peptides produced immune responses in most sheep; however combination of multiple peptides induced significant cell-mediated and humoural immune responses.

Nirmala (2008) stimulated whole blood with specific FMDV antigens (Type 'A', Type 'O' and Asia '1') to assess CMI in naïve, vaccinated and infected cattle. 20 µg of FMDV antigen was added in 100 µl of heparinated blood and incubated at 37^0C. After 48 and 72 hrs of incubation serum was harvested and used in Sandwich ELISA. IFN-γ specific CMI response was found only in FMDV infected cattle. Whole blood of naïve animals and vaccinated animals failed to respond to any of the three FMDV antigens. Priming with high dose of FMDV antigens may elicit T cell responses and regular vaccination with low dose of antigens may not elicit IFN-γ response in cattle.

Su and others (2008) studied effects of IL-6 and TNF-α on immune responses to FMDV. These two cytokines, thought to enhance the antigen-specific cell-mediated responses when used as molecular adjuvants along with FMDV VP1 DNA vaccine. Compared to the group immunized with VP1 alone, besides induction of maturation of the dendritic cells, co-inoculation with either IL-6/TNF-α resulted in higher ratio of IgG2a/IgG1, higher levels of expression of IFN-γ in $CD4^+$ and $CD8^+$ T cells, IL-4 in $CD4^+$ T cells and *in-vivo* antigen-specific cytotoxic response.

Summerfield and others (2008) reviewed on the topic of innate immune responses against FMDV. FMDV is sensitive to the action of IFN. To escape from the innate immune response FMDV can shut down cellular protein synthesis in susceptible epithelial cell which leads to evolution of FMDV.

Wang and others (2008) stated that the intranasal administration of FMDV DNA vaccine using chitosan as delivary vehicle and a construct expressing IL-15 as the molecular adjuvant enhanced mucosal and systemic immune responses in animals. Higher level of mucosal IgA and serum IgG was observed in the group vaccinated with molecular adjuvants. Intranasal delivery of the IL-15 construct with FMDV DNA vaccine significantly enhanced the CMI compared to the FMDV DNA vaccine alone. IL-15 as a

mucosal adjuvant enhances the antigen-specific mucosal and systemic immune responses which may confer protection against the FMDV initial infection.

Li and others (2009) reported that specific anti FMDV antibodies in serum were significantly increased in mice after oral administration of the herb *Rhizoma Atractylodis Macrocephalae*. Significant increase in splenocyte proliferation and production of IL-5 and IFN-γ was also noticed.

Mingala and others (2009) studied expression of cytokines in water buffalo after inactivated FMDV vaccination. Real-time PCR quantification indicated that IFN-γ, IL-10 and TNF-α was highest at three week post-vaccination than IL-2, IL-4 and IL-6.

Song and others (2009) concluded that co-administration with a mixture of oil and saponins extracted from ginseng stems and leaves in mice, FMDV antigen induced higher IgG responses, production IFN-γ and IL-5 by splenocytes and T and B lymphocyte proliferation.

Zhu and others (2010) indicated involvement of leader proteinase (L^{pro}) in the FMDV evasion of the innate immune response by inhibiting NF-κB dependent gene expression. L^{pro} of FMDV plays a critical role in viral pathogenesis. L^{pro} inhibits translation and transcription of some genes related to innate immune response. Using microarray technology upregulation of 39 out of 22,000 bovine genes were observed. These up-regulated genes corresponded to IFN-inducible genes, chemokines or transcription factors.

2.2 Related study

Tomar and others (1995) concluded that the 5 µg of Conconavalin A (Con A)/ml $^{-1}$ mitogen significantly stimulated T lymphocytes from goats which were maintained *in-vitro* for more than a month long duration.

Sharma and others (1996) showed that the IL-4 was involved in down regulation of antiviral cytokines and cytotoxic T lymphocyte responses.

Stabel (2000) compared cytokines expressed by PBMCs from healthy cows and cows subclinically and clinically infected with *Mycobacterium paratuberculosis*. Con A stimulated cells from subclinically infected cows secreted more cytokine than clinically infected cows suggesting that the activated T cells might have delayed the progression of Paratuberculosis.

Levy and Sastre (2001) reported detailed account on the antiviral pathway induced by IFN system and its importance to innate immune response. Viruses evolved with their hosts inventing strategies to survive and replicate even though IFN mediated defenses were existed.

Stordeur and others (2002) applied real-time PCR technology for quantification of cytokine mRNA using TaqMan probes and concluded that the hydrolysis probes as well as hybridization probes were equally efficient. Kinetics of IL-1Rα, IL-1β, IL-5, IL-13, TNF-α and IFN-γ were determined after stimulation of human PBMC by phytohaemagglutinin. The study had revealed that IFN-α induces IL-10 mRNA accumulation in human monocytes.

Werling and others (2002) reported that there was increase in IL-10 mRNA synthesis and reduction in IL-12p40 and IL-15 synthesis after *in-vitro* stimulation of monocyte-derived dendritic cells with live bovine respiratory syncytial virus (BRSV) than heat-inactivated virus in multiplex, real-time PCR.

Coussens and others (2004) reported that IL-10 gene expression was constantly increased in subclinically infected cattle after stimulation of peripheral blood mononuclear cells (PBMCs) by *Mycobacterium avium subsp. paratuberculosis.*

Novelli and Casanova (2004) indicated that mice lacking receptors for IFN-γ was more susceptible to viral infections.

Orsel and others (2007) showed that the vaccination of sheep lead to limitation of FMDV excretion and transmission. Risk of transmission from vaccinated carrier sheep is lower than non-vaccinated. FMDV transmission can occur in sheep without manifestations of clinical signs. Excretion level of infectious virus is low in vaccinated sheep. Sheep are unable to amplify and transmit the FMDV.

Schmidt (2007) listed out importance of TLRs in immune response and therapy. TLR immune receptors when activated by foreign particles (RNA or DNA) TLR recruits a secondary wave of B and T cells from the immune system. TLR4 is immune receptor for lipopolysaccharide endotoxin. Triggering TLR5 with fusion protein generates powerful immunological response. TLR7 is found nearly in all immune cells and it generates a much larger activation footprint than TLR9 which is found only in plasmocytes and dendritic cells. TLR now exploited by using drugs and vaccines targeting the TLR system. Vaccine combined with TLR produce good efficiency. TLR is also useful in topical,

oncology, inflammation and autoimmune diseases. As of now ten TLR signaling pathways are being explored.

Scott and Aronson (2008) mentioned that macrophage chemotactic protein 1 (MCP-1) is known to be induced by IFN-γ and can be produced by a variety of cells including macrophages, T cells and granulocytes. MCP-1 functions to recruit monocytes, T cells and NK cells to inflammatory sites. MCP-1 enhances macrophage infection by viral or bacterial pathogens.

Tizard (2008) gave detailed account on sickness behavior, its mechanism and significance. TNF-α, membrane associated or soluble is secreted in response to invading pathogen by macrophages and mast cells. It triggers the local release of chemokines and cytokines and promotes the adherence, migration, attraction and activation of leucocytes at the site of invasion. Then it facilitates the transition from innate to acquired immunity by enhancing antigen presentation by dendritic cells and T cell costimulation. A local increase of it causes the cardinal signs of inflammation, including heat, swelling, pain and redness.

MATERIALS AND METHODS

“Indeed one's faith in one's plans and methods is truly tested when the horizon before one is the blackest”

…Mahatma Gandhi

Chapter-3

MATERIALS AND METHODS

3.1 Blood, Serum and Plasma samples

Blood samples were collected asceptically in plastic/glass containers with or without anticoagulant (0.1 % Ethylenediaminetetraacetic acid (EDTA) or heparinated vacutainers, BD Vacutainer® with anticoagulant Sodium heparin 143 USP U) from the Jugular vein. Blood samples without anticoagulant were allowed to set for clotting to extract serum. Plasma samples were collected later wherever necessary. Detailed description for these samples is given below,

i) For preliminary experiments

To identify cytokines after *in-vitro* stimulation of Peripheral Blood Mononuclear Cells (PBMCs) by either mitogen, Conconavalin A (ConA) or specific Type of Foot-and-Mouth disease virus (FMDV), blood samples, with or without anticoagulant (0.1 % EDTA), were collected from (FMDV uninfected, apparently healthy and unvaccinated with no history of Foot-and-Mouth disease (FMD); Liquid phase blocking enzyme linked immunoassay (LPBE) titer below 1.5) Himalayan Holstein Friesian crossbred male cattle calf (No. – 380; *Bos taurus* x *Bos indicus*) and Himalayan Holstein Friesian crossbred dairy cows (No. – 6, 306, 333, 336, 337 and 354; *Bos taurus x Bos indicus*) residing at high altitude (more than 2000 m) from Dairy section of Indian Veterinary Research Institute (IVRI), Mukteshwar, Uttarakhand.

ii) From natural FMD outbreaks

Blood samples were collected from cattle experiencing FMD outbreaks at different places.

a) At Izatnagar, Uttar Pradesh

Blood samples (5 ml) were collected from seven adult (five to seven years old – No. 776, 811, 885, 1034, 1044, 1265, 1239; *Bos taurus* x *Bos indicus*)

Holstein Friesian crossbred breeding bulls of Germplasm center, IVRI on 9th October 2009 in heparinated vacutainers (BD Vacutainer® with anticoagulant Sodium heparin 143 USP U). Serum samples were also extracted from blood (4 ml) without anticoagulant. These bulls were vaccinated against FMD on 4th June 2009 (four months before blood collection). First clinical infection of FMDV in two breeding bulls (No. – 885 and 1265) was noticed on 24th August 2009. Subsequently, rest of the bulls caught the FMDV infection. 500 µl of whole blood was directly taken in RNAprotect® Animal blood tubes from each bull. After incubation at room temperature (rt) for 2 hours (hrs) as per manufacturer's directions, these tubes were stored at – 80 ^{0}C.

b) At Papum Pare, Arunachal Pradesh

500 µl of blood from clinically FMDV infected Mithun (*Bos gaurus*) was collected in RNAprotect® Animal blood tube and transported to Mukteshwar. It was donated by Dr. A. Sanyal and Dr. Sachin Pawar.

c) At Kashipur, Uttarakhand

Blood samples were collected with (10 ml) or without (10 ml) heparinated vacutainers (BD Vacutainer® with anticoagulant Sodium heparin 143 USP U) of 20 Holstein Friesian crossbred dairy cows (in-contact apparently healthy or clinically FMDV infected; *Bos taurus* x *Bos indicus*) from two closely located organized dairy cattle farms at Kashipur (Table 1). Except two dairy cows (R54 and R57) all were vaccinated against FMD by a commercially available vaccine. Serum samples were extracted. Dairy cows were experiencing natural clinical FMD outbreak which was confirmed by FMDV typing at the laboratory as Type 'A' FMDV infection (Figure 1). Antibiotics and other drugs were administerd to check secondary bacterial infections in these dairy cows. Dairy cow farms are of intensive rearing type which falls under common ownership, hence, intermingling or separation of these cows at circumstances was allowed to happen by the owner.

iii) From FMDV vaccination and/or challenge studies

Blood samples were collected from cattle calves kept in the Vaccine Potency testing during vaccination and challenge.

(A)

(B)

(C)

Figure 1. Foot-and-Mouth disease affected dairy cows with (A) Foot lesion (B) Profuse salivation and (C) Frothiness of saliva

a) At Hyderabad, Andhra Pradesh

Young crossbred cattle calves (8-10 months old, FMDV uninfected, apparently healthy and unvaccinated with no history of FMD) were used in the Vaccine Potency testing experiment against FMD at Indian Immunologicals Limited (IIL), Hyderabad. Blood samples (10 ml) were collected at different intervals (Table 2) in the heparinated vacutainers (BD Vacutainer® with anticoagulant Sodium heparin 143 USP U) from calves (No. – 202, 209, 211, 212, 257, 1004, 1022; *Bos taurus* x *Bos indicus*). These calves were vaccinated against FMD with monovalent inactivated vaccine (FMDV; Type 'O') and challenged with FMDV Type 'O' (R2/75).

b) At Mukteshwar, Uttarakhand

Blood samples with (10 ml) or without (10 ml) anticoagulant (heparinated vacutainers; BD Vacutainer® with anticoagulant Sodium heparin 143 USP U) were collected from young Holstein Friesian crossbred calves (FMDV uninfected and apparently healthy with no history of FMD; No. – 415, 423, 424, 425, 444; *Bos taurus* x *Bos indicus*; LPBE titers below 1.5 in most of the animals before vaccination) at different intervals before and after vaccination against FMD with trivalent inactivated vaccine (FMDV; Type 'O', Type A' and Type 'Asia 1') of Calf shed at Latoli farm, IVRI, Mukteshwar. Serum samples were also extracted and kept in the refrigerator at – 40 ^{0}C.

500 µl of blood samples were collected in RNAprotect® Animal blood tubes and blood samples (10 ml) without anticoagulant from Holstein Friesian crossbred dairy cows (FMDV uninfected and apparently healthy with no history of FMD; No. – 223, 766; *Bos taurus* x *Bos indicus*; LPBE titers 1.68 and 2.28, respectively) of Dairy Section, IVRI, Mukteshwar which were vaccinated against FMD with trivalent inactivated vaccine (FMDV; Type 'O', Type A' and Type 'Asia 1'). Serum samples were extracted. These tubes were allowed to incubate at rt for 2 hrs and later stored at – 80 ^{0}C.

500 µl of blood samples were also collected in RNAprotect® Animal blood tubes from Holstein Friesian crossbred young calves (FMDV uninfected and apparently healthy with no history of FMD; No. – 411, 418; *Bos taurus* x *Bos indicus*) vaccinated against FMD with trivalent inactivated vaccine (FMDV; Type

'O', Type A' and Type 'Asia 1') which are kept at experimental animal shed of Project Directorate on FMD (PDFMD). These tubes were allowed to incubate at rt for 2 hrs and later stored at – 80 ^{0}C.

3.2 Major equipment and accessories

i) At Mukteshwar

Refrigerators and freezers:

+ 4 to – 20 ^{0}C – Express-Cool, LG, Korea

– 40 ^{0}C – Heto lab equipment, Denmark

– 40 ^{0}C – Sanyo Biomedical freezer, USA

– 86 ^{0}C – Forma scientific, USA

Centrifuge machines:

Refrigerated centrifuge – SORVALL® RC 3 B plus, USA

Biofuge primo – Heraeus instruments, Germany

Biofuge fresco – Heraeus instruments, Germany

High-speed centrifuge for plate – 236HK, Hermle lab, GmBH, Germany

Balances:

Single pan analytical balance, for small quantities – Sartorius, UK

Single pan analytical balance, for large quantities – Citizen, India

Double pan balance – MN Evershine

Laminar flows:

Laminar flow clean air work station – Klenzaids, India

Biosafety cabinet class II – SCANLAF Mars 900, Denmark

Biosafety cabinet klasse II – HERA safe, Heraeus, Germany

Biosafety cabinet – Holten LaminAir Type S.2000 1.2, Denmark

Thermal cyclers:

PCR thermal cycler – Takara, Japan

Thermal cycler – Hybaid

PCR System – GeneAmp PCR system 9700, USA

Real time PCR – 7500 real time PCR system – AB Applied Biosystems, USA

Sequencers:

Capillary sequencer – 3130 Genetic Analyzer, USA

Gel sequencer – ALF express II amersham pharmacia biotech

Shaker-cum-incubators:

Incubator shaker – Innova 4000, New Brunswick lab scientific, USA

Refrigerated incubator shaker – Innova 4230, New Brunswick Lab scientific, USA

Gel documentation system – AlphaImager EP, Alpha Innotech, UK

QIAcube machine – Qiagen, Germany

Inverted microscope – Olympus CK 30, Japan

NanoDrop 1000 – Thermo Scientific Spectrophotometer, USA

Dry heating block – Hybaid omnigene

Hot water bath – Heto, Denmark

Incubator, 37 ^{0}C – Bharat instruments and chemicals, India

Vortex shaker – Spinix, India

Horizontal Electrophoresis units (HU10, HU13) – Scie-Plas, UK

Nunc-Immunocomb™ Wash 12

Haemacytometer – Sigma, USA

Cascada RO water purification system – Pall Corporation, USA

Milli Q water purification system – Millipore, USA

Ice machine – Icematic F125 compact

Pipettes (µl: 0.5-10, 5-50, 20-200 and 200-1000) – Finnpipette, Labsystems, Denmark

ELISA reader – Tecan Spectra, Germany

Microwave oven – BPL Sanyo

UV transilluminator – ULTRA-LUM (MUVB-20), USA

Spectrometer – ATI UNICAM UV2, USA

Room incubator – 37 ^{0}C

Cold room – + 4 ^{0}C

Cold room – – 20 ^{0}C

ii) At Hyderabad

Eppendorf Centrifuge (5810 R, Rotor – A-4-62 max 4000 rpm) – Germany

Honeywell DC 1040 – 70 ^{0}C Deep freeze AMRUTHA, USA

Voltas coldcell 4 ^{0}C, India

Elico digital pH meter, India

3.3 Biologicals

Virus – Ultrapurified FMDV Type 'A' (17/82)

Antigen – FMDV Type 'O', Type 'A' and Type 'Asia 1'

Coating serum – Raised in rabbits against each of FMDV Types ('O', 'A' and 'Asia 1')

Tracing serum – Raised in guinea-pigs against each of FMDV Types ('O', 'A' and 'Asia 1')

Coating 3AB3 antigen – 3AB3 protein available in the laboratory

Foetal Bovine serum (FBS) – HyClone, USA

Escherichia coli lysate – Available in the laboratory

Procaryotic host for cloning – *Escherichia coli* (JM 109) cells

Anti-guinea-pig horseradish peroxidase (HRPO) conjugate – DAKO, Denmark

Anti-bovine HRPO conjugate – DAKO, Denmark

3.4 Glasswares

Conical flasks (50 ml, 250 ml, 500 ml)

Measuring cylinders of various capacity

Glass vials

Syringes (20 ml)

Perspex plates

Glass Pasteur pipettes – Sigma, USA

Other miscellaneous sterile glasswares were procured from store section of the laboratory and utilized in the study whenever required.

Wherever possible, catalogue number (CN) and part number (PN) for particular item was mentioned in the information given below.

3.5 Plasticwares

Sterile pipette tips (10 µl, 20 µl 200 µl, 1000 µl) – Axygen, USA

MicroAmp™ optical adhesive film – AB Applied Biosystems, USA (PN 4311971)

MicroAmp™ optical 96-well plate – AB Applied Biosystems, Singapore (PN N801-0560)

MicroAmp™ optical 8-tube strip (0.2 µl) – AB Applied Biosystems, Singapore (PN 4316567)

MicroAmp™ 8-cap strip – AB Applied Biosystems, USA (PN 4323032)

24 well tissue culture plate – Cornig

96 well ELISA plate – Nunc, Denmark

Centrifuge tubes (1.5 ml, 2 ml) – Eppendorf, Germany

PCR tubes (0.2 µl, 0.5 µl) – Eppendorf, Germany

Centrifuge tubes (15 ml, 50 ml) – Nunc, Denmark

Cryo-vials (2 ml) – Greiner bio-one

Petriplates – Cornig

Pipettes (5 ml, 10 ml) – Cornig

Syringes (2 ml, 10 ml, 20 ml)

Measuring cylinders of various capacity (25 ml, 50 ml, 100 ml)

Other necessary plasticwares were procured from store section of the laboratory and utilized in the study whenever required.

3.6 Enzymes

Taq DNA polymerases – Fermentas, Qiagen and Biozymes

GoTaq® DNA polymerase (5 U/µl) – Promega, USA (CN M3005)

M-MLV reverse transcriptase (200 U/µl) – Promega, USA (CN M1705)

Restriction enzyme (EcoRI) – Promega, USA

Big Dye® enzyme for sequencing – AB, UK

3.7 Commercial kits, chemicals and miscellaneous

RNase away (250 ml) – M*β*P Molecular Bioproducts, USA

HiCare/Triclogel – HiMedia, India

Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) – Sigma, USA (E5134)

Sodium heparin vacutainer (10 ml) 143 USP U – BD Vacutainer®, USA (CN 367874)

Histopaque®-1077 – Sigma-Aldrich™, USA (CN 10771-500 ml)

Trypan blue – Fluka, Switzerland (CN 93590)

Ammonium chloride – Sigma, USA (CN 09718-250G)

Conconavalin A – Sigma, USA

RPMI (Rosewell Parker Memorial Institute) - 1640 media – Sigma®, USA

TRI Reagent™ – Sigma, USA (CN T9424)

TRIZOL® LS Reagent (200 ml) – Invitrogen, USA (CN 15596-018)

Nuclease free water – Amresco, USA

pGEM® - T Easy Vector System I (50 ng/µl) – Promega, USA (CN A1360)

AuPrep™ Plasmid extraction kit – Life Technologies™, USA

Zyppy™ Plasmid Miniprep kit – Zymo industries, USA (D4020)

RNeasy® Mini Kit (250) – Qiagen, Germany (CN 74106)

Gel extraction kit – Qiagen, Germany

QuantiTect® SYBR® Green RT-PCR kit – Qiagen, Germany (CN 204243)

QuantiTect® SYBR® Green PCR kit – Qiagen, Germany (CN 204145)

RNAprotect® Cell Reagent (250 ml) – Qiagen, Germany (CN 76526)

RNeasy® protect Animal blood kit (50) – Qiagen, Germany (CN 73224)

RNAprotect® Animal blood tubes (50 x 500 µl) – Qiagen, Germany (CN 76554)

Oligo $(dT)_{15}$ Primer (0.5 ug/µl) – Promega, USA (CN C1101)

Random Primers (0.5 µg/µl) – Promega, USA (CN C1181)

X-Gal (50 mg/ml) – Promega, USA (CN V3941)

Isopropyl-D-thiogalactopyrinoside (IPTG) (5 g dry powder) – Promega, USA (CN V3951)

Luria Bertani broth tablets (1.1g/tablet) – Sigma-Aldrich, USA (CN L7275)

Luria Bertani agar tablets (1.6 g/tablet) – Sigma®, USA (CN L7025)

Ethanol absolute GR for analysis – Merck, Germany (CN 1.00983.0511)

Isopropanol – Sigma®, USA (CN I-9516)

Chloroform – Sigma®, USA (CN C2432)

PCR Markers – Promega, USA (CN G3161)

100 bp DNA Ladder – SibEnzyme, Russia

1 kb DNA Ladder – SibEnzyme, Russia

GeneRuler™, 100 bp Plus DNA Ladder – Fermentas, USA (SM0321)

GeneRuler™, 1 kb DNA Ladder – Fermentas, USA (SM0311)

6x loading dye – Fermentas and Promega, USA

Agarose, low melting agarose – Sigma, USA (CN A-9414)

Agarose, preparative grade for small fragments – Promega, USA (CN V3841)

Ethidium bromide (Electran®) – BDH, UK (PN 44159)

Sucrose – Fluka, Switzeraland (CN 84099)

Bromophenol blue – BDH, England

Tris Acetate EDTA buffer (TAE) 10x – Promega, USA

Tris Borate EDTA buffer (TBE) 10x – Promega, USA

Trizma® base – Sigma®, USA (CN-T6066)

Polyethylene glycol 8000 – Sigma®, USA (CN P2139)

Dimethyl sulfoxide (DMSO) – Sigma®, USA (CN D-5879)

Glycerol – Merck, USA

Ampicillin, Sodium salt, 25g – Calbiochem®, Germany (CN 171254)

Big Dye® Terminator v1.1, v3.1 5x sequencing buffer – AB, UK (PN 4336697)

3130 POP-7™ – AB, UK (PN 4336697)

HiDi Formamide – AB, UK

Sodium acetate – Sigma®, Germany (CN S2889)

Hanahan's broth (SOB Medium) – Sigma®, USA (CN H-8032)

Boric acid, for molecular biology, approximately 99% – Sigma, USA (CN B6768)

D-Glucose – Glaxo laboratories, India (CN 1.15363.0500)

Magnesium chloride hexahydrate – Fluka, Germany (63068)

Carbonate-bicarbonate capsules – Sigma®, USA (C3041-100CAP)

Phosphate-citrate buffer tablets – Sigma®, USA (P-4809)

Sodium dihydrogen phosphate – Fluka, Germany (CN 71500)

Disodium hydrogen phosphate – Fluka, Germany (CN 71645)

Sodium chloride – Merck, India (CN 60640450001730)

Skim Milk Powder – Merck, Germany (CN 1.15363.0500)

Orthophenylenediamine dihydrochloride (OPD) – Sigma, USA (CN P1526)

Hydrogen peroxide – Merck, Germany (CN 1.07210.0250)

Sulfuric acid, 95-97 % – Merck, India

Sodium hydroxide – Merck, India

Hydrochloric acid min. 35 % – Merck, India

Tween 20® (Polysorbate - 20, Polyoxyethylene sorbitan monolaurate) – HiMedia, India

Gel sealing tape – Sigma®, USA (T-6656)

Parafilm – Sigma®, USA (P-7543)

Delicate task wipers – Kimwipes, Canada

pH papers (2 to 10.5 and 3.8 to 5.3) – Merck, India

Other miscellaneous materials available in the laboratory were utilized, wherever necessary.

3.8 Primers

A total of 116 primers for (96 different cytokines, chemokines, chemokine receptors, toll-like receptors (TLRs) and other genes; 2 for sequencing reactions) were used in the present investigation. Details of which are mentioned below.

Category A) - Readymade primers of 22 various bovine cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-18, IFN-γ, TNF-α, MCP-1, TGFβ-1, IGF-1, IGFBP-2, IGFBP-3, VEGF, Leptin, TLR2, TLR4, TLR9) (Table 3) were procured commercially from Pierce-Endogen (USA) for amplification of specific product during preliminary and other experiments as well. 500 pmole of forward and reverse primers were provided in single plastic tube along with separate vial of positive controls for each cytokine. Forward and reverse primer sequences were not disclosed by the manufacturer. But GenBank® accession number was provided for each primer. Position of the product size mentioned in the Table 3 was determined after sequencing of the amplified cytokine products.

Category B) - Custom oligonucleotide synthesized primers (Standard, 0.02 µmol) were obtained from Metabion GmBH (Germany) for a total of 92 bovine cytokines, chemokines, chemokine receptors and toll-like receptors (TLRs) and other genes (IL-1α, IL-1β, IL-2, IL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p35, IL-12p40, IL-13, IL-15, IL-16, IL-17A, IL-18, IL-19, IL-20, IL-21, IL-22, IL-25, IL-26, IL-27p28, IL-32, IL-34, IFN-α, IFN-αR1, IFN-β, IFN-γ, IFN-T, IRF-1, TNF-α, TNF-αSF1, TGFβ-1, TGFβ-2, TGFβ-3, GM-CSF, CSF, CCL3, CCL5, CCL8, CCR5, CXCL1, CXCL2, CXCL6, CXCL10, CXCR4, CD11a, CD11b, CD11c, CD14, CD18, CD68, Integrin β3, FasL, Galectin-3, Granzyme A, Granulysin, Perforin, Selenoprotein P, XCL1, MMR, MSR1, COX-2, iNOS, LIF, OSM, CT-1, L-Selectin, VCAM-1, ICAM-1, ICAM-3, BAX, BAD, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, 18S RNA, GAPDH-1, GAPDH-2, β-actin, CD4). Primers were chosen from either earlier published reports or designed with the help of National Center for Biotechnology Information (NCBI) Primer-Blast facility available on the internet. List of nucleotide sequences for primers along with further details was given (Table 4). Accession numbers were noted in the respective columns along with exact position of product. Primers were diluted as per the instructions of the manufacturer and kept at – 80 ^{0}C as a stock.

Category C) - To reconfirm the specific amplified products of cytokines and other genes by sequencing custom oligonucleotide synthesized primers of T7 promoter and M13R were made available in the laboratory and used in the present investigation.

Table 1. Status of FMDV infection in dairy cows

Cow No.	Status of FMDV infection at the time of blood collection
A-29*	In-contact apparently healthy
B-1*	In-contact apparently healthy
B-10*	Infected in September 2009 (Before 4 months)
B-16*	Infected in September 2009 (Before 4 months)
B-17*	Infected in December 2009 (Before 1 month)
B-40*	Infected in December 2009 (Before 1 month)
M-51*	Infected in September and re-infected in 20th December 2009
M-54*	In-contact apparently healthy
M-57*	Infected in December (Before 1 month)
A-12**	In-contact apparently healthy
B-4**	Infected 10 days before
B-19**	Infected first in September and re-infected in December
H-65**	Infected 2 days before
M-53**	In-contact apparently healthy
M-56**	Infected first in September and re-infected in December
R-54**	Infected 7 days before
R-57**	First infection on 4th January 2010 (2 days before)
T-61**	Infected 5 days before
T-69**	Infected 8 days before
T-70**	Infected in December (Before 1 month)

*Sona farm, Hempur, Kashipur

(History – The herd is comprised of 74 dairy cows. After 34 clinically FMDV infected dairy cows were introduced to the herd, subsequent flaring up of the FMD outbreak was noticed)

** Raghusa Agro farm limited, Rampura, Kashipur

(History – The herd is comprised of 100 clinically FMDV infected dairy cows. One dairy cow was died)

Table 2. Collection of blood samples from calves used in Vaccine Potency testing

Calf No.	Date of vaccination	10 DPV	15 DPV	21 DPV / 0DPI	3 DPC /DPI	5 DPC /DPI	12 DPC /DPI
209	24-01-2010	03-02-2010	08-02-2010	15-02-2010	18-02-2010	20-02-2010	27-02-2010
211	24-01-2010	03-02-2010	08-02-2010	15-02-2010	18-02-2010	20-02-2010	27-02-2010
212	24-01-2010	03-02-2010	08-02-2010	15-02-2010	18-02-2010	20-02-2010	27-02-2010
257	24-01-2010	03-02-2010	08-02-2010	15-02-2010	18-02-2010	20-02-2010	27-02-2010
1004	24-01-2010	03-02-2010	08-02-2010	15-02-2010	18-02-2010	20-02-2010	27-02-2010
202*	Not vaccinated	-	-	15-02-2010	18-02-2010	20-02-2010	Died
1022*	Not vaccinated	-	-	15-02-2010	18-02-2010	20-02-2010	27-02-2010

*Control FMDV uninfected and unvaccinated calves experimentally infected with FMDV (Type 'O' - R2/75) at the time of challenge, Calf No. 202 died before 12 DPC, rest five calves were used in vaccination and challenge study

DPV-Days post vaccination

DPI-Days post infection

DPC-Days post challenge

Table 3. Primers for bovine cytokines obtained from Pierce-Endogen

Sr. No.	Item	Product size, bp	Positive control, size, bp	Catalogue number	Accession number and position of product
1	IL-1β	420	325	PS0003	M37211 (414-833)
2	IL-2	345	330	PS0006	M13204 (147-491)
3	IL-4	289	330	PS0007	M77120 (171-459)
4	IL-5	308	345	PS0023	Z67872
5	IL-6	491	325	PS0004	X57317 (217-707)
6	IL-8	209	336	PS0014	AF232704 (171-379)
7	IL-10	330	336	PS0012	U00799 (98-427)
8	IL-12p35	457	345	PS0017	U14416
9	IL-12p40	336	345	PS0018	U11815
10	IL-18	347	345	PS0016	AF124789 (374-720)
11	IFN-γ	509	325	PS0002	M29867 (53-560)
12	TNF-α	360	325	PS0001	AF348421 (339-699)
13	MCP-1	209	345	PS0021	M84602 (134-342)
14	TGFβ-1	264	345	PS0025	M36271 (672-935)
15	IGF-1	317	330	PS0009	X15726
16	IGFBP-2	365	324	PS0019	NM_174555
17	IGFBP-3	365	345	PS0020	NM_174556
18	VEGF	419	336	PS0015	M32976
19	Leptin	349	336	PS0011	U43943
20	TLR2	503	324	PS0047	NM_174197 (1346-1848)
21	TLR4	473	324	PS0048	NM_174198 (1349-1821)
22	TLR9	377	324	PS0049	NM_183081 (1951-2327)

Table 4. List of primers used to amplify specific products of cytokines, chemokines, chemokine receptors, toll-like receptors and other genes

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
1	IL-1α[L]	F-5'- CCA CTT CGT GAG GAC CAG ATG AAT-3'	124	24	M37120.1 (178-302)
		R-5'- TCT TCA GAA TCT TCC CAC TGG CTG-3'		24	
2	IL-1β[L]	F-5'-TCC ATG GGA GAT GGA AAC ATC CAG-3'	194	24	NM_174093 (197-390)
		R-5'-GAC GTT TCG AAG ATG ACA GGC TCT-3'		24	
3	IL-2[K]	F-5'-TTT TAC GTG CCC AAG GTT AA-3'	217	20	M12791 (207-423)
		R-5'- CGT TTA CTG TTG CAT CAT CA-3'		20	
4	IL-2Rα[A]	F-5'-TAG CCC AGT CCT CCA AGC TA-3'	128	20	NM_174358.2 (1523-1650)
		R-5'-GCT CAA CAG TTC CAT GGG TT-3'		20	
5	IL-3[A]	F-5'-TTT CCG GAT GAA ATT GGA AG-3'	116	20	NM_173920 (381-496)
		R-5'-TGA GGC TTC AGA GAG GGA AC-3'		20	
6	IL-4[K]	F-5'-CAA AGA ACA CAA CTG AGA AG-3'	181	20	M77120 (245-425)
		R-5'-AGG TCT TTC AGC GTA CTT GT-3'		20	
7	IL-5[C]	F-5'-CTG GTG GCA GAG ACC TTG ACA CT-3'	320	23	Z67872.1 (85-404)
		R-5'-CAA CTT TCC ATT GTC CAC TCT GTG-3'		24	
8	IL-6[K]	F-5'-TCC AGA ACG AGT ATG AGG-3'	236	18	X57317 (428-663)
		R-5'-CAT CCG AAT AGC TCT CAG-3'		18	

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
9	IL-8[L]	F-5’-GGT GCA GAA GGT TGT GCA GGT ATT-3’	176	24	NM_173925.2 (325-500)
		R-5’-ACA CCA GAC CCA CAC AGA ACA TGA-3’		24	
10	IL-9 [A]	F- 5’-GCA CGA GAT TCC ACC TGA TT-3’	149	20	XM_864128 (176-324)
		R - 5’-ACT TTC CAG CAG AGC CTT CA-3’		20	
11	IL-10 [K]	F- 5’-TGC TGG ATG ACT TTA AGG G-3’	186	19	U00799 (209-394)
		R - 5’-AGG GCA GAA AGC GAT GAC -3’		19	
12	IL-12p35[C]	F- 5’-CTT TCT TCA AAT GCA GCA TTG G-3’	89	23	AJ271034.1 (596-685) (271)
		R - 5’-GGG TCT GGG TGA TAC AAC GAA-3’		21	
13	IL-12p40[K]	F-5’-AAC CTG CAA CTG AGA CCA TT-3’	186	20	U11815 (721-906)
		R-5’-ATC CTT GTG GCA TGT GAC TT-3’		20	
14	IL-13[A]	F-5’-CCT GTG CTG TTC TAG GCT CC-3’	111	20	NM_174089.1 (51-161)
		R-5’-CCT TGA GGG CTG TAG CAG AA-3’		20	
15	IL-15[We]	F-5’-GGC TGG CAT TCA TGT CTT CA-3’	74	20	NM_174090 (84-157)
		R-5’-CAT ACT GCC AGT TTG CTT CTG TTT-3’		24	
16	IL-16[C]	F-5’- GAG GGC GGT CCC AGA AGT-3’	73	18	NM_001075253 (2255-2327)
		R- 5’- CTC TCT AGA TGC AGT CTG TCG TTT GT- 3’		26	
17	IL-17A[A]	F-5’-TGA GTC TGG TGG CTC TTG TG-3’	138	20	NM_001008412.1 (102-239)

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
		R-5’-GCC TTC TGG AGT TTG TGC TC-3’		20	
18	IL-18^{L}	F-5’-TTC TGC TCT CCA ATG CTT TCA GCG-3’	156	24	BC102879.1 (38-194)
		R-5’-TCT GCA GCC ATC TTT ATG CCT GTG-3’		24	
19	IL-19^{A}	F-5’-CCC TTG AAT TTC TGC GAG AG -3’	196	20	XM_606029.3 (32-227)
		R-5’- ATT CCT CGG AAG CTC TCC TC-3’		20	
20	IL-20^{A}	F-5’-GAG AGG TTC TGG TCT TCC CC-3’	149	20	XM_593569.3 (282-430)
		R-5’- CCC GAA TCT CTG AAA ATC CA-3’		20	
21	IL-21^{A}	F-5’-ACT GTG AGC GGT CAG CTT TT-3’	132	20	NM_198832.1 (206-337)
		R-5’- TCC CTG TAT TTG TGG CAG CTT TT-3’		20	
22	IL-22^{A}	F-5’-GCA GAA ATC TGT GGG CTC TC -3’	128	20	NM_001098379.1 (79-207)
		R-5’- AGT CGG ACT CGT TGA GCC TA-3’		20	
23	IL-25^{A}	F-5’-ACC ACA ACC AGA CCG TCT TC-3’	100	20	XM_605190.2 (452-551)
		R-5’- GCC AAG GAG ACA CGG TAG AG-3’		20	
24	IL-26^{A}	F-5’-TCG CTG CAG TTC TAT CCA GA-3’	156	20	XM_001250651.1 (529-684)
		R-5’- AGC CCA GTG GAA TGA CTG AC-3’		20	
25	IL-27p28^{A}	F-5’- CAG CCT TTT GCT GCT CTT CT-3’	111	20	XM_001788526.1 (36-146)
		R-5’- ACC TTG AAC TCC CTC TGC AA-3’		20	

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
26	IL-32^{A}	F-5'-GCA GGA TCT CAG GTC CCT T-3'	177	19	XM_001790355.1 (27-203)
		R-5'- CTC CGT CTC ATC TAG GGC TG-3'		20	
27	IL-34^{A}	F-5'-AGG GAC ACA GAG CCT GAG AA-3'	165	20	NM_001100324.1 (1404-1568)
		R-5'-CTG ACT CAG GAG AAG GTC GG-3'		20	
28	IFN-α^{z}	F-5'-GTG AGG AAA TAC TTC CAC AGA CTC ACT-3'	118	27	EU276064.1 (473-580)
		R-5'-TGA RGA AGA GAA GGC TCT CAT GA-3'		23	
29	IFN-αR1^{L}	F-5'- TCC ACA TGG TAT GAG GTT GAG CCA-3'	157	24	NM_174552.2 (514-670)
		R-5'- AGC TTG AAC GAT CCA TAG CCC ACA-3'		24	
30	IFN-β^{z}	F-5'-CCT GTG CCT GWT TTC ATC ATG A-3'	97	22	M15477.1 (192-288)
		R-5'-GCA AGC TGT AGC TCC TGG AAA G -3'		22	
31	IFN-γ^{L}	F-5'-GAA TGG CAG CTC TGA GAA ACT GGA-3'	171	24	M29867 (408-578)
		R-5'-CGG CCT CGA AAG AGA TTC TGA CTT -3'		24	
32	IFN-T^{A}	F-5'- TCC CCG GAA ACT AGA ATT CAC-3'	111	20	NM_001015511.2 (3-113)
		R-5'-CCA GGG CCA TCA GTA GAG AG-3'		20	
33	IRF-1^{L}	F-5'- ATT GTA GTG GAC AGC GCA GAC AGA-3'	141	24	CK960990
		R-5'-ATT GTA GTG GAC AGC GCA GAC AGA-3'		24	
34	TNF-α^{L}	F-5'- CAT TGC AGT CTC CTA CCA GAC CAA-3'	121	24	Z14137 (5740-5860)

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
		R-5'-TCC TCC CTG GTA GAT GGG TTC ATA-3'		24	
35	TNF-αSF1[A]	F-5'- GGA GCT TGG GTG GAT GAC TA-3'	200	20	NM_001013401 (945-1144)
		R-5'-TCA GAG CCT CGC TTT CTC TC-3'		20	
36	TGFβ-1[C]	F-5'-CTG AGC CAG AGG CGG ACT AC -3'	262	20	M36271 (67-328)
		R-5'-TTG CTG AGG TAG CGC CAG GAA TTG-3'		24	
37	TGFβ-2[A]	F-5'- GAA CTT GAA CTC AAA CGG GC-3'	135	20	NM_001113252 (2176-2310)
		R-5'-GGG CAG TTG GTA CAA AGC AT-3'		20	
38	TGFβ-3[A]	F-5'- CTC TCT GGT CTG CTC CTT GG-3'	116	20	BC149207 (2157-2272)
		R-5'-GAC CCA AAC TCC AGT CGT GT-3'		20	
39	GM-CSF [T]	F-5'- AAT GAC ACA GAA GTC GTC TCT GAA A-3'	87	25	NM_174027.2 (173-259)
		R-5'- CAG GCC GTT CTT GTA CAG CTT-3'		21	
40	CSF[A]	F-5'-ATT TTG ACT CCT CGT GTG GG-3'	158	20	NM_174028.1(1130-1287)
		R-5'-AGA CAT ACG ACC ATC TGC CC-3'		20	
41	CCL3[L]	F-5'-TCA AGC CTG GTG TCA TCT TCC AGA-3'	82	24	AY050252 (192-273)
		R-5'-ATG TAT TCC CTG GAC CCA GTC CTC A-3'		25	
42	CCL5[L]	F-5'-AAC GCT TTG GAG TTG AGC TAG GGT-3'	82	24	BC102064.1 (317-398)
		R-5'-TGC TTA GGA CAA GAG CGA GAA GCA-3'		24	

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
43	CCL8[L]	F-5’-TGC ATG TAG GAT GGT GAG GTC CTT-3’	94	24	NM174007.1 (655-748)
		R-5’-AGC ACA CAT CCA CTT ACA GGA GCA-3’		24	
44	CCR5[L]	F-5’-ACA TGC TGG TTG TCC TCA TCC TGA-3’	119	24	NM_001011672 (655-)
		R-5’-AGC CCA GAA TGG GAT GGT GAT GAT-3’		24	
45	CXCL1[L]	F-5’-ACA TCC AGA GCG TGA AGG TGA CAA-3’	93	24	NM175700.1 (191-283)
		R-5’-TGA GAC ACA CTT CCT GAC CAG TCT-3’		24	
46	CXCL2[L]	F-5’-AGA AGC TCT TGG ATG GCT GTT CCA-3’	91	24	NM174299.2 (392-482)
		R-5’-AGA TGG CCT TAG GAG GTG GTG ATT-3’		24	
47	CXCL6[L]	F-5’-TTG TGA GAG AGC TGC GTT GTG TGT-3’	139	24	NM174300.2 (174-312)
		R-5’-TCC CTT CCA TTC TTC AAG GTG GCT-3’		24	
48	CXCL10[T]	F-5’- AAG TCA TTC CTG CAA GTC AAT CCT-3’	103	24	EU276062.1 (152-254)
		R-5’- TTG ATG GTC TTA GAT TCT GGA TTC AG-3’		26	
49	CXCR4[L]	F-5’-ACC TGT GGC TAG TGG TGT TTC AGT-3’	113	24	AF399642.1 (625-737)
		R-5’-TTT GGA GTG GGA CAG CTT GGA GAT-3’		24	
50	CD11a[L]	F-5’-TGA ACT CCA TGA GGG TGA GGA TCA-3’	102	24	BI681282.1 (11-112)
		R-5’-AGT GAA ACT GTG CCA AGC ACC TAC-3’		24	
51	CD11b[L]	F-5’-AGG AAA TCT CGA CAG GAG CTC GAT-3’	139	24	AJ535320 (79-217)

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
		R-5’-TCT GCG TAC CCT CAA TCG CAA AGA-3’		24	
52	CD11c^{L}	F-5’-TGT CAA CCT GAG CTT CTT AGG GCT-3’	158	24	NM_001102496 (2457-2614)
		R-5’-TTG CTA ACG TGC GTC GAT AGG ACA-3’		24	
53	CD14^{L}	F-5’-TCT AGC GCC GTT CAG TGT ATG GTT-3’	81	24	NM_174008.1 (221-301)
		R-5’-TCC CTT GAG AAA CTG TTC CAG GCT-3’		24	
54	CD18^{L}	F-5’-AAC TGG CAG AAA GCA ACA TCC AGC-3’	133	24	EU860996.1 (929-1061)
		R-5’-TCC ACC ACG TTC CTG GAA TCT TCA-3’		24	
55	CD68^{L}	F-5’-TGA TGA GAG GCA GCA AGA TGG ACT-3’	164	24	CK774183
		R-5’-GGG CCA TAG CTT CAG TTG CAG AAA-3’		24	
56	Integrin β3^{L}	F-5’-TCC CAC TTG CTG GTG TTT ACC ACT-3’	137	24	CK838238 (17-153)
		R-5’- TCC ATG GTG GTG GAG GCA GAA TAA-3’		24	
57	FasL^{L}	F-5’-CCA GCC AAA GGC ATA CAG CAT CAT-3’	160	24	AB035802.1 (11-170)
		R-5’-ACC AGG GCA ATT CCA TAG GTG TCT-3’		24	
58	Galectin-3^{L}	F-5’- TCG CAT TGG GCT TTA CTG TAC CCA-3’	97	24	CK849687 (474-570)
		R-5’- TCC GGA CCA CTG AAT GTG CCT TAT-3’		24	
59	Granzyme A^{L}	F-5’- ATG ACT CAG TCT TTG CTG ATC GGG-3’	121	24	CK950789.1
		R-5’- AGA AGC ATC TTG CAG CCC TCT GAA-3’		24	

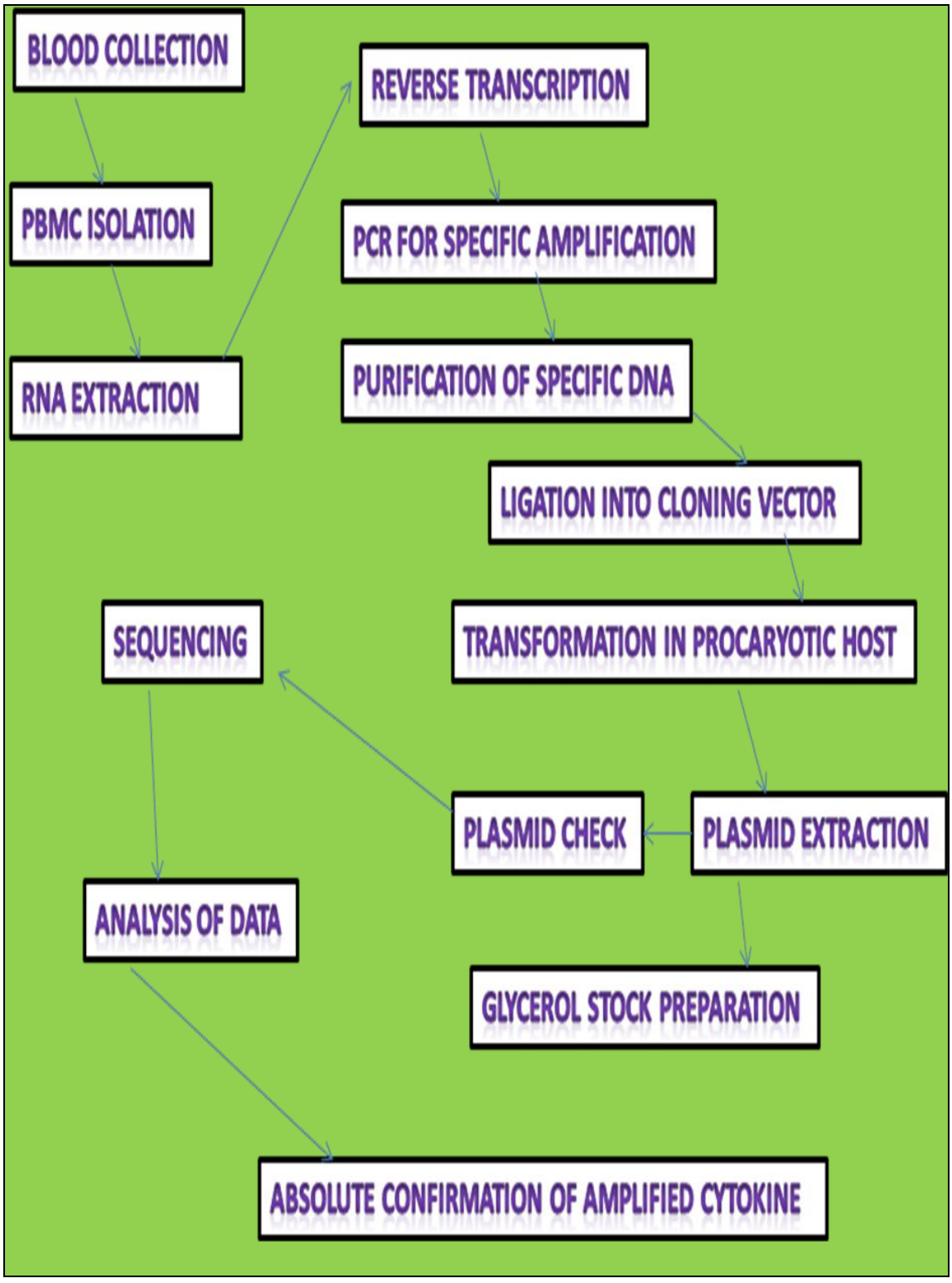
Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
60	Granulysin[L]	F-5'- ACG CTG TGG TTC TTG TCG GAG AAT-3'	128	24	NM_001075143 (204-331)
		R-5'- ACA GAC CTT TCA GCA GCC TCA TCT-3'		24	
61	Perforin[L]	F-5'- TCC TTT CCA GTT TGG CAC AGA GGT-3'	178	24	CK771825 (465-642)
		R-5'- TGG ACC ACG GTC TCC TGA AAT TCT-3'		24	
62	Selenoprotein P[L]	F-5'- TTC AGG TCT TCA TCA CCA CCA CCA-3'	199	24	AB032826.1 (720-918)
		R-5'- GCA ACA GCA GCT ACT CAA AGC AGA-3'		24	
63	XCL1[L]	F-5'- AAT CTG TGC TGA TCC TCA AGC TGC -3'	147	24	NM175716.2 (241-383)
		R-5'- AAA GTG CTG GAA GGT CAC TAC CCA-3'		24	
64	MMR[L]	F-5'- GTG TCA CCT GAG GTT ATA TGT ACT TGA C-3'	126	28	BF440340 (216-341)
		R-5'- AGA AGC AAC CAG CTA AGT GTC CTG-3'		24	
65	MSR1[L]	F-5'- TCT GTG AAG TTC GAT GCT CGC TCA-3'	174	24	NM_174113.1(104-277)
		R-5'- TTT CAG GAG CTG AGC TGC CAC TAT-3'		24	
66	COX-2 [T]	F-5'- GCA CAA ATC TGA TGT TTG CAT TC-3'	80	23	AF004944 (224-303)
		R-5'- AGC TGG TCC TCG TTC AAA ATC T-3'		22	
67	iNOS [T]	F-5'- GGC CCA GGA AAT GTT CGA A -3'	82	19	AJ699400 (126-206)
		R-5'- ACA GTG ATG GCC GAC CTG AT-3'		20	
68	LIF[A]	F-5'-CTT CTG GGA GAC TAC AGC CG-3'	111	20	NM_173931.1 (967-1077)

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
		R-5'-GGT CGC CAG AGA CAA CTT TC-3'		20	
69	OSM[A]	F-5'-GAC TTC CTG CAG ACC CTC AA-3'	138	20	NM_175713.3 (271-408)
		R-5'-CAT GCA GTG GAT GTT GTT CC-3'		20	
70	CT-1[A]	F-5'-CTC ACC CCT TCC CCA CTT-3'	102	18	XM_592709.4 (51-152)
		R-5'-TGC TGC ACA TAT TCC TGG AG-3'		20	
71	L-Selectin[A]	F-5'-CTC CCA TCC CTC AAC TTT CA-3'	160	20	NM_174182 (1429-1588)
		R-5'-ATT GAA GCT GGA AGC CAG AA-3'		20	
72	VCAM-1[T]	F-5'- TTG GAT GGT GTT TGC AGT TTC T-3'	97	22	AB052747 (122-218)
		R-5'- AGT CAG TGA AAC AGA GTC ACC AAT CT-3'		26	
73	ICAM-1[L]	F-5'- TGA AGA ACT CAA CCT GAG CTG CCT-3'	146	24	NM_174348.2 (934-1079)
		R-5'- TCG ACT GCA CTG TGA ACA TGA CCT-3'		24	
74	ICAM-3[L]	F-5'- TGA AGA ACT CAA CCT GAG CTG CCT-3'	169	24	NM_174349.1 (501-669)
		R-5'- ATT GGT GCC ATG GTC CTC TCT TCT-3'		24	
75	BAX[A]	F-5'-CTC CCC GAG AGG TCT TTT TC-3'	130	20	NM_173894 (260-389)
		R-5'- GGC ACC TTG GTG CAC AG-3'		17	
76	BAD[A]	F-5'-CTT TTC TGC AGG CCT TAT GC-3'	151	20	NM_001035459 (641-791)
		R-5'-GGT AAG GGC GGA AAA ACT TC-3'		20	

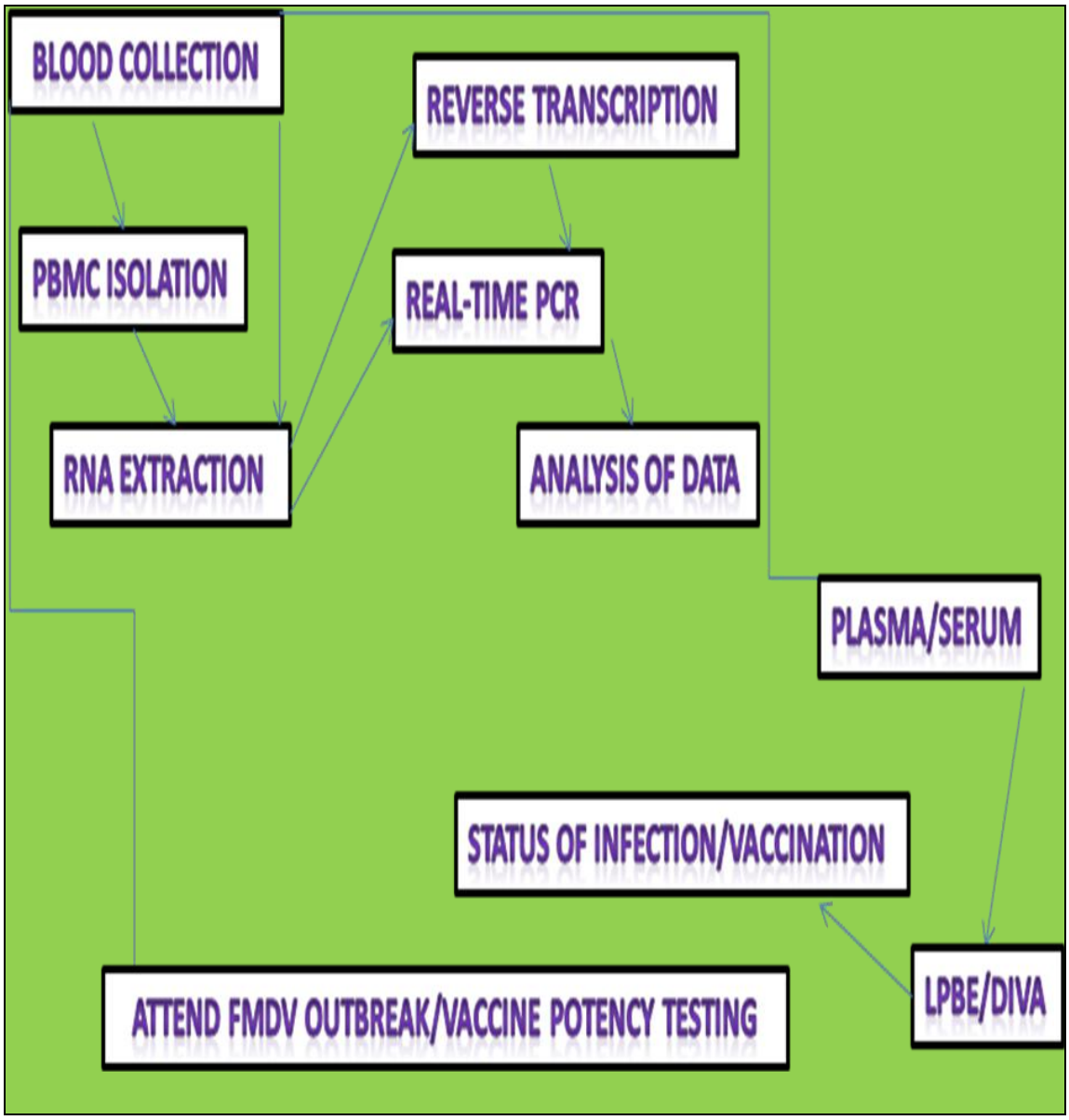
Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
77	TLR1 [M]	F-5’- CCC ACA GGA AAG AAA TTC CA -3’	208	20	AY957622 (203-410)
		R-5’- GGA GGA TCG TGA TGA AGG AA -3’		20	
78	TLR2 [M]	F-5’- ACG ACG CCT TTG TGT CCT AC -3’	192	20	AY957623 (313-502)
		R-5’- CCG AAA GCA CAA AGA TGG TT -3’		20	
79	TLR3[M]	F-5’- GAG GCA GGT GTC CTT GAA CT -3’	329	20	AY957624 (1-327)
		R-5’- GCT GAA TTT CTG GAC CCA AG -3’		20	
80	TLR3[Z]	F-5’- TGA ACC ATG CAC TCT GTT TGC -3’		21	
		R-5’- CTG GAC CCA AGT GCT ACT TTC AA -3’		23	
81	TLR4[M]	F-5’- ACT GAC GGG AAA CCC TAT CC -3’	208	20	AY957625 (118-325)
		R-5’- CAG GTT GGG AAG GTC AGA AA -3’		20	
82	TLR5 [M]	F-5’- AAA ACC ACA TCG CCA ACA TC -3’	191	20	AY957626 (87-277)
		R-5’- CAT CAG ATG GAA CTG GGA CA -3’		20	
83	TLR6 [M]	F-5’- CAA AGC AGG GAA CAA TCC AT -3’	206	20	AY957627 (117-322)
		R-5’- CCA CAA TGG TGA CAA TCA GC -3’		20	
84	TLR7 [M]	F-5’- ACT CCT TGG GGC TAG ATG GT -3’	180	20	GU817062.1 (93-272)
		R-5’- GCT GGA GAG ATG CCT GCT AT -3’		20	
85	TLR8 [M]	F-5’-TCC ACA TCC CAG ACT TTC TAC GA-3’	151	23	AY957629 (97-247)

Sr. No.	Item	Primer sequence	Product size, bp	Primer length	Accession number of nucleotide sequence and position of product
		R-5’-GGT CCC AAT CCC TTT CCT CTA -3’		21	
86	TLR9 [M]	F-5’-CTC GTA TCC CTG TCG CTG AG -3’	210	20	AY957630 (59-268)
		R-5’-CAC CTC CGT GAG GTT GTT GT -3’		20	
87	TLR10 [M]	F-5’-TCT GCC TGG GTG AAG TAT GA -3’	190	20	EU006654.1 (1946-2135)
		R-5’-AAT GGC ACC ATT CAG TCT GG -3’		20	
88	18S RNA [L]	F- 5’-CTG AGA AGA CGG TCG AAC TTG ACT-3’	90	24	DQ222453.1 (1789-1878)
		R-5’-TCC GTT AAT GAT CCT TCC GCA GGT-3’		24	
89	GAPDH-1 [T]	F-5’- GCG ATA CTC ACT CTT CTA CCT TCG A -3’	82	25	U85042.1 (827-908)
		R-5’- TCG TAC CAG GAA ATG AGC TTG AC-3’		23	
90	GAPDH-2 [T]	F-5’- GGC GTG AAC CAC GAG AAG TAT AA -3’	119	23	DQ403066.1 (319-437)
		R-5’- CCC TCC ACG ATG CCA AAG T-3’		19	
91	β-actin	F- 5’-CGA TGA AGA TCA ART CAT TGC-3’	153	21	BC147868 (1068-1221)
		R- 5’-AAG CAT TTG CGG TGG AC-3’		17	
92	CD4 [A]	F-5’-TAC CAG GGA ACA AAA CCT GC-3’	152	20	NM_001103225 (2442-2593)
		R-5’-GTT CAG TTT CCA GCC AGC TC-3’		20	
93	T7 promoter	5’-GTA ATA CGA CTC ACT ATA GGG C-3’		22	
94	M13R	5’-GGA AAC AGC TAT GAC CAT G-3’		19	

	Primers designed with the help of NCBI Primer-Blast: [A]; Primers reported by: [C] - Coussens *et al*., 2004; [K] - Konnai *et al.*, 2003; [L] - Lahmers *et al.*, 2006; [M] - Menzies and Ingham, 2005; [T] - Taubert and Hermosilla, 2008; [We] - Werling, 2002; [Z] - Zhang *et al.*, 2006; Other primers or sequences – provided by laboratory



Flowchart 1. Action plan for cytokine identification



Flowchart 2. Action plan to study level of cytokine expression in Foot-and-Mouth disease virus infection and vaccination

Action plans for cytokine identification and studying expression level of cytokines in PBMCs of normal (stimulated or unstimulated) and Foot-and-Mouth disease virus (FMDV) infected cattle and also from those vaccinated against Foot-and-Mouth disease (FMD) were given (Flowchart 1 and Flowchart 2). Descriptions of different methods used in the study are given below.

3.9 Liquid phase blocking enzyme linked immunoassay (LPBE) test

The test is routinely performed in the laboratory and used in the present investigation. LPBE is used in the quantification of protective antibody level against FMD in the animals following vaccination against FMD. Homologous antibodies present in the serum block the antigen from being detected by guinea-pig serum samples.

3.9.1 Procedure for LPBE

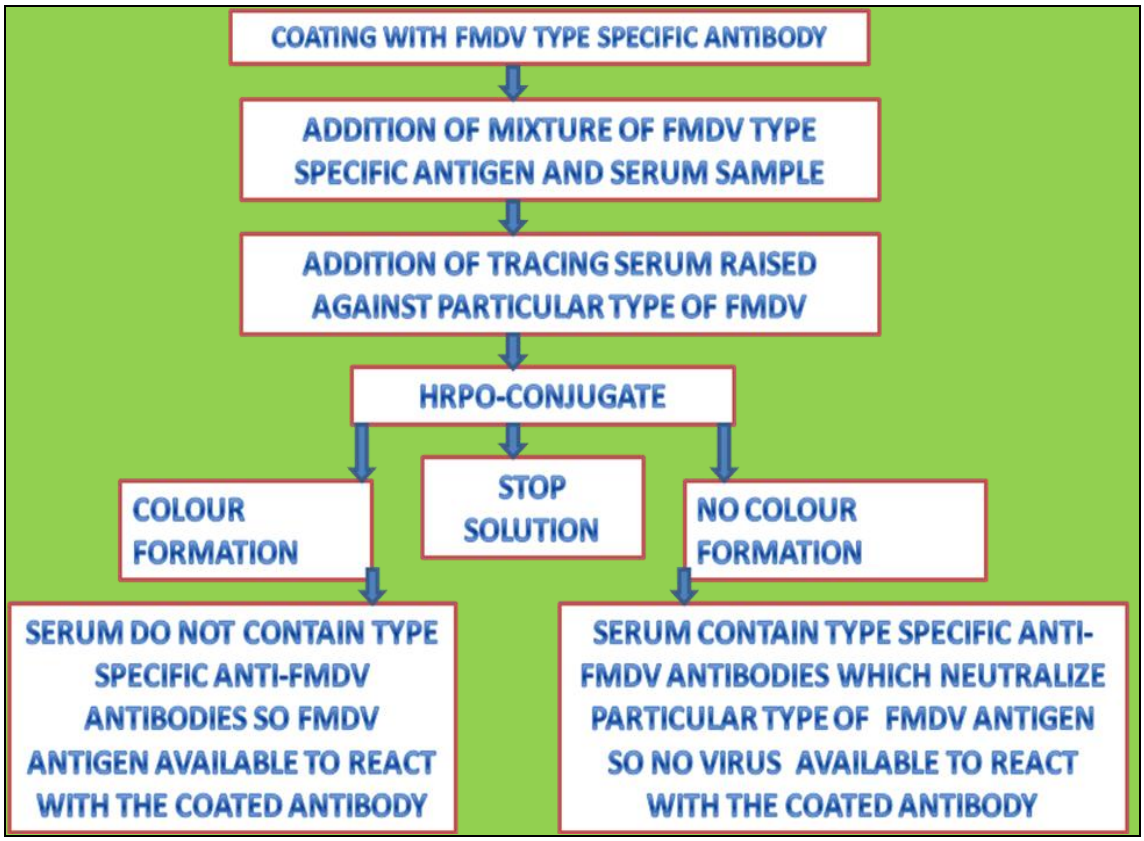
The procedure is described below and action plan is given (Flowchart 3).

3.9.1.1 Coating of plates with Type specific antibodies

1. 50 µl of diluted serum containing antibody against single Type of FMDV (Type - 'O', 'A' and 'Asia 1'- raised in rabbits) (dilution protocol mentioned in the appendix) was added in 96 well enzyme linked immunoassay (ELISA) plates.
2. These plates were incubated at 37 ^{0}C for 1 hour (hr) then transferred at 4 ^{0}C till its use.

3.9.1.2 Preparation of antigen-antibody mixture

1. Two-fold dilutions (starting from 1:32 to 1:256) of serum samples were prepared in low binding Perspex plates with Phosphate buffer saline containing 0.05 % Tween-20 (PBS-T).
2. 75 µl of each dilution of serum samples were transferred to four separate Perspex plates.
3. Equal volume of diluted FMDV antigen (for each Type of FMDV separately) was added in diluted serum samples.
4. Antigen control was kept for each Type of FMDV with equal quantity of PBS-T and antigen without test serum. These Perspex plates were kept at 4 ^{0}C for



Flowchart 3. Action plan for liquid phase blocking enzyme linked immunosorbentassay

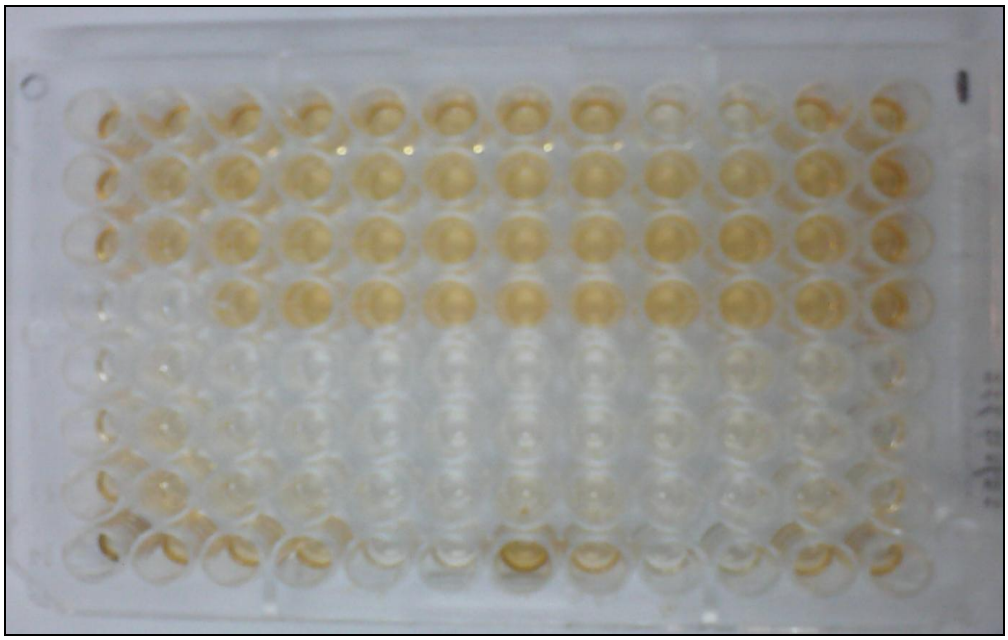


Figure 2. Testing of serum samples in liquid phase blocking enzyme linked immunoassay

overnight incubation.

3.9.1.3 Addition of antigen-antibody mixture into coated plates

1. Coated plates were removed from 4 ^{0}C and brought to room temperature (rt).
2. These plates were washed with washing buffer containing 0.1 % Tween-20, thrice, with a gap of 3 min in each wash at rt.
3. Carefully, 50 µl of antigen-antibody mixture was added in duplicate wells.
4. Controls were kept for each Type of FMDV. Background control was also kept to test any non-specific binding between coating and tracing serum.
5. These plates were incubated for 1 hr at 37 ^{0}C and washed thrice as per mentioned earlier.
6. Tracing serum for each Type of FMDV was added and the plates were incubated for 1 hr.
7. These plates were washed as earlier and 50 µl of anti-guinea-pig HRPO conjugate in blocking buffer (1:3000) was added in each well. These plates were incubated at 37 ^{0}C for 1 hr.
8. After washing as earlier, 50 µl of substrate was added in each well and these plates were incubated at 37 ^{0}C for 15 min.
9. Wells showing formation of yellow colour, visibly, were considered negative for presence of antibody against FMDV (Figure 2).
10. Reaction was stopped with 50 µl of 1 M H_2SO_4.
11. These plates were read in ELISA reader to obtain optical density (OD) values at 492 nm wavelength (reference 620 nm) and hard copy of the reading was taken.
12. Calculated Log_{10} values were recorded. Value less than 1.5 was considered unprotective.

3.10 Differentiation from infected and vaccinated animals (DIVA) test

The DIVA test developed in the laboratory was employed in the detection of infected animals. Antibody against NSP (3AB3) is produced in FMDV infected cattle (8-10 days after FMDV infection) but FMDV free animals which have been vaccinated with inactivated purified vaccine do not produce antibody against NSP. This discriminatory production of antibody to NSP after FMDV vaccination

and infection is deployed to differentiate vaccinated and infected animals. In the test, anti-NSP (3 AB3) antibody present in the serum sample is measured against purified recombinant 3AB3 antigen.

3.10.1 Procedure for DIVA

1. All reagents were brought to rt.
2. 50 µl of diluted 3AB3 protein was added in each well of the 96 well ELISA plates for coating and incubated overnight at 4 ^{0}C.
3. Plates were kept at 37 ^{0}C for 15 min.
4. Test and control serum samples were diluted in diluent buffer (1:20) in Perspex plates.
5. Each coated plate was washed thrice with the gap of 3 min at rt.
6. 100 µl of diluted serum samples were added in duplicate wells of 3AB3 coated ELISA plate.
7. Positive, negative and background (100 µl of diluent buffer) controls were also kept.
8. These plates were incubated for 1 hr at 37 ^{0}C with intermittent shaking.
9. Plates were washed thrice with 3 min gap at rt in each subsequent wash.
10. 50 µl of anti-bovine HRPO conjugate diluted in the diluent buffer (1:2000) was added in each well.
11. Plates were incubated for 1 hr at 37 ^{0}C with intermittent shaking.
12. Plates were washed thrice with 5 min gap in each subsequent wash at rt.
13. 50 µl of freshly prepared substrate solution was added in each well.
14. Plates were incubated for 15 min at 37 ^{0}C without shaking.
15. Colour reaction was stopped with 50 µl stop solution.
16. OD was measured at the wavelength of 492 nm (reference 620 nm).

Test to negative control OD ratio (T/N) was calculated by the following formula,

T/N = (mean OD of test sample – mean OD of background) / (mean OD of negative control – mean OD of background)

Samples exhibiting T/N values more than 2, between 1.75 to 2, and less than 1.75 were considered as positive, negative and suspicious for reactivity to 3 AB3 NSP.

3.11 Preliminary experiments for identification of cytokines, chemokines, chemokine receptors, toll-like receptors and others

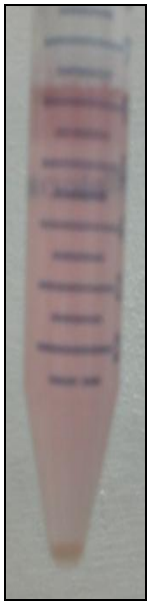
3.11.1 Isolation of peripheral blood mononuclear cells (PBMCs)

1. Anti-coagulant added (0.1 % EDTA) blood sample (5 ml) (Animal No. 380) was diluted with equal amount of PBS (5 ml).
2. Diluted blood sample (7 ml) was carefully and slowly pipetted on the inner-side of the wall of the 15 ml sterile pyrogen free conical polypropylene centrifuge tubes with already added 3 ml of Histopaque®-1077 solution (Boyum, 1968).
3. After balancing, tubes were kept in the centrifuge at 400 x g (1300 revolutions per minute, rpm) for 30 min at rt in SORVALL® RC 3 B plus centrifuge with rotor H 4000.
4. On centrifugation, whitish circular band of PBMCs was observed in between upper plasma and lower red cellular layer (Figure 3).
5. Band of PBMCs was transferred with the help of glass Pasteur pipette to another 15 ml centrifuge tube with 5 ml of RPMI-1640 and centrifuged at rt for 10 min at 250 x g (900 rpm).
6. Resulting cell pellet was again washed as per above and finally the cell pellet was dissolved in 2 ml of PBS (Stock of PBMCs).

3.11.2 Vital cell counting

1. Vital cell counting was performed as per the method described by Sigma.
2. In short, 500 µl of 0.4 % Trypan blue was added in 500 µl of stock of PBMCs (200 µl of cell mixture from Stock and 300 µl of PBS) at the final dilution of 1:5.
3. The mixture of cells and Trypan blue was allowed to stand for 10 min at rt.
4. 10 µl of this mixture was loaded over white blood cells (WBCs) counting chambers of the Haemacytometer by pipette.
5. Dead cells were stained blue and not counted. Unstained viable cells were counted from all four WBC counting chambers.
6. After determining the average count per square, No. of cells/ml was calculated using following formula,

(A)



(B)

Figure 3. Band (A) and pellet (B) of peripheral blood mononuclear cells obtained after treatment of whole blood with Histopaque

No. of cells/ml= the average count per square x dilution factor x 10^4

3.11.3 Stimulation of PBMCs with mitogen, Conconavalin A

1. The PBMCs were diluted in RPMI-1640 medium with 10 % Foetal Bovine Serum (FBS) to 10^4 cells/ml.
2. Wells of a 24 well tissue culture plate was seeded with 1 ml of diluted cells.
3. Simultaneously, mitogen, ConA was added into each well at the final concentration, 5 ng/ml.
4. At 4 hr, 8 hr and 24 hr intervals, the ConA stimulated cells were harvested from four wells.
5. Cell suspensions from each well for single treatment was pooled in 2 ml eppendorf tubes and centrifuged at 2000 rpm (380 g) for 10 min.
6. After dissolving cell pellet in 500 µl of Tri reagent LS (Sigma) tubes were kept at – 80 ^{0}C till its treatment.

3.11.3.1 RNA Extraction from mitogen stimulated PBMCs

1. Extraction of RNA from Con A stimulated PBMCs was carried out as per the method of Chomczynski and Sacchi, 1987 following manufacturer's instructions (Tri reagent LS).
2. First, samples were removed from freeze and kept at rt for 5 min to thaw.
3. Chloroform (100 µl) was added in each tube before centrifugation at 12000 g for 15 min.
4. Resulting supernatant was collected; Isopropanol (250 µl) was added into each tube and allowed to incubate for 5-10 min.
5. Thereafter, tubes were centrifuged at 12000 g for 10 min at 4 ^{0}C.
6. Resulting supernatant was removed and RNA pellet was washed with 75 % ethanol (1 ml).
7. To wash RNA pellet, ethanol added tubes were vortexed and then centrifuged at 7500 g for 10 min at 4 ^{0}C.
8. Ethanol was discarded and RNA pellet was allowed to dry for 5 min by slanting the eppendorf tubes on tissue paper in the laminar flow.

9. 30 µl Nuclease Free Water (NFW) was added, after short spin the RNA samples were kept at – 80 ^{0}C till its treatment.

3.11.3.2 Reverse transcription (RT)

1. Complementary DNA (cDNA) was prepared as per the following method for a total volume of 25 µl reaction.
2. In 0.5 ml properly labeled PCR eppendorf tubes, after addition of extracted RNA – 8 µl, Oligo $dT_{(15)}$ – 2 µl (1 µg), Random primers – 2 µl (1 µg), tubes were incubated at 70 ^{0}C for 10 min in the water bath.
3. Immediately, tubes were placed on ice for 5 min.
4. RT Master Mix was prepared as per given below.

RT Master Mix:

5x RT buffer	–	5 µl	x	'X'
dNTP (10 mM/µl)	–	2 µl	x	'X'
MMLV-RT (200 U/µl)	–	1 µl	x	'X'
NFW	–	5 µl	x	'X'

'X'- Total number of reactions.

As a general rule, to make-up the loss of reaction volume during pipetting, the total number of reactions was considered as few more than the actual reactions required for preparing the Master Mix of any kind. Contols were kept wherever required.

5. After short spin, 13 µl of this RT Master Mix was added into each tube.
6. Tubes were again placed back in the water bath at 42 ^{0}C for 1 hr.
7. Tubes were placed in dry heat at 95 ^{0}C for 10 min to inactivate RT enzyme.
8. Finally, short spin was given.
9. Tubes were stored at – 20 ^{0}C.

3.11.3.3 Polymerase chain reaction (PCR)

1. Commercially procured primers were used for specific amplification of IL-2, IL-4, IL-10 and IFN-γ.
2. Positive controls were also kept.
3. 3 µl of cDNA was used as a template in PCR reaction volume of 25 µl.

4. In properly labeled 0.2 ml PCR eppendorf tube PCR Master Mix was added (22 µl for each reaction).
5. The PCR Master Mix was prepared as follows.

PCR Master Mix:

10 x buffer	–	2.5 µl	x	'X'
dNTP (10 mM/ µl)	–	1.0 µl	x	'X'
$MgCl_2$ (25 mM/µl)	–	1.5 µl	x	'X'
Primer set (approx. 8.33 pmole/µl)	–	1.0 µl	x	'X'
NFW	–	15.75 µl	x	'X'
HotStar Taq DNA polymerase, (5 U/µl)	–	0.25 µl	x	'X'

'X'- Total number of reactions.

6. Template was added and the tubes were kept in the thermocycler.

Cycling conditions:

Steps	**Temp**	**Time**	**Remark**
I) Initial activation	95 ^{0}C	15 min	-
IIa) Denaturation	95 ^{0}C	30 s	35 cycles
IIb) Annealing	50 ^{0}C	30 s	
IIc) Extension	72 ^{0}C	1 min	
III) Final extension	72 ^{0}C	10 min	-
IV) Hold	4 ^{0}C	Infinite	-

3.11.3.4 Gel electrophoresis

1. 1 % agarpose gel was prepared in TBE.
2. Before stacking the gel Ethidium bromide (0.5 µg/ml) was added in the gel.
3. 5 µl of amplified cytokine DNA products were loaded in the well using 6x loading dye.
4. Suitable PCR marker was also kept to identify the size of the amplified product.
5. Gel documentation system was used to visualize and document the picture.

3.11.4 Stimulation of PBMCs with specific FMDV

PBMCs were isolated and stimulated with FMDV (Type 'A').

3.11.4.1 Isolation of PBMCs and stimulation with FMDV and mitogen

1. PBMCs were isolated from anti-coagulant added (0.1 % EDTA) blood of six cows (No. – 6, 306, 333, 336, 337 and 354) from Dairy section, Mukteshwar as per the method described earlier.
2. Cells were counted by vital staining with Trypan blue as per described earlier.
3. Cells were diluted at final concentration of 2.8 x 10^5 cells/ml in RPMI-1640 with 10 % FBS.
4. In the wells of 24 well tissue culture plates, 1 ml of this cell suspension was added.
5. PBMCs of each animal were treated with either ConA (5 ng/ml final concentration) or 12.5 µg of ultrapurified FMDV antigen (Type A - 17/82 – measured by spectrometer).
6. Cell control was kept from single animal.
7. The cells were pelleted after 8 hr post stimulation as per described earlier.
8. 500 µl of Trizol LS (Invitrogen) was added in pelleted PBMCs from each well.
9. These cells were completely lysed by pipetting and kept at – 80 ^{0}C till its treatment.

3.11.4.2 RNA extraction

1. RNA was extracted as per Chomczynski and Sacchi, 1987 following manufacturer's instructions (Trizol LS).
2. Pelleted PBMCs in Trizol LS (500 µl) were allowed to homogenize at rt to allow complete dissocation of nucleoprotein complexes by keeping them at rt for 10 min.
3. 150 µl of chloroform was added into each tube containing Trizol LS and were shaken vigorously for 15 s and allowed to incubate at rt for 10 min.
4. The PBMC + Trizol + chloroform mixture was centrifuged at 10,000 g for 15 min at rt.
5. Upper colourless aqueous phase, which was approx 70 % of volume of Trizol, was pipetted out in a 1.5 ml eppendorf tube.
6. RNA in the aqueous phase was precipitated by adding 320 µl isopropyl alcohol.

7. Samples were allowed to incubate for 10 min and centrifuged at 10000 g for 10 min at rt.
8. Supernatant was discarded, 700 µl of ethanol (75 %) was added, vortexed for 10 s and centrifuged (7500 g for 10 min).
9. Ethanol was discarded and RNA pellet was dried as per earlier.
10. RNA was dissolved in 35 µl NFW.
11. After short spin, tubes were kept at – 80 ^{0}C till its treatment.

3.11.4.3 <u>Reverse Transcription (RT)</u>

cDNA was prepared as per the method mentioned earlier except total reaction volume was half, 12.5 µl and random primers were not used.

Proportionately other reagents were reduced or increased.

3.11.4.4 <u>Polymerase chain reaction (PCR)</u>

1. Specific primers were used to amplify particular cytokine product (Pierce-Endogen).
2. Positive and negative controls were also kept.
3. 1 µl of cDNA was used as a template in total PCR reaction volume of 12.5 µl.
4. In properly labeled 0.2 µl PCR eppendorf tube, 11.5 µl PCR Master Mix for each reaction was added
5. PCR Master Mix was prepared as follows,

PCR Master Mix:

5 x buffer	–	2.5 µl	x	'X'
dNTP mix (10 mM/µl)	–	0.5 µl	x	'X'
$MgCl_2$ (25 mM/µl)	–	0.75 µl	x	'X'
Primer set (approx. 8.33 pmole/µl)	–	0.5 µl	x	'X'
GoTaq polymerase (5 U/µl)	–	0.125 µl	x	'X'
NFW	–	7.125 µl	x	'X'

Cycling conditions were followed as per mentioned earlier except initial activation at 95 ^{0}C for 2 min and slight modifications in annealing temperatures, if necessary.

3.11.4.5 Gel electrophoresis

Gel electrophoresis was performed and visualized amplified products were documented as per mentioned earlier.

3.12 Amplification of DNA product for purification

1. Primers by Pierce-Endogen and Custom synthesized were used to amplify specific cytokine or other interested target DNA product using polymerase chain reaction technique with the total volume of reaction of 50 µl.
2. Taq DNA polymerase was used according to manufacturer's instructions.
3. Cycling conditions were used as per mentioned earlier with the annealing temperatures between 50-60 ^{0}C.
4. Suitable low melting agarose gel (2%) was employed in the gel electrophoresis using TAE buffer.
5. Ethidium bromide was added in the gel before stacking. Electrophoresis was performed at 90 V for 40 min.
6. After completion of electrophoresis, gel was visualized using Ultraviolet (UV) transilluminator for specific amplified product.
7. Located product was carefully cut on the UV transilluminator using fresh razor blade.

3.13 Gel extraction and purification of DNA product

1. Amplified DNA product in the gel was extracted using Gel extraction kit (Qiagen) with manual operation or by the machine, QIAcube.
2. Fed protocol in the QIAcube machine for short fragment was used to extract and purify the DNA.
3. Mostly, manual method was used which was described here.
4. In eppendorf tube (1.5 ml) containing 600 µl of buffer QG, gel slab with specific DNA product visualized on UV transilluminator in ethidium bromide stained gel was cut precisely and put into QG buffer.
5. Tubes containing sliced gel slabs in QG buffer were kept immediately at 50 ^{0}C for 10 min in hot water bath.
6. 500 µl, mix of QG buffer with solubilized gel was transferred on gel extraction

columns mounted on collection tubes and centrifuged at 12000 rpm for 1 min.

7. Discarded the flowthrough from the column, added rest of the mixture and centrifuged.
8. Added 600 µl buffer QG to columns, kept for 2 min at rt and centrifuged at 12000 rpm (13684 g) for 1 min.
9. Buffer PE, 750 µl was added and kept for 2 min and centrifuged at 12000 rpm for 1 min.
10. Empty spin was given at 12000 rpm for 3 min and collection tubes were discarded.
11. 30 µl of elution buffer was added in each tube, kept for 10 min and centrifuged at 12000 rpm for 5 min.
12. Eluted DNA was stored after it was measured by NanoDrop at – 80 ^{0}C.

3.14 Molecular cloning of purified products

3.14.1 Ligation of purified DNA to the vector

1. To clone the desired purified DNA products T-A cloning was employed using pGEM® - T Easy Vector System I according to manufacturer's instructions with slight modifications.
2. Insert volume was calculated according to the formula given by the manufacturer, wherever needed.

$$\frac{\text{ng of vector (50 ng)} \times \text{kb size of insert (x)}}{\text{kb size of vector (3 kb)}} \times \text{insert: vector molar ratio (3:1)} = \text{ng of insert}$$

3. In the 0.2 ml PCR tube following items were added,

pGEM®-T Easy Vector (50 ng/µl)	– 1 µl
2x Rapid Ligation buffer	– 5 µl
T4 DNA ligase 3 Weiss units/µl	– 1 µl
Insert (Purified DNA)	– 'X' µl
NFW (to make-up the volume 10 µl)	– 'X' µl

4. The tubes containing the insert and vector left for 1 hr at rt.

5. Thereafter, these tubes were incubated at 4 ^{0}C overnight.
6. The ligation mixture was used freshly in the transformation whenever possible. Otherwise, the ligation mix was stored at – 80 ^{0}C till its use.

3.14.2 Transformation

1. Stock of single cell colony of prokaryotic host *Escherichia coli* (JM 109) grown in Luria Bertani (LB) broth was kept at 4^{0}C. 50 µl of this stock was taken into 5 ml of freshly prepared LB broth placed in 50 ml glass conical flask (1:100).
2. Flask was kept in shaker cum incubator for overnight incubation at 37 ^{0}C at 180 rpm.
3. Next day, 500 µl of these refreshed *Escherichia coli* (JM109) cells were put into 50 ml of LB broth placed in 500 ml glass conical flask.
4. Flask was kept in shaker cum incubator for incubation at 37 ^{0}C at 180 rpm for 2 hours till visible slight turbidity/OD reaches to 0.4 to 0.6.
5. Grown culture was taken in 50 ml centrifuge tube and left on ice in the plastic thermos with lid closed for 20-30 min.
6. Meanwhile new microtips of all capacity and 1.5 ml labeled eppendorf tubes wrapped in alluminium foil were kept at – 40 ^{0}C.
7. Bacterial culture kept on ice was centrifuged for 20 min at 4 ^{0}C for 2000 rpm.
8. Supernatant was discarded; 2 ml of transformation storage solution (TSS) was added in cell pellet while the tube was placed on ice. Gently twice or thrice it was pipette up and down.
9. The tube left on ice with closed lid for 2 hr.
10. 200 µl of competent cells were pipetted in chilled eppendorf tubes placed on ice and 4 µl of ligation mixture was added in the tubes.
11. After gentle tapping with fingers for few seconds tubes were kept on ice for 1 hr.
12. Heat shock was given to the cells for increase in pore size which facilitate entry of DNA into the cells.
13. The eppendorf tubes containing mixture of cells in TSS and ligation mixture was placed in hot water bath at 42 ^{0}C for 55 s.
14. After removing from hot water bath the tubes were immediately placed on the ice for 5 min.

15. 800 µl of solution of competence (SOC) was added in each tube and tubes were placed in shaker cum incubator for 1 and half hr.
16. Autoclaved LB agar was allowed to cool and just before solidification of the agar, IPTG (0.5 mM), X-Gal (80 µg/ml) and Ampicillin (100 µg/ml) were added. Agar plates were prepared and the culture grown in SOC was poured over it by the pipette and allowed to absorb for 15 min in the laminar flow in the fashion that the lids of the plates were open.
17. These lid closed agar plates were kept in the room incubator at 37 ^{0}C for overnight.
18. Next day the plates were observed for presence or absence of white and blue colonies. If no white colonies were observed then the procedure was repeated.

3.14.3 Growth and preservation of transformed cells

1. Several white colonies from the plates were either directly used to grow in LB broth with 100 µg/µl antibiotic, Ampicillin or streaked on separate IPTG-X-Gal-LB agar containing Ampicillin plates and then the colony from this plate was used to grow.
2. 3 ml of LB broth with antibiotic was added in 15 ml centrifuge tubes/conical flasks.
3. Individual white colony picked up by sterile microtip which was left into the broth in the tube.
4. These centrifuge tubes were kept in the shaker-cum-incubator at 180 rpm overnight.
5. These grown cells were used to extract the plasmid DNA with the insert.
6. To preserve the cells, in a new set of centrifuge tubes, 3 ml of LB broth with antibiotic was added.
7. Earlier grown transformed cells in LB broth were used at the dilution of 1:100 to add in the new set of centrifuge tubes/conical flaks.
8. These new tubes were kept in the shaker-cum-incubator at 180 rpm for 4 hr.
9. Sterile glycerol was added into this grown bacterial culture at the final concentration of 15 %, carefully. Then it was vortexed for 30 s to ensue complete mixing.

10. Appropriate volume of grown cells with 15 % glycerol were added in the 2 ml properly labeled cryo-vials and kept at – 80 ^{0}C.
11. Freshening of the preserved cloned cells was carried out whenever possible.

3.14.4 Plasmid extraction from transformed cells

1. Plasmid was extracted by using manufacturer's instructions (AuPrep, Zyppy™ Plasmid Miniprep kit). Mostly, Zyppy™ Plasmid Miniprep kit was employed by using classical centrifuge-based procedure for processing 3 ml of bacterial culture.
2. Overnight grown transformed bacterial culture in LB broth with antibiotic was added to 1.5 ml eppendorf centrifuge tubes.
3. The tubes with the culture were centrifuged at 10000 g for 30 s. Supernatant was discarded and again rest of the bacterial culture was added in the 1.5 ml centrifuge tube and it was centrifuged again to pellet the cells.
4. Supernatant was discarded. In each tube 600 µl of NFW was added to resuspend the bacterial cells completely. 100 µl of completely dissolved 7x Lysis buffer (blue) was added in the bacterial cells resuspended in NFW and mixed by inverting the tubes 6 times.
5. After adding the Lysis buffer the solution changed from opaque to clear blue indicating complete bacterial lysis. 350 µl of Neutralization buffer (yellow) containing RNase A was put in every tube with lysed bacterial culture and mixed by pipetting or inverting the tubes.
6. Blue colour of the solution was changed to yellow after complete neutralization.
7. Yellow precipitates formed in the tubes were pelleted by centrifugation at 10000 g for 4 min.
8. Approx. 900 µl of supernatants were added into the Zymo-Spin™ IIN columns placed into collection tubes and centrifuged for 15 s at 10000 g.
9. Flow-through in the collection tubes was discarded and the columns were placed back into the collection tubes.
10. 200 µl of Endo-Wash buffer was added in the each column and centrifuged for 15 s at 10000 g.
11. 400 µl of Zyppy™ wash buffer with ethanol was added in the each column and

centrifuged for 30 s at 10000 g.

12. Columns were transferred into clean 1.5 ml centrifuge tubes and 30 µl of Zyppy™ Elution buffer was added directly to the column matrix.
13. Columns were allowed to stand at rt for 1 min.
14. Columns containing elution buffer were centrifuged at 10000 g for 15.
15. The tubes containing eluted plasmids were properly labeled.
16. Plasmid DNA concentration was measured using NanoDrop and these tubes were kept at – 80 ^{0}C till its use.

Plasmids were checked in 1 % agarose gel using TBE buffer as per mentioned earlier and documented with the use of gel documentation system.

3.14.5 Checking the plasmid for insert

Plasmids with inserts were checked either by conducting polymerase chain reaction using bacterial colony or plasmid (colony/plasmid PCR) or by treating the plasmids with restriction enzyme, whenever possible.

3.14.5.1 Colony or Plasmid PCR

1. 200 µl transformed bacterial cultures grown in LB broth with 100 µg/ml antibiotic were taken in the 0.5 ml centrifuge tubes and centrifuged at 2000 g for 2 min at rt.
2. Resulting bacterial pellets were resuspended in 100 µl of NFW and placed in the hot water bath at 90 ^{0}C for 10 min.
3. Later the tubes were centrifuged at 4000 g for 2 min.
4. Resulting supernatants (2 µl) or extracted plasmids (1 µl) were used in the PCR (12.5 µl total volume of the PCR reaction) to amplify specific products by using specific primers of interest as per mentioned earlier.
5. Gel electrophoresis was done in 1% agarose gel as per mentioned earlier and results were documented.

3.14.5.2 Restriction enzyme treatment

1. After adding the following items in the 0.5 ml tubes for each plasmid the tubes were placed in the hot water bath for 2 hr at 37 ^{0}C.

EcoRI	–	0.75 µl
10x buffer	–	1.00 µl
Plasmid	–	8.25 µl

2. 1 % agarose gel with ethidum bromide was prepared in the TBE.
3. Gel was stacked and 5 µl of restriction enzyme treated plasmids were loaded in the wells cut into the gel.
4. Suitable PCR marker was loaded in a well.
5. The gels were visualized for specific release of the inserts and documented using gel documentation system.

3.15 Sequencing

1. To absolute confirmation of cloned DNA inserts in the plasmids, sequencing was carried out with respect to the manufacturer's protocol.
2. Both, gel (ALF express II amersham pharmacia biotech) or capillary method (3130 Genetic Analyzer, USA) was employed to sequence targeted cytokine using extracted plasmids with inserts.
3. Capillary method of sequencing was employed in most of the cases and is described, in short.
4. After adding following items per well into MicroAmp™ optical 96-well plate, it was sealed with MicroAmp™ optical adhesive film.
5. Plasmid DNA concentration was measured by NanoDrop and about 200 ng of plasmid DNA was used.

Sequencing reaction:

Big Dye® enzyme Terminator v1.1, v3.1 5x sequencing buffer	– 0.5 µl
Big Dye® Terminator v1.1, v3.1 5x sequencing buffer	– 1.75 µl
Primer M13R/T7 promoter	– 1.5 µl
NFW	– 4.25 µl
Template (plasmid DNA-about 200 ng)	– 2 µl

6. The plate was kept in the thermocycler and cycling conditions were used as under.

Cycling conditions:

Steps	Temp	Time	Remark
I) Initial activation	95 ^{0}C	1 min	-
IIa) Denaturation	96 ^{0}C	10 s	25 cycles
IIb) Annealing	45-50 ^{0}C	05 s	
IIc) Extension	60 ^{0}C	4 min	
III) Hold	4 ^{0}C	Infinite	-

(Annealing temp 45 ^{0}C for M13R and 50 ^{0}C for T7 promoter)

7. After PCR reaction was completed, purification of sequencing extension products was carried out as follows.
8. 12 µl of Mix I (10µl of Milli-Q water and 2 µl of 125 mM EDTA (pH 8.0) was added into each well with subsequent addition of 52 µl of Mix II (2µl of Sodium acetate, pH 4.6 and 50µl of absolute ethanol).
9. After sealing the plate, contents in the plate were mixed by inverting and the plate was left to incubate for 15 min at rt.
10. The plate was centrifuged for 30 min at 3000 g. Supernatant was discarded by inverting the plate on paper towels.
11. Plate was centrifuged in inverted position at 180 g for short duration to remove residual supernatant.
12. After adding 100 µl of 70 % ethanol the plate was centrifuged at 3000 g for 5 min and the step was repeated.
13. Again the plate was centrifuged in inverted position at 180 g for short duration to remove residual supernatant.
14. 10 µl of Hi-Di formamide was added in each well and the plate was placed in the centrifuge and short spin was given.
15. Sequencing products were denatured at 95 ^{0}C for 5 min, immediately placed on ice and short spin was given.
16. Electrophoresis was carried out by placing the plate between plate base and plate retainer and loaded in ABI 3130 genetic analyzer.
17. Data collection software (v3.0- Applied Biosystems) was used to control and monitoring of the run.
18. KB base caller v1.4 software was used to generate DNA sequence data.

3.16 Investigation of FMD outbreak at Izatnagar and Papum Pare and Vaccination at Mukteshwar

3.16.1 Purification of total RNA > 200 nucleotides from blood preserved in RNAprotect® Animal blood tubes using RNeasy protect Animal blood kit

1. Red blood cells (RBCs) lysing reagent in these tubes disrupts RBCs and stabilizes intracellular RNA to arrest gene expression profile.
2. Extraction of RNA from the blood was performed as per the method given by the manufacturer.
3. Tubes with lysed blood samples were first incubated at rt and centrifuged for 3 min at 5000 x g to pellet the cells.
4. Resulted supernatant was removed by pipette and 1 ml RNase-free water was added to dissolve the pellet by vortexing.
5. After adding 240 µl of RSB in the pellet, the tubes were vortexed to dissolve the pellet.
6. These samples were taken into 1.5 ml collection tubes.
7. 200 µl of RBT buffer and 20 µl of Proteinase K were added into the tubes.
8. Tubes were vortexed for 5 s and incubated for 10 min at 55 ^{0}C at 400 rpm. After incubation the shaker-cum-incubator was set to 65 ^{0}C.
9. The samples were taken into Qiashredder spin columns (lilac) attached on 2 ml collection tubes.
10. These tubes were centrifuged at 10,000 g for 3 min.
11. The resulted flow-through was taken in the 1.5 ml collection tubes.
12. 240 µl of absolute ethanol was added in the tubes and mixed by vortexing.
13. The mixtures were taken into RNeasy MinElute spin column (pink) attached on 2 ml collection tube.
14. Lids were closed and the tubes were centrifuged for 1 min at 8000 g.
15. Resulted flow-through for each treatment was discarded.
16. 350 µl of buffer RW1 was added to the column.
17. After closing the lid, these tubes were centrifuged for 15 s at 8000 g. Flow-through was discarded.

18. DNase I incubation mix (80 µl) was added onto each RNeasy MinElute spin column membrane and incubated at rt for 15 min.
19. RW1 (350 µl) was added into the RNeasy MinElute spin columns, lids were closed and these tubes were centrifuged for 15 s at 8000 g.
20. Flow-through was discarded. 500 µl of RPE buffer was added to the RNeasy Min Elute spin columns.
21. Lids were closed and the tubes were centrifuged for 15 s at 8000 g.
22. Flow-through was discarded.
23. 500 µl of 80 % ethanol was added to the RNeasy MinElute spin columns.
24. Lid closed tubes were centrifuged for 2 min at 8000 g.
25. RNeasy Min Elute spin columns were placed in new 2 ml collection tubes.
26. Lid opened spin columns were centrifuged at 10000 g for 5 min.
27. Flow-through and collection tubes were discarded.
28. RNeasy MinElute spin columns were placed in new properly labeled 1.5 ml collection tubes.
29. 30 µl of REB buffer was added onto the spin column membranes.
30. Lids were closed and the tubes were centrifuged for 1 min at 8000 g.
31. Eluted RNA was incubated at 65 ^{0}C for 5 min in the shaker-cum-incubator without shaking.
32. The tubes with eluted RNA were placed on ice immediately after incubation.
33. The eluted RNA was stored at – 80^{0}C.

3.16.2 Measurement and dilution of RNA

1. RNA was measured using NanoDrop 1000.
2. 1 µl of RNA samples were placed over sensor point on Nanodrop after blanking with diluents.
3. Readings were noted on the label pasted on each eppendorf tube containing RNA.
4. Extracted RNA was diluted with NFW to the concentration of 5 ng/ µl in separate eppendorf tubes.

3.16.3 Real-time PCR

1. Real-time PCR was performed using the 7500 real time PCR system (AB Applied Biosystems, USA).
2. One-step real-time PCR was performed using quantitation by comparative CT (ΔΔ) type of experiment (Livak and Schmittgen, 2001; Zhang *et al.*, 2002) as per manufacturer's directions.
3. Following directions given by the manufacturer (QuantiTect® SYBR® Green RT-PCR kit), the cycling conditions were fed to amplify specific product and collection of data.
4. Melt curve analysis was performed as per manufacturer's indications.

Cycling conditions:

Steps	Temp	Time	Remark
I) Holding	50 ^{0}C	30 min	RT enzyme acts (RNA to cDNA)
II) Holding/Denaturation	95 ^{0}C	15 min	RT enzyme denatured
IIIa) Denaturation	94 ^{0}C	15 s	45 cycles
IIIb) Annealing	52 ^{0}C	30 s	
IIIc) Extension	72 ^{0}C	30 s	
Melt curve analysis			

5. 10 ng of total RNA was used in the reaction to study IL-2 mRNA expression.
6. Total RNA was used in real time PCR using Qiagen one-step RT PCR kit.
7. 2x Quanti Tect SYBR Green RT-PCR Master Mix contains HotStarTaq® DNA polymerase, Quanti Tect SYBR Green RT-PCR Buffer, dNTP mix including dUTP, SYBR Green I, ROX passive reference dye and 5 mM $MgCl_2$. Quanti Tect RT Mix is a mixture of Omniscript® reverse transcriptase and Sensiscript® reverese transcriptase.
8. 18 µl of Master Mix was added in each well of MicroAmp™ optical 96-well plate.
9. 2 µl of diluted RNA was mixed with the master mix (prepared as per mentioned later) in the well. β-actin was used as endogenous control. Negative controls were also used in the reaction.
10. One of the RNA sample from cattle (LPBE titer lower than 1.5) was used as a reference sample in the experiment.
11. After short spin at 1200 g for 3 min the plate was kept in the slab provided by the

manufacturer and the run was started.

Master Mix:

2x Quanti Tect SYBR Green RT-PCR Master Mix	–	10.0 µl	x	'X'
IL-2 Primer F (5 pmole/µl)	–	1.0 µl	x	'X'
IL-2 Primer R (5 pmole/ µl)	–	1.0 µl	x	'X'
Quanti Tect RT Mix	–	0.2 µl	x	'X'
RNase free water	–	7.0 µl	x	'X'

'X' – total no of reactions including negative controls and few more reactions to make-up the loss during pipetting.

12. After the run was completed, the experiment was saved and the gene expression data generated by the software supplied with the machine was recorded and analyzed.

3.17 Investigation of FMD outbreak at Kashipur

3.17.1 Storage, transportation, PBMC isolation from blood samples collected at Kashipur

1. Blood samples collected in the heparinated Vacutainer from 20 dairy cows of two adjacent farms at Kashipur were brought to the laboratory at +4 ^{0}C after 4 hr of journey and kept overnight at 4 ^{0}C till further processing for PBMC isolation.
2. PBMC isolation was done with earlier method but with slight modifications.
3. Here, instead of dilution of blood with PBS, dilution of blood samples was made with RPMI-1640 with 5 % FBS.
4. Only, 3 ml of blood samples with anti-coagulant were diluted with equal amount of dilution media. Rest of the procedure was similar as per mentioned earlier.
5. Though, in some cases, undiluted blood samples (3 ml of anticoagulated blood) were used to isolate PBMCs.
6. Pelleted cells were directly used for RNA extraction.

3.17.2 RNA extraction (RNeasy Mini method)

1. Total RNA from cell samples was extracted by the protocol given by the manufacturer (Qiagen).

2. 600 µl of buffer RLT containing β-mercaptoethanol (ME) (10 µl in 1 ml RLT) was added in each tube (freshly prepared cells) and vortexed well to disrupt cells.
3. Lysate was homogenized using column.
4. Lysate was pipetted on QIAshredder spin column placed in a 2 ml collection tube and centrifuged at 10000 rpm for 2 min.
5. Spin column was thrown out and collection tubes were marked carefully.
6. 600 µl of 70 % ethanol was added into the homogenized lysates and mixed well by pipetting.
7. 600 µl of ethanol-lysate mix was added on the RNeasy spin column placed in a 2 ml collection tube.
8. After closing the lid it was centrifuged at 9000 rpm for 15 s.
9. Flow-through was discarded and RNeasy spin column reloaded with ethanol-lysate mix.
10. Flow-through was discarded again and 700 µl of RW1 buffer was added on spin column and after closing lid it was centrifuged at 9000 rpm for 15 s.
11. Flow-through was discarded again.
12. Added 500 µl buffer RPE to RNeasy spin column, lid was closed and it was centrifuged at 9000 rpm for 15 s.
13. Decanted flow-through and again 500 µl of buffer RPE was added to RNeasy spin column.
14. Closing the lid gently column was centrifuged at 9000 rpm for 2 min.
15. Flow-through along with tube was discarded and column with closed lid was kept in a new 2 ml collection tube and centrifuged at 10000 rpm for 1 mt.
16. RNeasy column was kept on 1.5 ml collection tube.
17. 50 µl of RNase free water was pipetted carefully on the membrane of the column.
18. Lid was closed and it was centrifuged at 9000 rpm for 1 min. Eluted RNA was stored at – 80 ^{0}C.

3.17.3 Measurement and dilution of RNA

RNA was measured as per earlier mentioned. The RNA was diluted with NFW to the concentration of 10 ng/µl.

3.17.4 Real-time PCR

1. One-step real-time PCR was performed as per mentioned earlier using comparative CT (ΔΔ) type of experiment.
2. Rest of the procedure for run and gene expression data recording was followed as per mentioned in earlier experiment.
3. 20 ng of total RNA was used in the reaction to study expression level of IFN-α, IFN-β, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-16, IL-21, IL-27p28 and TLR7. Concentractions of $MgCl_2$ for IL-10 and IL-12p40 were 5 mM and 4 mM respectively (Konnai *et al.*, 2003).
4. 2 µl of the diluted total RNA was used in performing real time PCR with the use of QuantiTect® SYBR® Green RT-PCR kit.
5. 18 µl of final Master Mix was added in each well of MicroAmp™ optical 96-well plate. β-actin was used as endogenous control.
6. Negative controls were kept in the experiment.
7. One of the RNA sample from in-contact healthy cow was used as a reference sample in the experiment.
8. Melt curve analysis was performed and outliers were negated in general for analysis.

Cycling conditions:

Steps	Temp	Time	Remark
I) Holding	50 ^{0}C	30 min	RT enzyme acts (RNA to cDNA)
II) Holding/Denaturation	95 ^{0}C	15 min	RT enzyme denatured
IIIa) Denaturation	94 ^{0}C	15 s	50 cycles
IIIb) Annealing	55 ^{0}C	30 s	
IIIc) Extension	72 ^{0}C	30 s	
Melt curve analysis			

Master Mix:

2x Quanti Tect SYBR Green RT-PCR Master Mix	–	10.0 µl	x	'X'
Primer F (5 pmole/ µl)	–	0.4 µl	x	'X'
Primer R (5 pmole/ µl)	–	0.4 µl	x	'X'
Quanti Tect RT Mix	–	0.2 µl	x	'X'
RNase free water	–	7.0 µl	x	'X'

'X' – total no of reactions including negative controls and few more reactions to make-up the loss during pipetting.

3.18 Investigation of vaccination and challenge study at Indian Immunologicals Limited (IIL), Hyderabad

3.18.1 Isolation and preservation of PBMCs in RNAprotect solution

1. Blood samples were collected in heparinated Vacutainer and processed at IIL, Hyderabad for PBMCs isolation as per procedure mentioned earlier but without diluting the blood. Resulting plasma samples were collected and kept at -70 ^{0}C.
2. PBMCs were isolated from total of 9 ml of blood.
3. PBMCs were diluted in 200 µl of dilution medium (RPMI-1640 with 5% FBS) and pipetted in four labeled 1.5 ml eppendorf tubes in equal quantity.
4. In each tube 500 µl of RNA protect solution was added to seize and preserve mRNA expression level in PBMC.
5. Eppendorf tubes with cells preserved in RNA protect solution was placed in duly labeled polythene bags and kept at – 70 ^{0}C

3.18.2 Transportation of samples

Cells preserved in RNA protect solution can be transported at 4 ^{0}C for few days. Even though, dry ice was commercially procured and placed in thermocol box. Labeled and duly checked polythene bags containing eppendorf tubes with cells preserved in RNA protect solution and plasma samples was kept in thermocol box. This box was sealed and labeled properly with clear cut identity of both parties. Samples were brought to its destination and stored at – 80 ^{0}C in the refrigerator after 48 hrs of journey.

3.18.3 RNA extraction

1.5 ml eppendorf tubes containing cells preserved in 500 µl of RNA protect cell reagent were taken out from – 80 ^{0}C and allowed to thaw completely at rt. Mix was centrifuged at 5000 g for 5 min. Resulting supernatant was discarded carefully. Before

adding RLT cell pellet was loosened by tapping bottom of the tube with fingers. Rest of the procedure was followed as per mentioned earlier for RNeasy kit.

3.18.4 Measurement of RNA

RNA was measured as per earlier method.

3.18.5 Assessment of mRNA expression level of cytokines, chemokines, toll-like receptors and other genes after 3 days post FMDV infection: Real-time PCR using total RNA

1. Total RNA extracted from one control calf (1022) (before and 3 days post FMDV infection) kept in the Vaccine Potency testing at Hyderabad was diluted to 10 ng/µl and used in the total of 10 µl reaction per well in one step real-time PCR.
2. The test was performed as per mentioned earlier using comparative CT (ΔΔ) type of experiment.
3. 30 ng of diluted RNA was used in the reaction to study expression levels of different cytokines, chemokines, toll-like receptors and others.
4. 7 µl of Master Mix was added in each well of MicroAmp™ optical 96-well plate with slight modification.
5. Master Mix was prepared as follows.

Master Mix:

2x Quanti Tect SYBR Green RT-PCR Master Mix	–	5.0 µl	x	'X'
Primer F (5 pmole/ µl)	–	0.4 µl	x	'X'
Primer R (5 pmole/ µl)	–	0.4 µl	x	'X'
Quanti Tect RT Mix	–	0.1 µl	x	'X'
RNase free water	–	1.1 µl	x	'X'

'X' – total no of reactions including negative controls and few more reactions to make-up the loss during pipetting.

6. 3 µl of diluted RNA for each interested cytokine or items was mixed with the master mix in the well.
7. β-actin, GAPDH was used as endogenous controls in the study.
8. Negative controls were also kept in the experiment.
9. Rest of the procedure for recording of data was followed as per mentioned in

earlier experiment.

10. Total RNA sample obtained before FMDV infection was used as a reference sample.
11. Melt curve analysis was performed and outliers were negated.

Cycling conditions:

Steps	Temp	Time	Remark
I) Holding	50 ^{0}C	30 min	RT enzyme acts (RNA to cDNA)
II) Holding/Denaturation	95 ^{0}C	15 min	RT enzyme denatured
IIIa) Denaturation	94 ^{0}C	15 s	} 45 cycles
IIIb) Annealing	55 ^{0}C	30 s	
IIIc) Extension	72 ^{0}C	30 s	
Melt curve analysis			

3.18.6 Reverse transcription (RT)

1. A total of 500 ng/0.5 µg RNA was used to synthesis cDNA (25 µl of reaction).
2. In 0.5 ml labeled PCR tubes, volume of total RNA corresponding to about 500 ng of total RNA was pipetted.
3. Volume of total RNA was adjusted to 8 µl with nuclease free water.
4. Later in these tubes 2 µl (1 µg) of Oligo dT_{15} was added which make up volume in the tube to 10 µl.
5. These PCR tubes were kept in water bath at 70 ^{0}C for 10 min.
6. After the incubation, tubes were placed on ice for 5 min.
7. 15 µl of RT master mix containing enzyme was added in each tube.
8. RT Master Mix was prepared as follows.

RT Master Mix:

5x RT buffer	– 5 µl
dNTP (10 mM/µl)	– 2 µl
MMLV-RT (200 U/µl)	– 1 µl
NFW	– 7 µl

9. These tubes were again kept in water bath at 42 ^{0}C for 1 hour.
10. Residual enzyme in mix was inactivated by placing these tubes in heating block at 95 ^{0}C for 10 min.

11. Tubes containing cDNA were kept at – 80 ^{0}C.

3.18.7 Assessment of mRNA expression level of IFN-γ in cattle calves used in the vaccination and challenge study of Vaccine Potency testing: Real-time PCR using cDNA

1. Two-step real-time PCR was performed as per mentioned earlier using comparative CT (ΔΔ) type of experiment.
2. 1µl cDNA was used to perform the experiment.
3. QuantiTect® SYBR® Green RT-PCR kit of which 2x Quanti Tect SYBR Green RT-PCR Master Mix was used in the experiment for a total reaction volume of 20 µl, the use of Quanti Tect RT Mix was avoided in the reaction.
4. 19 µl of Master Mix was added in each well of MicroAmp™ optical 96-well plate.

Master Mix:

2x Quanti Tect SYBR Green RT-PCR Master Mix	– 10.0 µl	x	'X'
IFN-γ Primer F (5 pmole/µl)	– 0.8 µl	x	'X'
IFN-γ Primer R (5 pmole/µl)	– 0.8 µl	x	'X'
RNase free water	– 7.4 µl	x	'X'

'X' – total no of reactions including negative controls and few more reactions to make-up the loss during pipetting.

5. cDNA sample from young calves at 10 days post vaccination or 0 days post infection was used as a reference standard. β-actin was used as endogenous control. Negative controls were kept in the experiment.
6. Rest of the procedure was followed as per mentioned in earlier experiment.
7. Gene expression data generated by the software was recorded and analyzed.

Cycling conditions:

Steps	Temp	Time	Remark
I) Holding	95 ^{0}C	15 min	Activation of HotStarTaq DNA polymerase
IIa) Denaturation	94 ^{0}C	15 s	} 45 cycles
IIb) Annealing	55 ^{0}C	30 s	
IIc) Extension	72 ^{0}C	30 s	
Melt curve analysis			

3.19 Investigation of vaccination at Mukteshwar

3.19.1 Isolation of PBMCs and RNA extraction

1. PBMCs were isolated from 3 ml blood obtained from young calves maintained at Latoli farm, Mukteshwar before and after vaccination against FMD with a commercially available trivalent (Type 'O', 'A' and Asia '1') inactivated vaccine.
2. PBMCs were isolated as per earlier procedure.
3. RNA was extracted from freshly isolated PBMCs isolated from 3 ml of blood by using commercially available kit (RNeasy® Mini Kit) as per mentioned earlier.

3.19.2 Isolation of WBCs and RNA extraction

1. WBCs were isolated after treatment of 1 ml blood with 1 ml of 0.85 % Ammonium chloride (Hempel *et al.*, 2002).
2. Mixture of blood and Ammonium chloride in 15 ml centrifuge tube was kept at rt for 1 hr.
3. The mixture was centrifuged at rt for 800 g for 30 min to obtain cell pellet with stains of rbcs.
4. Again 1 ml of Ammonium chloride was added in the cell pellet to lyse the rbcs.
5. After centrifugation at rt for 800 g for 10 min, the cell pellet obtained was washed with RPMI-1640 containing 5% FBS.
6. RNA was extracted by using commercial kit (RNeasy® Mini Kit).

3.19.3 Measurement of RNA

Total RNA was measured as per mentioned earlier.

3.19.4 Reverse transcription (RT)

RT reaction was performed to prepare cDNA from 500 ng of total RNA extracted from PBMCs and WBCs as per mentioned earlier (total volume of reaction 25 µl).

3.19.5 Real-time PCR

1. Two-step real-time PCR was performed using comparative CT (ΔΔ) type of experiment.

2. QuantiTect® SYBR® Green PCR kit was used to perform the experiment using 1 µl of transcribed cDNA from PBMCs and WBCs in a total reaction volume of 20 µl.
3. 2x Quanti Tect SYBR Green PCR Master Mix contains HotStar Taq DNA polymerase, QuantiTect SYBR Green PCR buffer, SYBR Green I and ROX passive reference dye.
4. 19 µl of Master Mix was added in the wells of MicroAmp™ optical 96-well plate.

Master Mix:

2x Quanti Tect SYBR Green PCR Master Mix	– 10.0 µl	x	'X'
IFN-γ Primer F (5 pmole/µl)	– 0.8 µl	x	'X'
IFN-γ Primer R (5 pmole/µl)	– 0.8 µl	x	'X'
RNase free water	– 7.4 µl	x	'X'

'X' – total no of reactions including negative controls and few more reactions to make-up the loss during pipetting.

5. cDNA sample from young calves at 0 DPV was used as a reference standard.
6. β-actin was used as endogenous control.
7. Negative controls were kept in the experiment.
8. Rest of the procedure was followed as per mentioned in earlier experiments.
9. Gene expression data generated by the software was recorded and analyzed.

Cycling conditions:

Steps	**Temp**	**Time**	**Remark**
I) Holding	95 ^{0}C	15 min	Activation of HotStarTaq DNA polymerase
IIa) Denaturation	94 ^{0}C	15 s	35 cycles
IIb) Annealing	55 ^{0}C	30 s	
IIc) Extension	72 ^{0}C	30 s	
Melt curve analysis			

RESULTS

“The discovery of new dish does more for human happiness than the discovery of star”

…Anthelme Brillat-Savarin

Chapter-4

RESULTS

4.1 Preliminary experiments

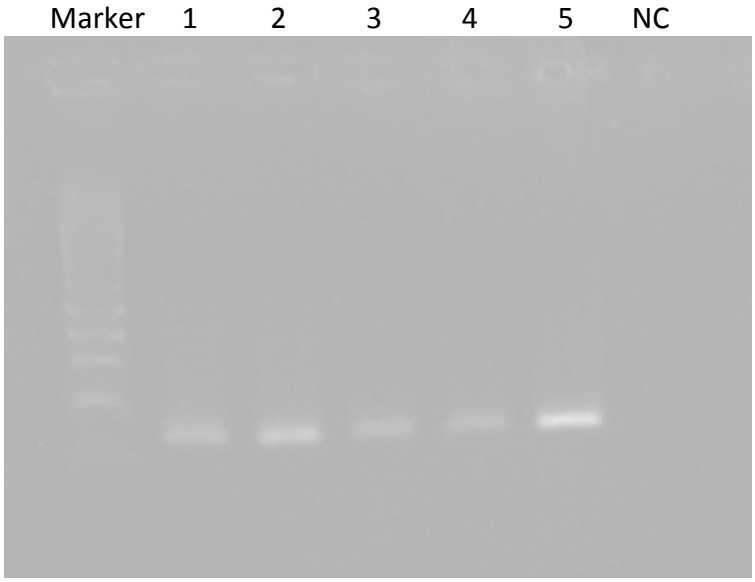
Preliminary experiments were carried out to identify amplification of specific cytokines and others in mitogen and Foot-and-Mouth disease virus (FMDV) stimulated/unstimulated peripheral blood mononuclear cells (PBMCs)**.**

4.1.1 Identification of cytokines after *in-vitro* stimulation of peripheral blood mononuclear cells with mitogen, Conconavalin A (Con A)

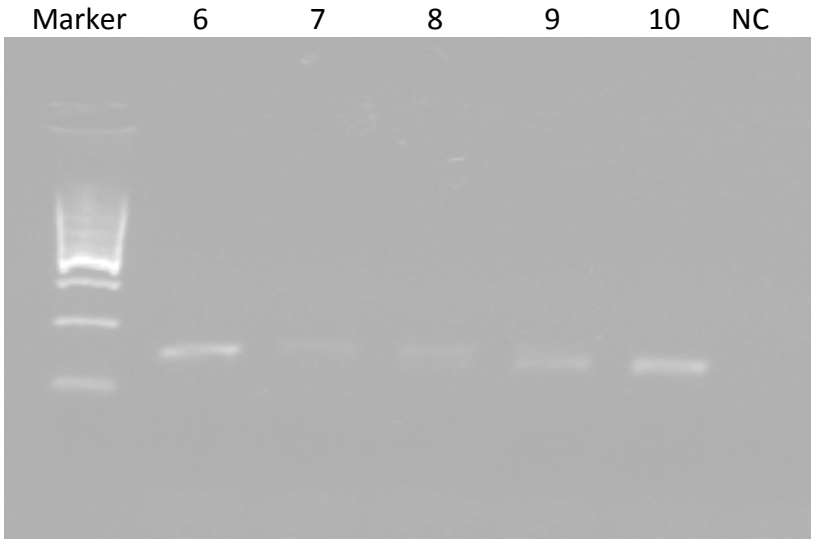
Before using prepared complementary deoxyribonucleic acid (cDNA) for amplification of specific cytokines and others the cDNA was checked for successful β-actin amplification (Figure 4). Interleukin-2 (IL-2) was first detected in ConA stimulated PBMCs after 4 hours (hrs) post stimulation. While IL-4 and Interferon (IFN)-γ was first detected in ConA stimulated PBMCs after 8 hrs post stimulation. IL-10 amplification was weak (Figure 5). Hence, for further amplification of cytokine, cDNA from only ConA stimulated PBMCs after 8 hrs post stimulation was used. Of 22 cytokines, chemokines, toll-like receptors and others tested (Table 5), 14 were amplified after ConA stimulation of peripheral blood mononuclear cells. Positive and negative controls were kept in the experiments which behaved truly.

4.1.2 Identification of cytokines after *in-vitro* stimulation of peripheral blood mononuclear cells with specific FMDV and mitogen

Except IL-5, IGFBP-2 and IGFBP-3 other 19 cytokines were amplified (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-18, IFN-γ, TNF-α, MCP-1, TGFβ-1, IGF-1, VEGF, Leptin, TLR2, TLR4, TLR9) and intensity of band was recorded (Table 6). Specific pattern of upregulation of cytokines, chemokines, toll-like receptors and others after Type 'A' FMDV stimulation was not found at significant level when compared to mitogen stimulated PBMCs. Representative amplified products of cytokines, chemokines and toll-like receptors and others along with their positive controls are shown (Figure 6, Figure 7, Figure 8).



(A)

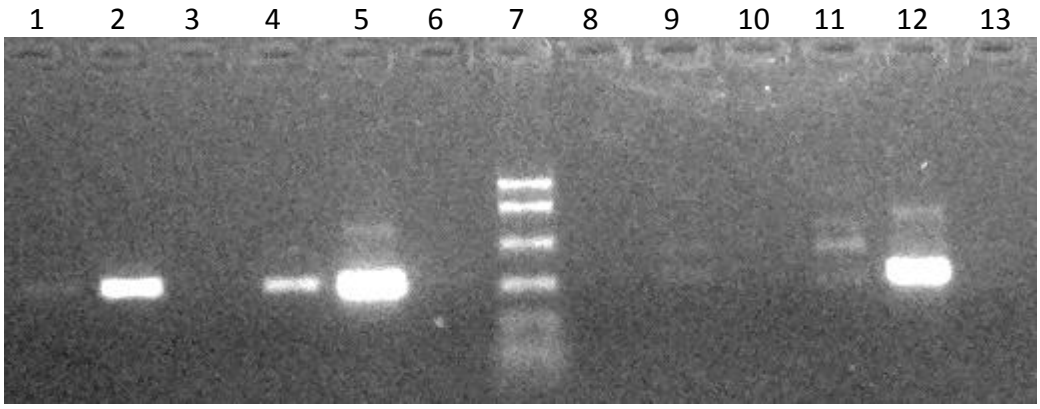


(B)

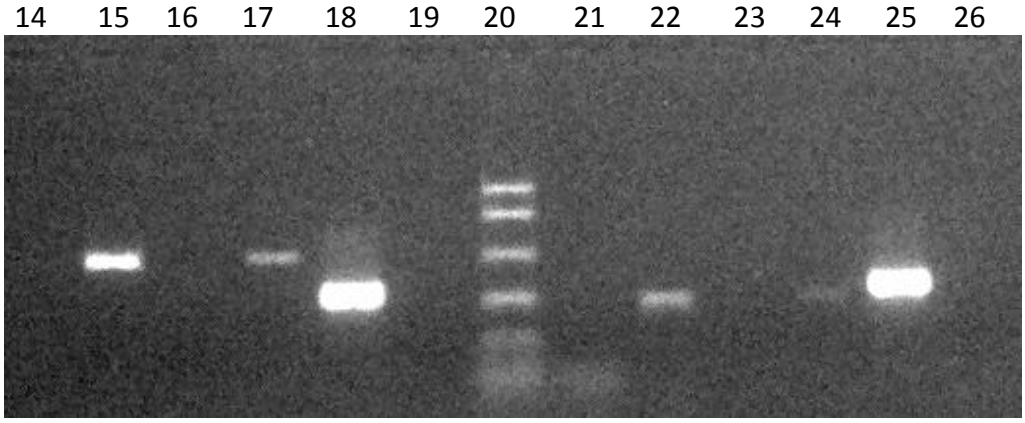
Figure 4 (A, B). Agarose gel electrophoresis of amplified products

Lanes: 1 to 10 - β-actin (153 bp); NC-Negative controls;

Marker - 100 bp DNA Ladder, SibEnzyme



(A)



(B)

Figure 5 (A, B). Agarose gel electrophoresis of cytokine products amplified after stimulation of peripheral blood mononuclear cells with mitogen

Lanes:

1, 2, 4 - IL-2 (345 bp) amplification after 4, 8 and 12 hours respectively;

Positive (Lane 5 - 330 bp) and Negative controls (Lanes 3, 6) for IL-2;

8, 9, 11 - IL-10 (330 bp) amplification after 4, 8 and 12 hours respectively;

Positive (Lane 12 - 336 bp) and Negative controls (Lanes 10, 13) for IL-10;

14, 15, 17 - IFN-γ (509 bp) amplification after 4, 8 and 12 hours respectively;

Positive (Lane 18 - 325 bp) and Negative controls (Lanes 16, 19) for IFN-γ;

21, 22, 24 - IL-4 (289 bp) amplification after 4, 8 and 12 hours respectively;

Positive (Lane 25 - 330 bp) and Negative controls (Lanes 23, 26) for IL-4;

7, 20 - PCR Markers, Promega

Table 5. Detection of cytokines and others in mitogen stimulated peripheral blood mononuclear cells

No.	Item	Annealing temp	Product Size, bp	Band intensity	Result
1	IL-1β	50 ^{0}C	420	Strong	Positive
2	IL-2	50 ^{0}C	345	Strong	Positive
3	IL-4	50 ^{0}C	289	Strong	Positive
4	IL-5	-	308	Invisible	Negative
5	IL-6	50 ^{0}C	491	Weak	Positive
6	IL-8	50 ^{0}C	209	Medium	Positive
7	IL-10	55 ^{0}C	330	Weak	Positive
8	IL-12p35	-	457	Invisible	Negative
9	IL-12p40	-	336	Invisible	Negative
10	IL-18	50 ^{0}C	347	Weak	Positive
11	IFN-γ	55 ^{0}C	509	Strong	Positive
12	TNF-α	50 ^{0}C	360	Strong	Positive
13	MCP-1	50 ^{0}C	209	Strong	Positive
14	TGFβ-1	50 ^{0}C	264	Strong	Positive
15	IGF-1	-	317	Invisible	Negative
16	IGFBP-2	-	365	Invisible	Negative
17	IGFBP-3	-	365	Invisible	Negative
18	VEGF	-	419	Invisible	Negative
19	Leptin	-	349	Invisible	Negative
20	TLR2	50 ^{0}C	503	Weak	Negative
21	TLR4	50 ^{0}C	473	Strong	Positive
22	TLR9	50 ^{0}C	377	Strong	Positive
23	β-actin	55	153	Strong	Positive

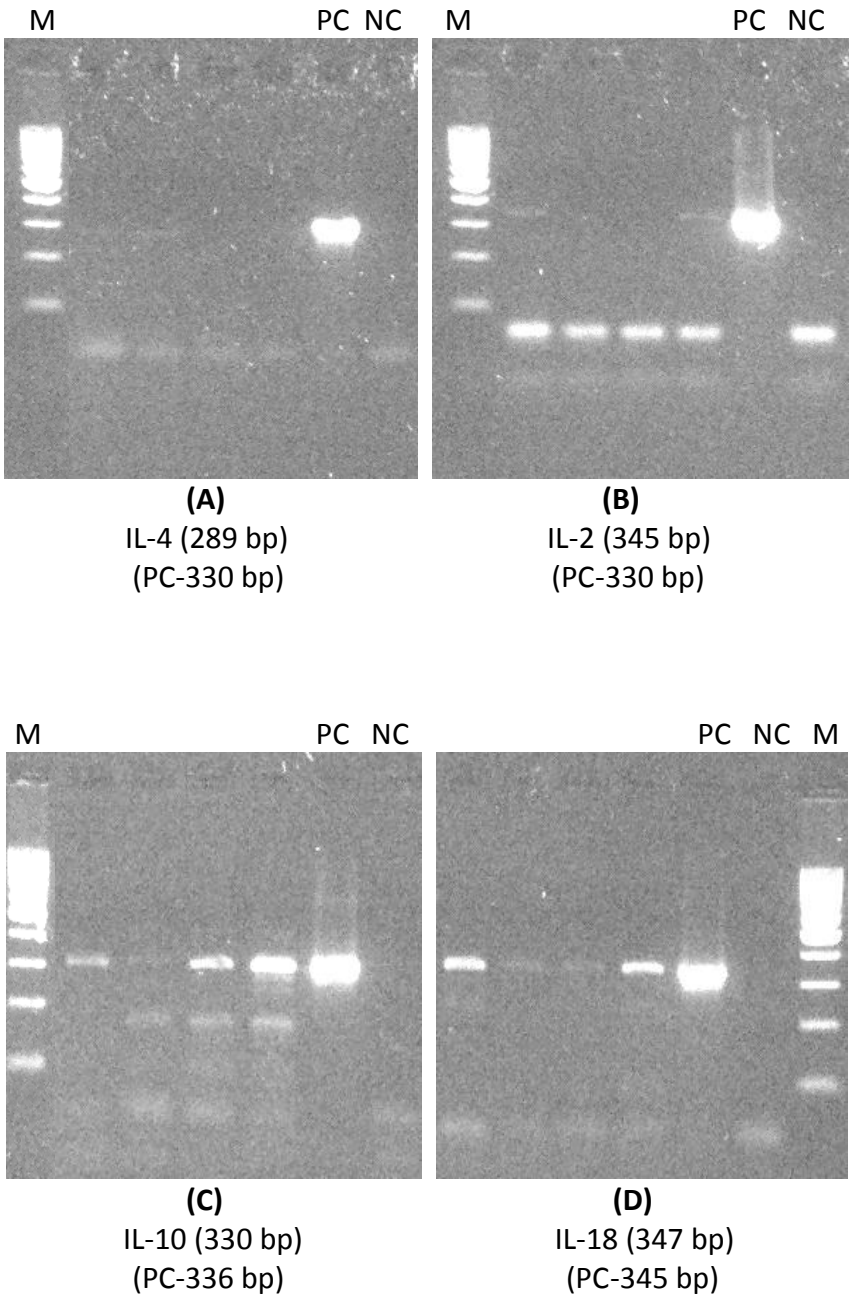
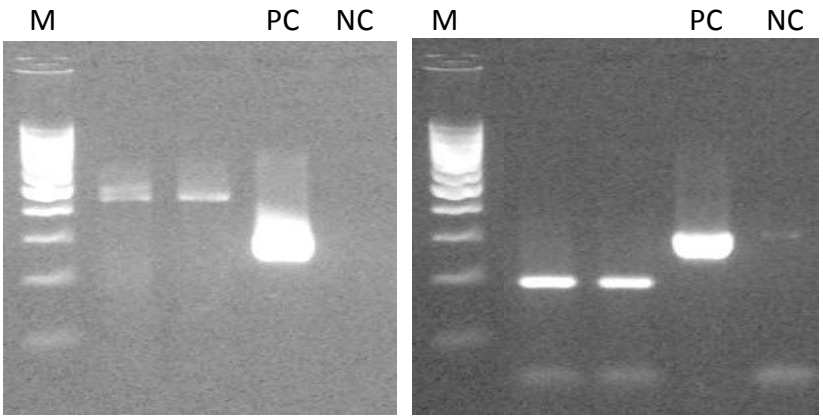


Figure 6 (A, B, C, D). Agarose gel electrophoresis of amplified products

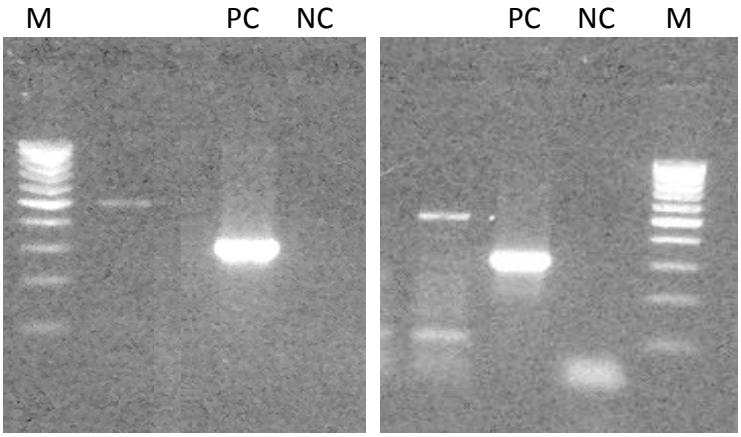
PC - Positive control; NC - Negative control;

M - GeneRuler™ 100 bp Plus DNA Ladder, Fermentas



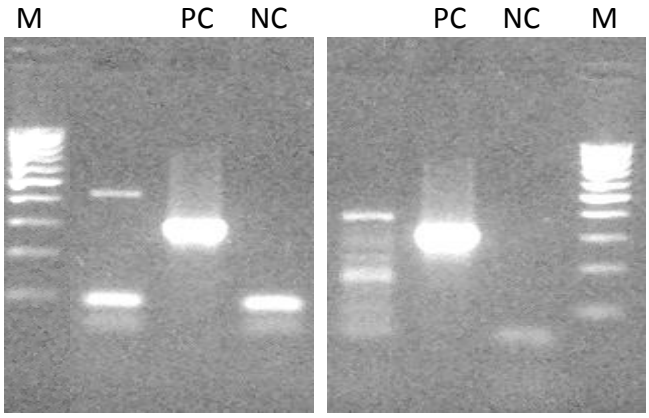
(A)
IL-6 (491 bp)
(PC-325 bp)

(B)
IL-8 (209 bp)
(PC-336bp)



(C)
IFN-γ (509 bp)
(PC-325 bp)

(D)
TLR2 (503 bp)
(PC-324 bp)



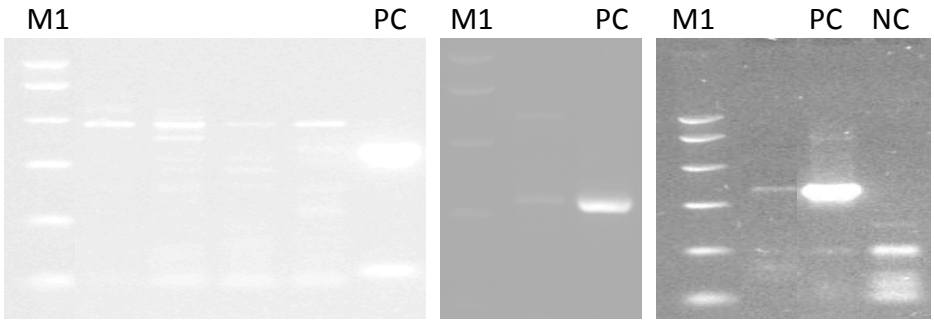
(E)
TLR4 (473 bp)
(PC-324 bp)

(F)
TLR9 (377 bp)
(PC-324 bp)

Figure 7 (A, B, C, D, E, F). Agarose gel electrophoresis of amplified products

PC - Positive control; NC - Negative control;

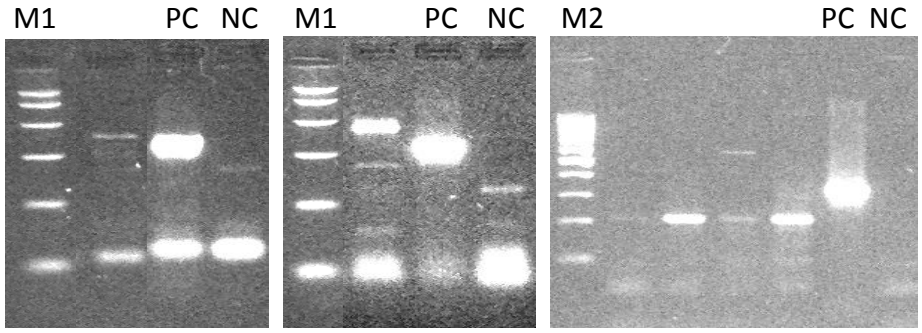
M - GeneRuler™ 100 bp Plus DNA Ladder, Fermentas



(A) IL-12p35 (457 bp) (PC-345bp)

(B) IL-12p40 (336 bp) (PC-345bp)

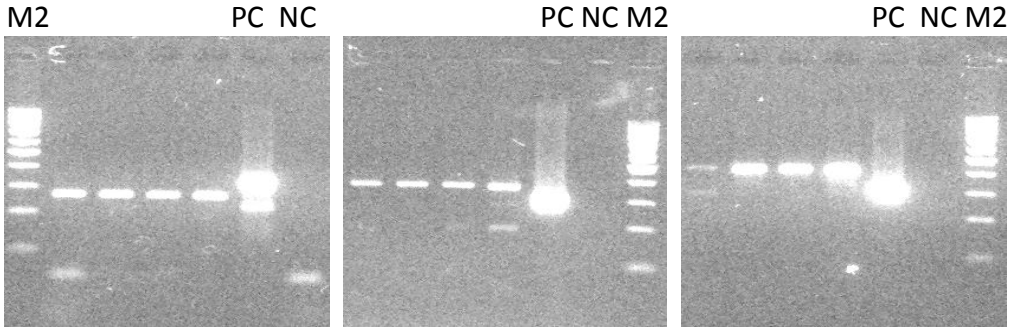
(C) Leptin (349 bp) (PC-336bp)



(D) IGFBP-3 (365 bp) (PC-345 bp)

(E) VEGF (419 bp) (PC-336 bp)

(F) MCP-1 (209 bp) (PC-345 bp)



(G) TGFβ-1 (264 bp) (PC-345 bp)

(H) TNF-α (360 bp) (PC-325 bp)

(I) IL-1β (420 bp) (PC-325 bp)

Figure 8 (A, B, C, D, E, F, G, H, I). Agarose gel electrophoresis of amplified products

PC - Positive control; NC - Negative control;

M1 - PCR Markers, Promega; M2 - GeneRuler™ 100 bp Plus DNA Ladder, Fermentas

Table 6. Detection of cytokines and others in mitogen, virus stimulated and unstimulated peripheral blood mononuclear cells

	Cow No.	337		306		354		336		333		6		337
No.	Item	ConA	FMDV	ConA	FMDV	ConA	FMDV	ConA	FMDV	ConA	FMDV	ConA	FMDV	CC
1	IL-1β	++	+	++	++	+++	+++	#	#	++	++	+++	+++	++
2	IL-2	-	-	-	+	+	-	#	#	+	-	-	-	+
3	IL-4	+	-	+	-	-	-	#	#	-	-	-	-	-
4	IL-5	-	-	-	-	-	-	-	-	-	-	-	-	-
5	IL-6	++	+	++	++	+++	++	#	#	++	++	++	++	++
6	IL-8	+++	+	++	++	++	++	#	#	++	++	++	++	+++
7	IL-10	+	+	++	+	++	++	#	#	++	++	+	++	++
8	IL-12p35	-	-	+	-	++	+	+++	+	++	+++	++	+++	+
9	IL-12p40	-	-	-	+	+	+	-	+	+	-	-	-	-
10	IL-18	+	+	++	-	+	-	#	#	++	++	+	++	++
11	IFN-γ	-	-	-	-	+	++	++	++	++	++	+	+	-
12	TNF-α	++	++	+++	++	+++	++	+++	++	+++	++	++	++	++
13	MCP-1	++	+	+++	++	+++	++	+++	++	+++	++	++	++	++
14	TGF-β1	++	++	+++	-	++	+++	++	++	+++	++	+++	++	++
15	IGF-1	-	-	-	-	-	-	-	-	-	-	-	-	-
16	IGFBP-2	-	-	-	-	-	-	-	-	-	-	-	-	-
17	IGFBP-3	-	+	+	-	+	-	+	+	+	-	-	-	-
18	VEGF	++	-	++	+	++	++	++	++	++	++	++	++	-
19	Leptin	-	-	-	-	-	-	-	+	-	+	-	-	-
20	TLR2	++	+	++	-	+++	++	+++	+++	+++	+++	++	++	++
21	TLR4	+	+	+	_	+++	++	+++	++	+++	+++	++	++	++
22	TLR9	++	++	++	-	+++	++	+++	++	++	++	++	++	++

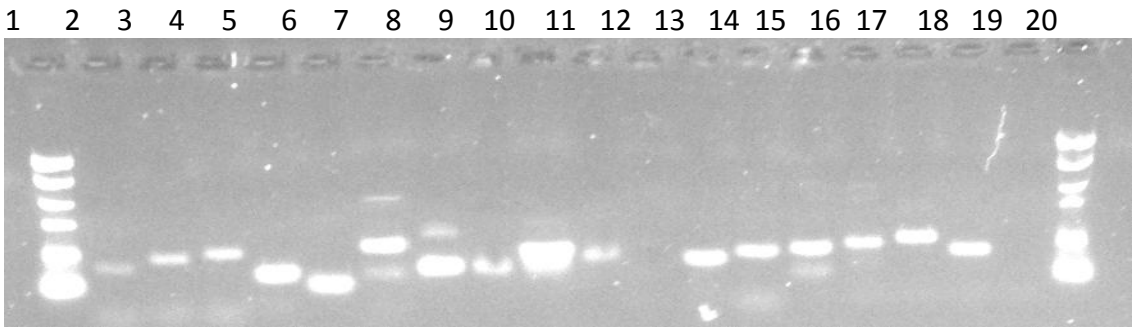
ConA-Conconavalin A; #-Unavailable; FMDV-Foot-and-Mouth disease virus; CC-unstimulated cell control

4.2 Purification, cloning and sequencing of amplified products

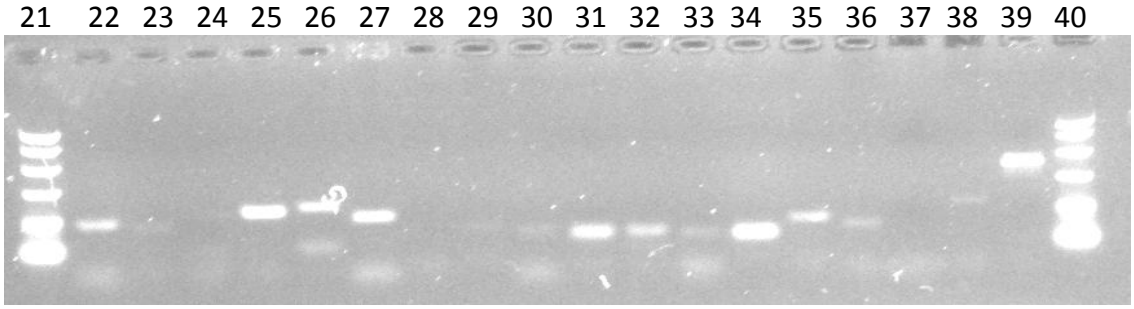
Preliminary experiments showed that cytokines and others are amplified from cDNA prepared from unstimulated PBMCs of FMDV free, unvaccinated and apparently healthy cattle, also (Table 6). Hence, for amplification of rest of the cytokines, chemokines, chemokine receptors, toll-like receptors and others this cDNA was used. Primers were used as per instructions given by the reporters in their publications with slight modifications. Primers were also designed using NCBI Primer-blast website. To use designed primers for amplification of specific products, annealing temperatures between, 50-55 ^{0}C were used. Amplified products of cytokines and others (Figure 9) were gel purified (Figure 10, Figure 11, Figure 12, Figure 13, Figure 14, Figure 15, Figure 16, Figure 17, Figure 18) ligated in pGEMT-Easy vector system I, transformed in the prokaryotic host (*Escherichia coli* cells - JM109). Bacterial colonies or/and plasmids purified from transformed cells were tested in polymerase chain reaction (PCR) or/and restriction enzyme, EcoRI treatment wherever possible to confirm the specific inserts in the plasmids (Figure 19, Figure 20, Figure 21, Figure 22, Figure 23, Figure 24). Plasmids with cloned inserts for 64 cytokines, chemokines, chemokine receptors, toll-like receptors and others were used in sequencing (gel or capillary method) and transformed bacterial cells were preserved in 15% glycerol (Table 7). Cloned inserts for 61 cytokines and others were successfully sequenced to generate electropherogram and nucleotide sequence data. Electropherogram data of cloned products in the vector is depicted (Plate 1 to 61). Plasmids with IL-20, IL-21 and IL-25 inserts, though tested positive in PCR (Figure 24) failed to generate sequencing data.

4.2.1 Processed nucleotide sequence data of cloned products

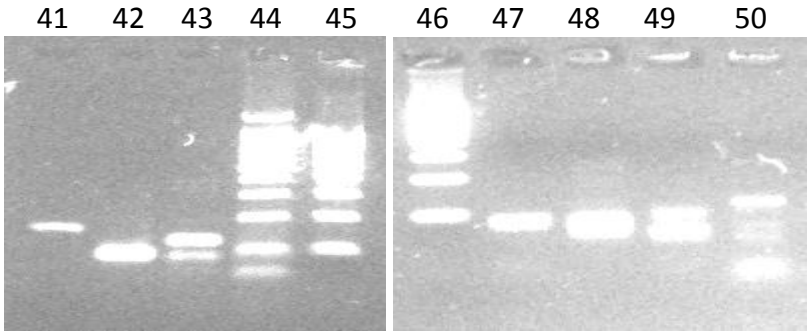
Processed partial nucleotide sequences in the bacterial clones of bovine cytokines and others were found patched between two ends of cloning vector (Plate 1 to 61). With the help of NCBI website blast was performed. Except few discripancies all other sequences were closely matched to that of sequences available. MCP-1 sequence revealed extra stretch of nucleotides at 5'. Another CD4 clone when sequenced proved to be Bovine isovaleryl dehydrogenase (IVD). Lab designation was given to each of the clone starting from the abbreviated name of the laboratory. The processed sequences for cytokines, chemokines, chemokine receptors and toll-like receptors were given.



(A)



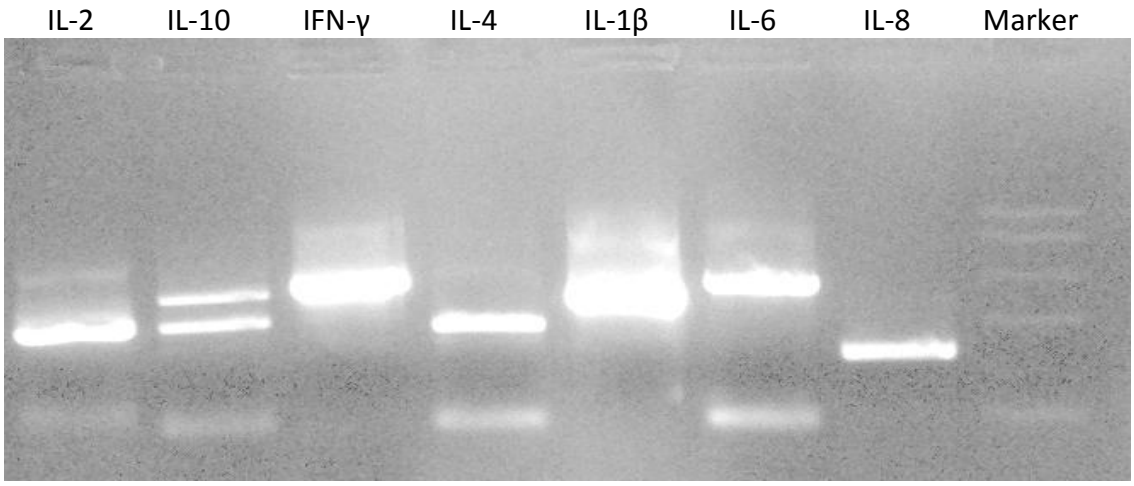
(B)



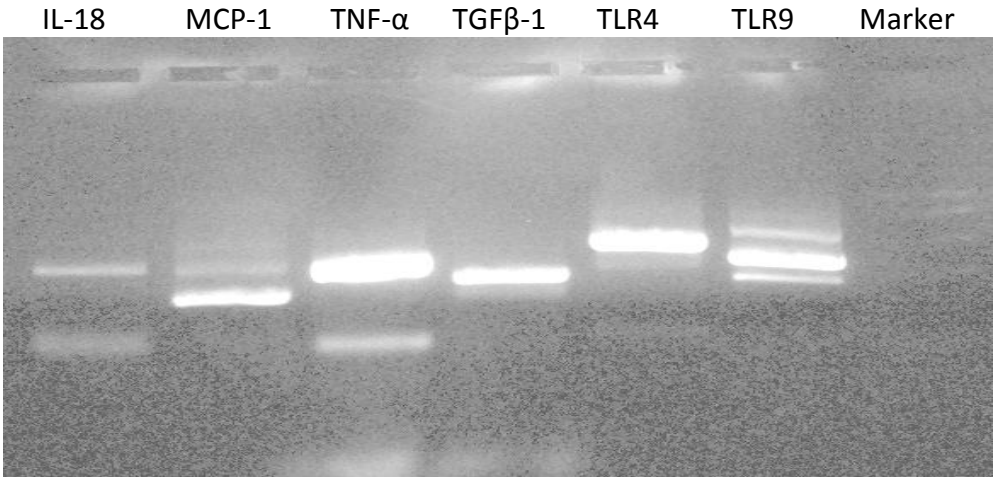
(C) **(D)**

Figure 9 (A, B, C, D). Agarose gel electrophoresis of amplified products

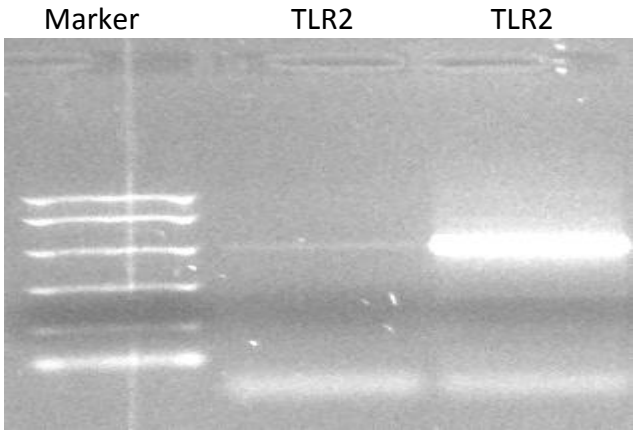
Lanes: 2-CD11a (102 bp); 3-CD11b (139 bp); 4-CD11c (158 bp); 5-CD14 (81 bp); 6-CD18 (133 bp); 7-CD68 (164 bp); 8-CXCL1 (93 bp); 9-CXCL2 (91 bp); 10-CXCL6 (139 bp); 11-CXCR4 (113 bp); 12-FasL (160 bp); 13-Galectin-3 (97 bp); 14-Granzyme A (121 bp); 15-Granulysin (128 bp); 16-ICAM-1 (146 bp); 17-ICAM-3 (169 bp); 18-IFN-αR1 (157 bp); 19-IRF1 (141 bp); 22-Integrin β3 (137 bp); 23-MMR (127 bp); 24-MSR1 (174 bp); 25-Perforin (178bp); 26-Selenoprotein P (199 bp); 27-XCL1 (147 bp); 28-VCAM-1 (97 bp); 29-CXCL10 (103 bp); 30-GM-CSF(87 bp); 31-COX-2 (80 bp); 32-iNOS (82 bp); 33-IL-15 (74 bp); 34-IL-16 (73 bp); 35-TLR3; 36-IFN-β (97 bp); 37-IL-12p35 (89 bp); 38-IL-12p40 (186 bp); 39-VEGF (419 bp); 41-β-actin (153 bp); 42-18S RNA (90 bp); 43-IL-1α (124 bp); 47, 48 -CCL5 (82 bp); 49-CCL8 (94 bp); 50-CCR5 (119 bp); 1, 20, 21, 40, 44 - PCR Markers, Promega; 45, 46 - 100 bp DNA Ladder, SibEnzyme



(A)



(B)

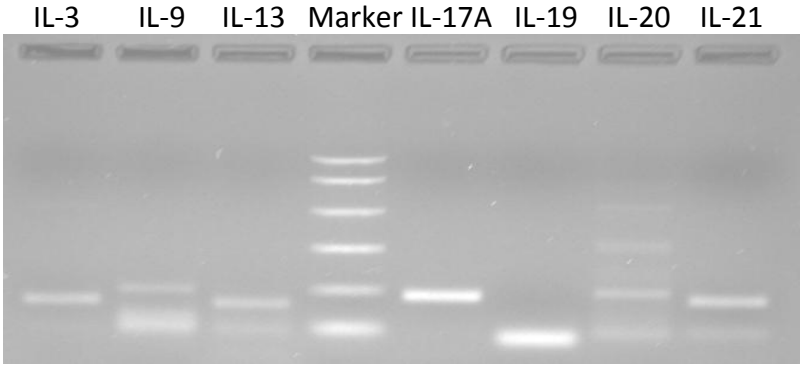


(C)

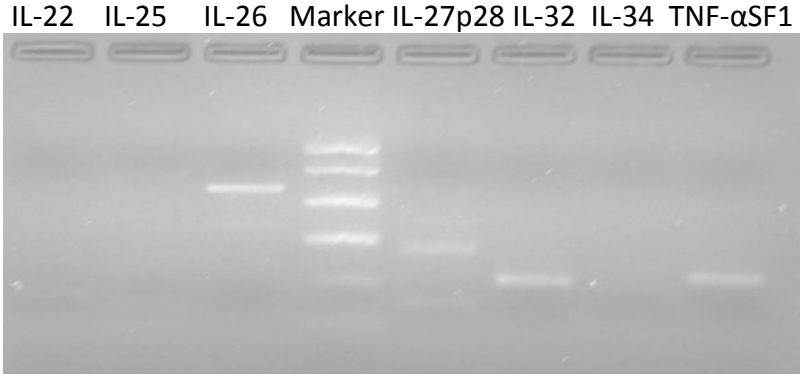
Figure 10 (A, B, C). Agarose gel electrophoresis of amplified products used in purification

Marker - PCR Markers, Promega;

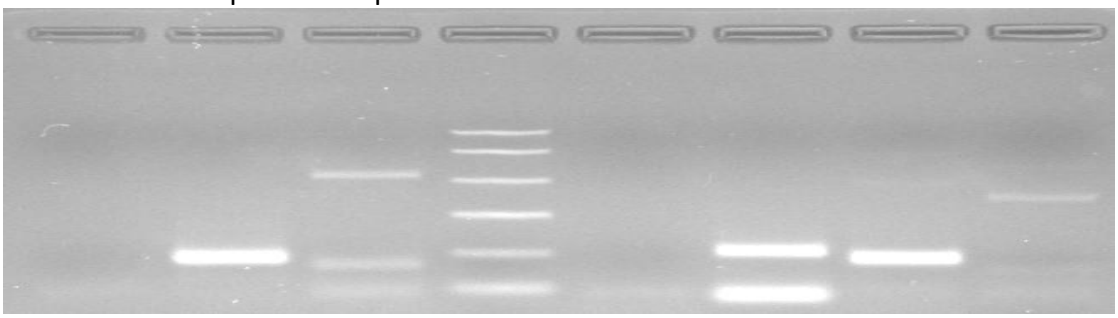
Note: Poor quality of Marker is regretted



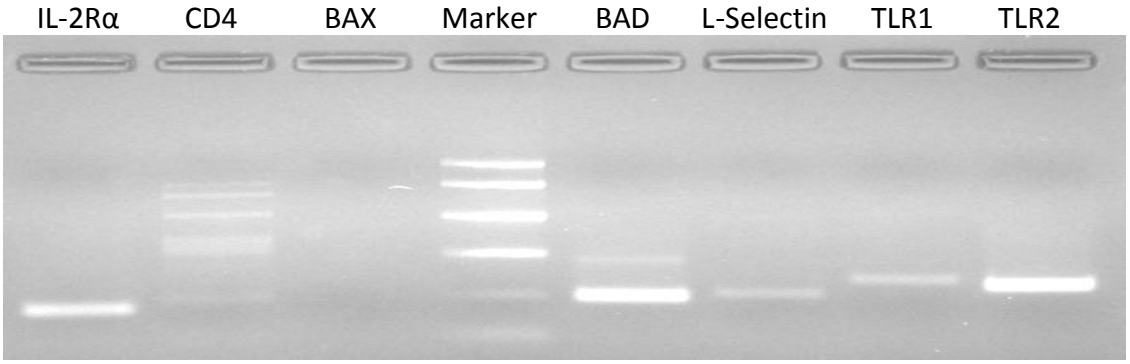
(A)



(B)



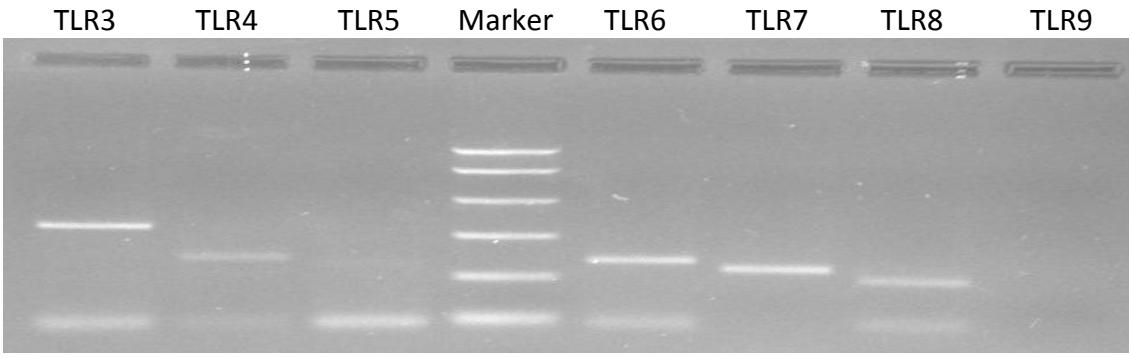
(C)



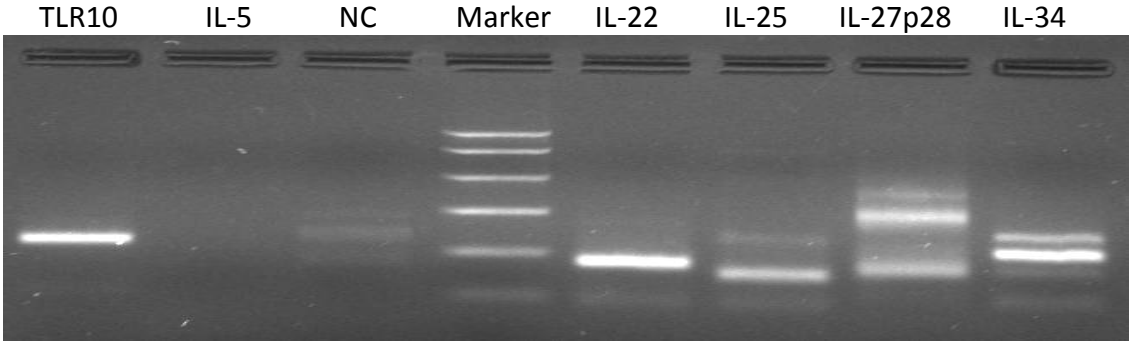
(D)

Figure 11 (A, B, C, D). Agarose gel electrophoresis of amplified products used in purification

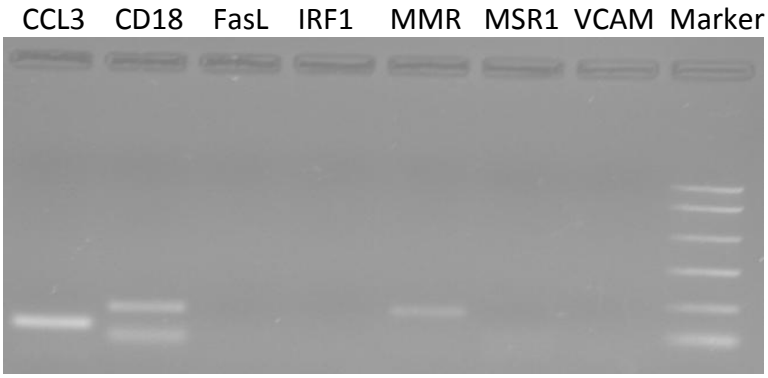
Marker - PCR Markers, Promega



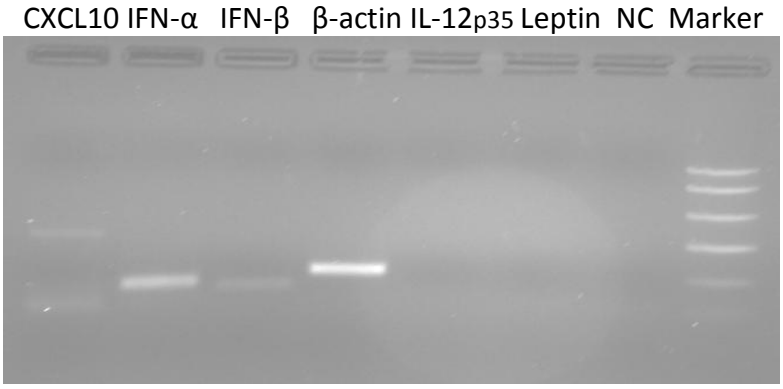
(A)



(B)



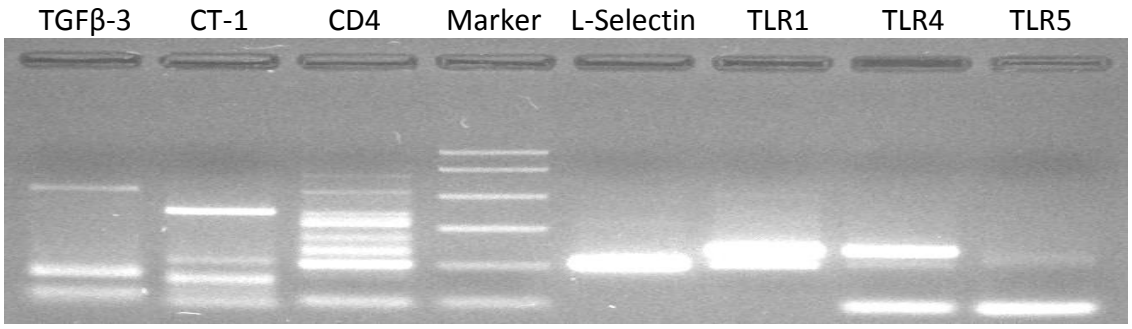
(C)



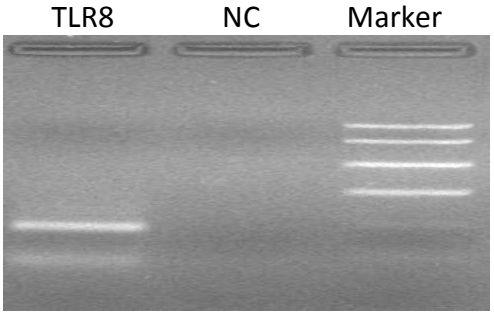
(D)

Figure 12 (A, B, C, D). Agarose gel electrophoresis of amplified products used in purification

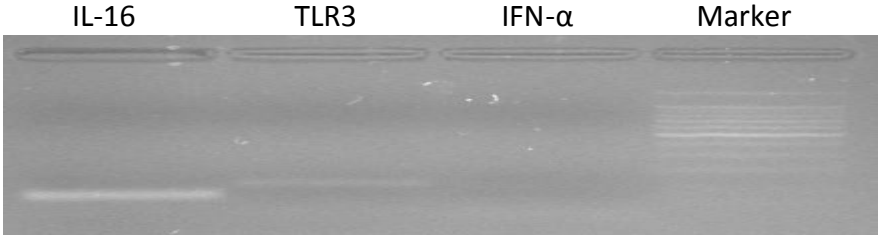
Marker - PCR Markers, Promega



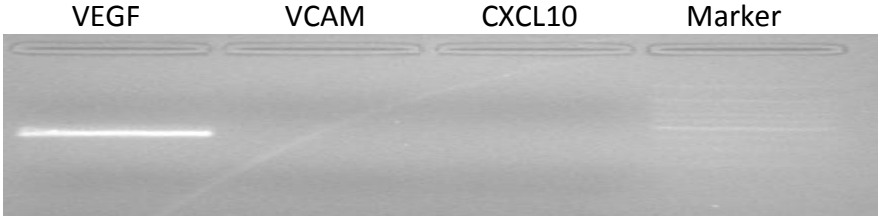
(A)



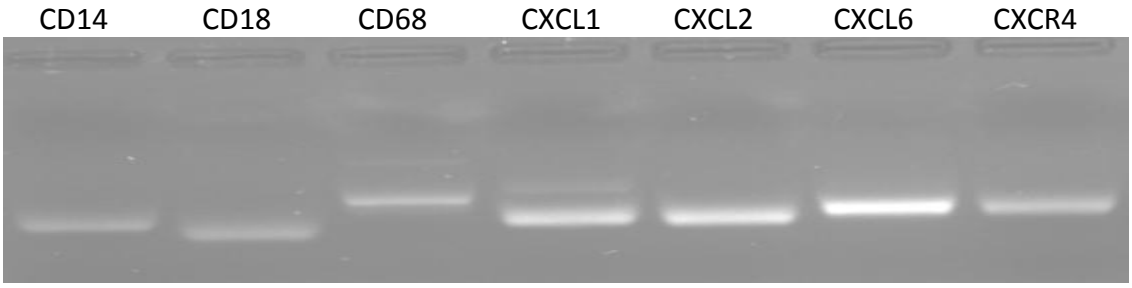
(B)



(C)



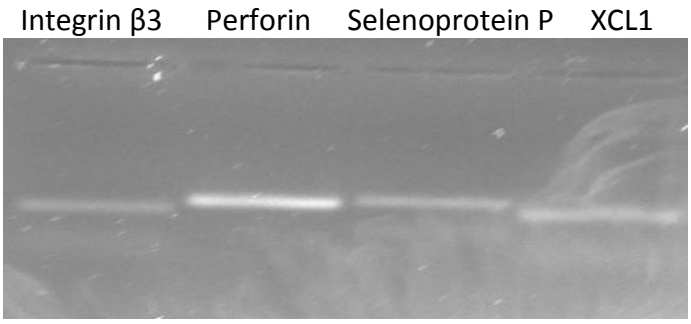
(D)



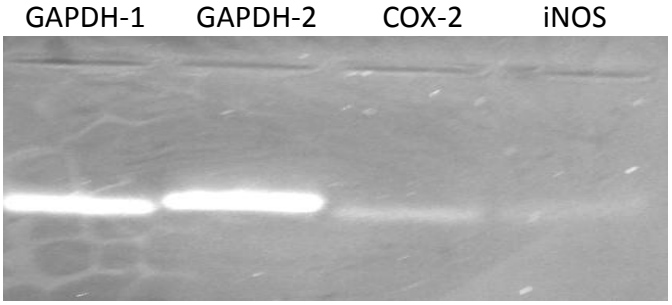
(E)

Figure 13 (A, B, C, D, E). Agarose gel electrophoresis of amplified products used in purification

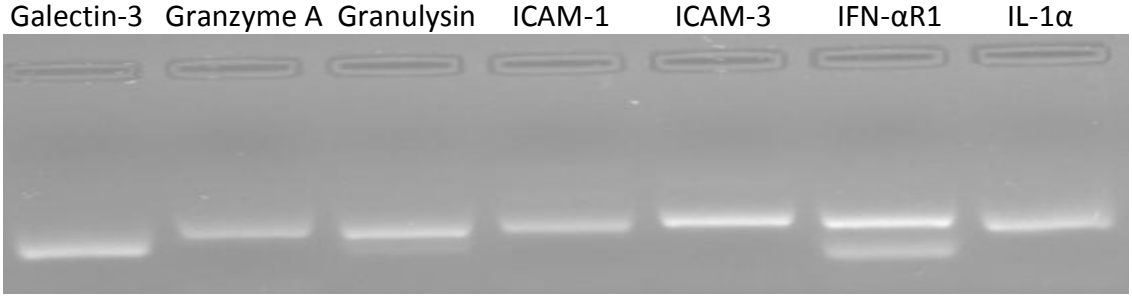
NC - Negative control; Marker - PCR Markers, Promega



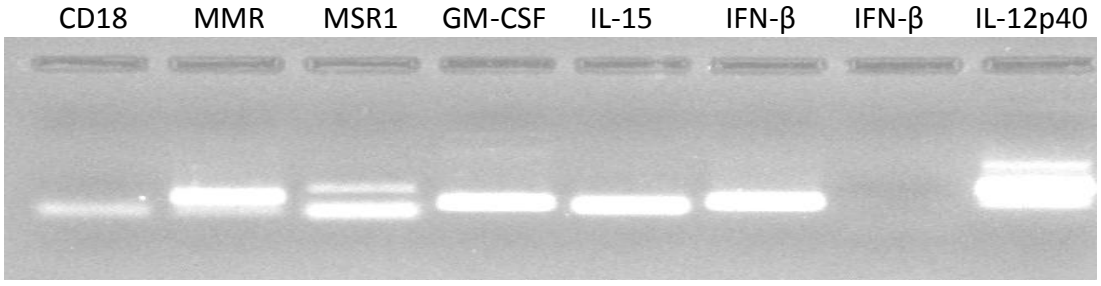
(A)



(B)

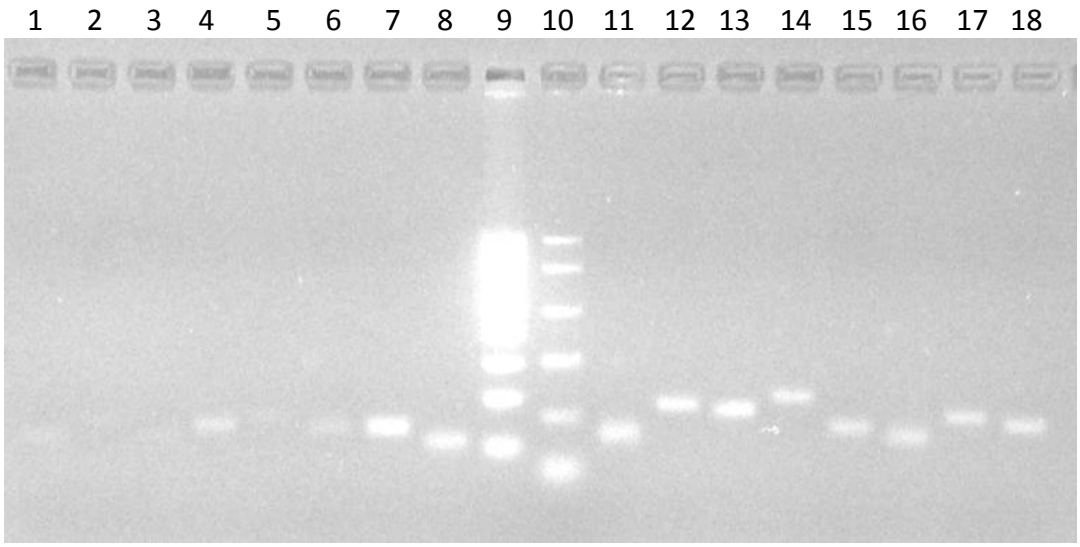


(C)

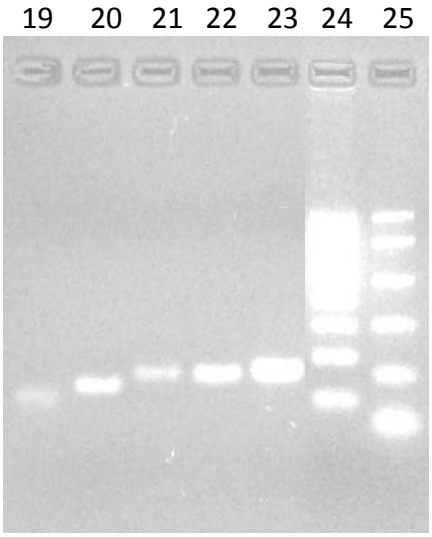


(D)

Figure 14 (A, B, C, D). Agarose gel electrophoresis of amplified products used in purification



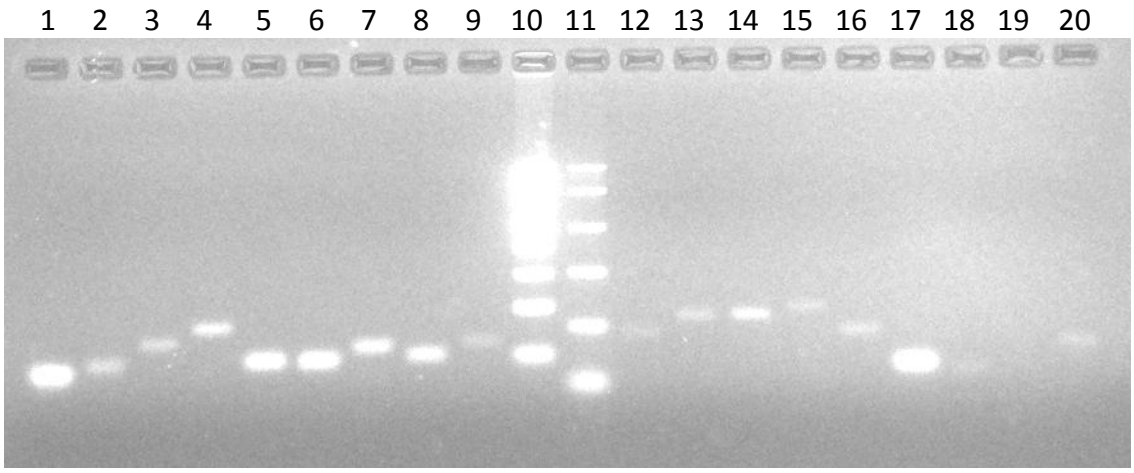
(A)



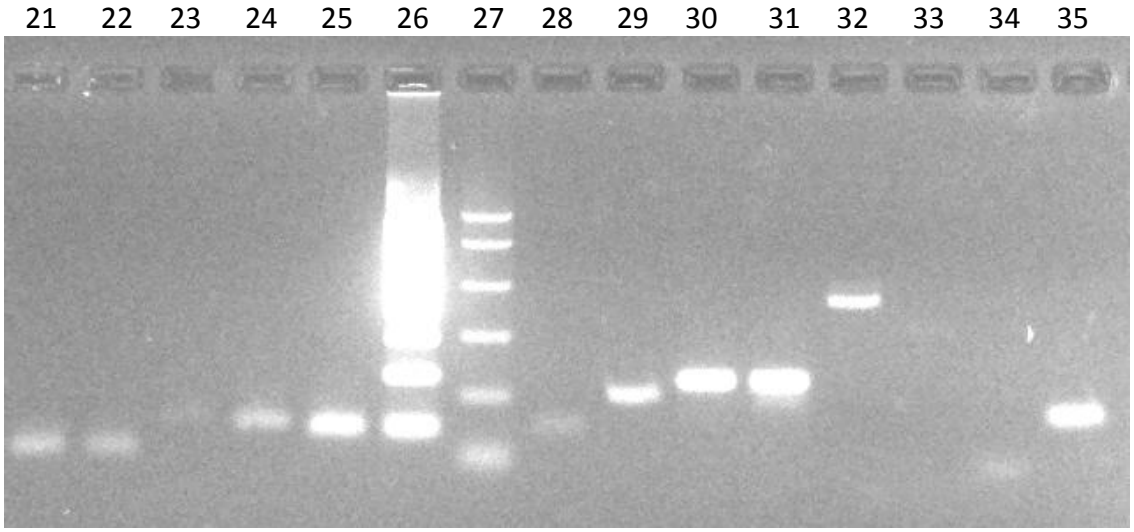
(B)

Figure 15 (A, B). Agarose gel electrophoresis of purified products

Lanes: 1- IL-3 (116 bp); 2- IL-9 (149 bp); 3- IL-13 (111 bp);
4- IL-17A (138 bp); 5- IL-20 (149 bp); 6-IL-21 (132 bp);
7-IL-22 (128 bp); 8-IL-25 (100 bp); 11-IL-27p28 (111 bp);
12-IL-32 (177 bp); 13-IL-34 (165 bp); 14-TNF-αSF1 (200 bp);
15-TGFβ-2 (135 bp); 16- TGFβ-3 (116 bp); 17- CSF (158 bp);
18- OSM (138 bp); 19-CT-1 (102 bp); 20-IL-2Rα (128 bp);
21-CD4 (152 bp); 22-BAD (151 bp); 23-L-Selectin (160 bp);
9, 24 -100 bp DNA Ladder, SibEnzyme;
10, 25 - PCR Markers, Promega



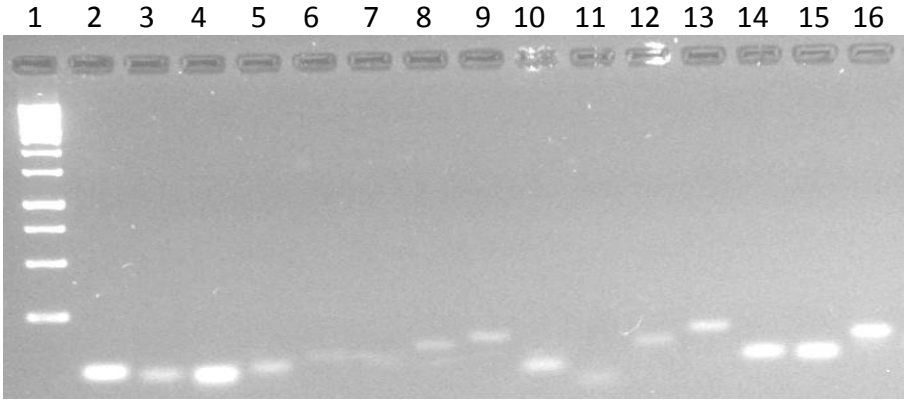
(A)



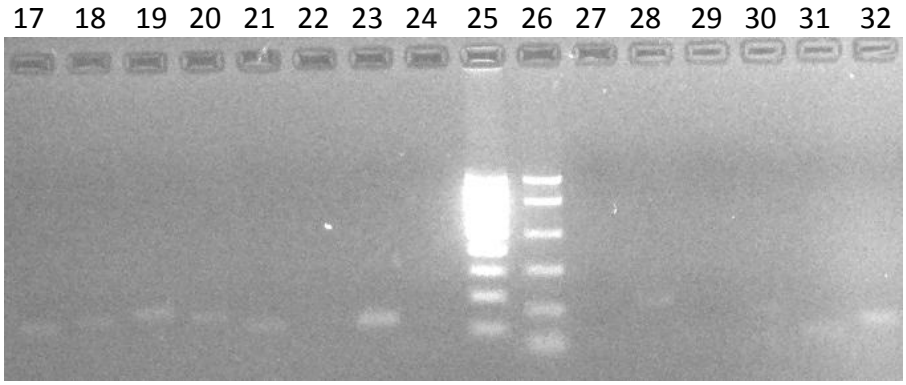
(B)

Figure 16 (A, B). Agarose gel electrophoresis of purified products

Lanes:1- CCL5 (82 bp); 2- CCL8 (94 bp); 3-CD18 (133 bp); 4-CD68 (164 bp); 5-CXCL1 (93 bp); 6-CXCL2 (91 bp); 7- CXCR4 (113 bp); 8- Galectin-3 (97bp); 9- Granzyme A (121bp); 12- Integrin β3 (137 bp); 13- MSR1 (174 bp); 14- Perforin (178bp); 15- Selenoprotein P (199 bp); 16- XCL1 (147 bp); 17- GM-CSF(87 bp); 18-COX-2(82 bp); 19-iNOS (82 bp); 20- MMR (127 bp); 21-IL-15 (74 bp); 22-IL-16 (73 bp); 23-TLR3; 24-IFN-α (118 bp); 25, 28- IFN-β (97 bp); 29-β-actin (153 bp); 30, 31-IL-12p40 (186 bp); 32-VEGF (419 bp); 33-CXCL10 (103 bp); 34-CD18 (133 bp); 35-MMR (127 bp); 10, 26 - 100 bp DNA Ladder, SibEnzyme; 11, 27 - PCR Markers, Promega



(A)



(B)

Figure 17 (A, B). Agarose gel electrophoresis of purified products

Lanes: 2-18S RNA (90 bp); 3-CCL3 (82 bp); 4-CCL5 (82 bp); 5-CCL8 (94 bp); 6-CCR5 (119 bp); 7-CD11a (102 bp); 8-CD11b (139 bp); 9-CD11c (158 bp); 10-CD14 (81 bp); 11,12-CD18 (133 bp); 13-CD68 (164 bp); 14-CXCL1 (93 bp); 15-CXCL2 (91 bp); 16-CXCL6 (139 bp); 17-Granulysin (128 bp); 18-ICAM-1 (146 bp); 19-ICAM-3 (169 bp); 20-IFN-αR1 (157 bp); 21-IL-1α (124 bp); 22-Integrin β3 (137 bp); 23, 24-MMR (127 bp); 27-MSR1 (174 bp); 28-Perforin (178 bp); 29-Selenoprotein P (199 bp); 30-XCL1 (147 bp); 31-GAPDH-1 (82 bp); 32-GAPDH-2 (100 bp); 1 - GeneRuler™ 1 kb DNA Ladder, Fermentas; 25 - 100 bp DNA Ladder, SibEnzyme; 26 - PCR Markers, Promega

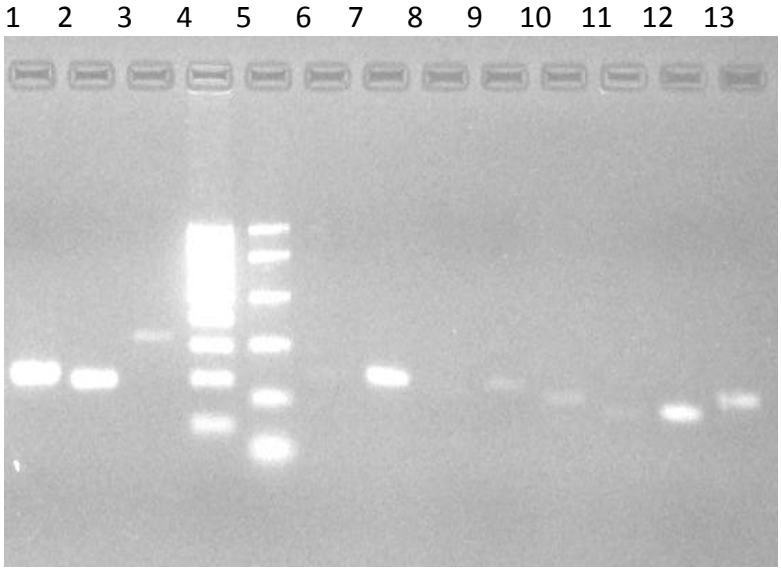


Figure 18. Agarose gel electrophoresis of purified products

Lanes: 1- TLR1 (208 bp);

2-TLR2 (192 bp);

3-TLR3 (329 bp);

6-TLR4 (208 bp);

7-TLR4 (208 bp);

8-TLR5 (191 bp);

9-TLR6 (206bp);

10-LR7 (180 bp);

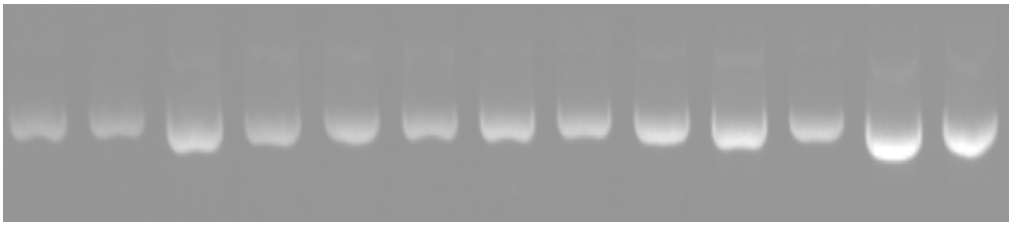
11-TLR8 (150 bp);

12-TLR8 (150 bp);

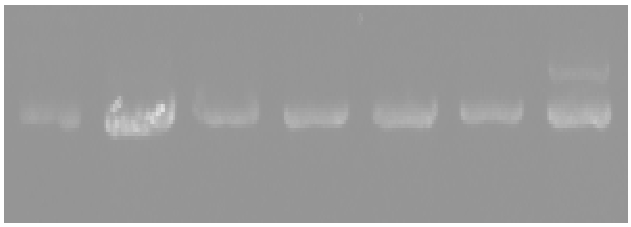
13-TLR10 (190 bp);

4 - 100 bp DNA Ladder, SibEnzyme;

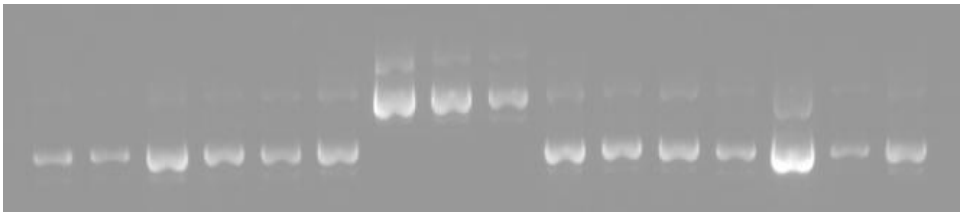
5 - PCR Markers, Promega



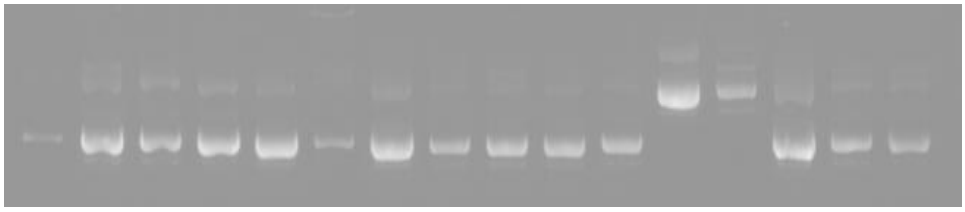
(A)



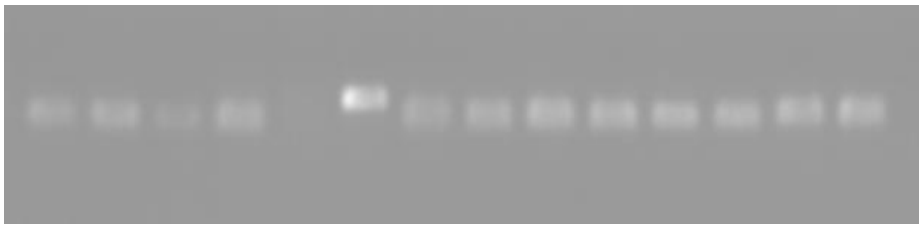
(B)



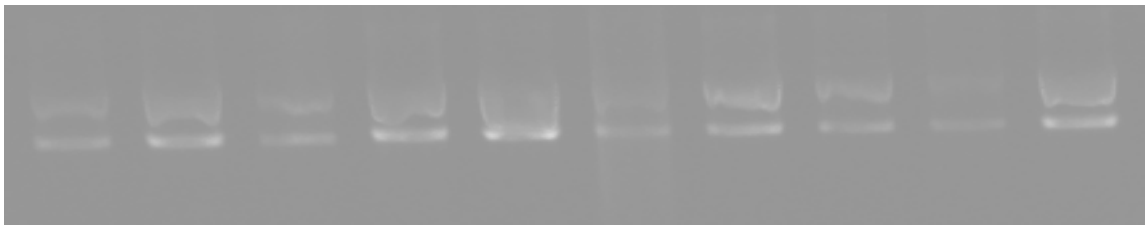
(C)



(D)

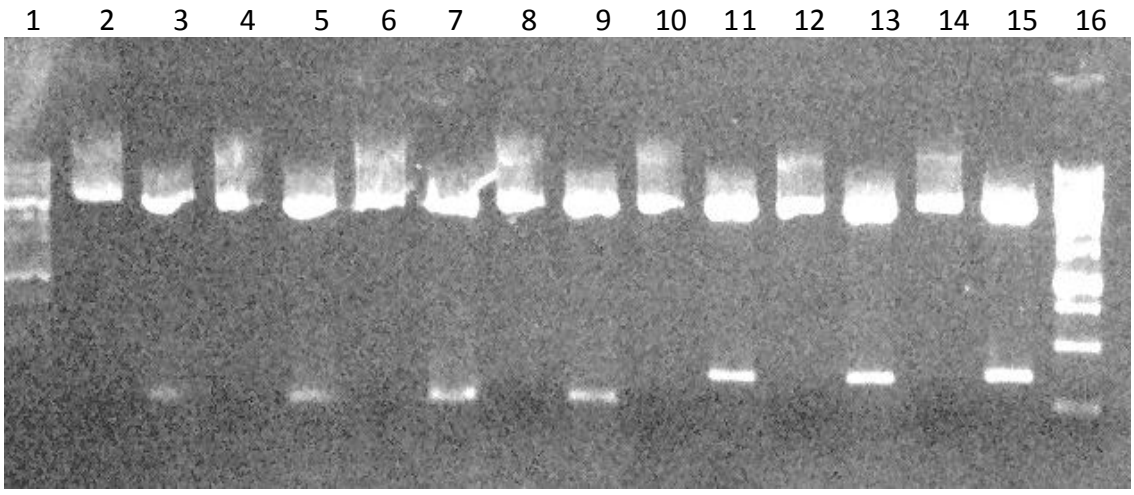


(E)

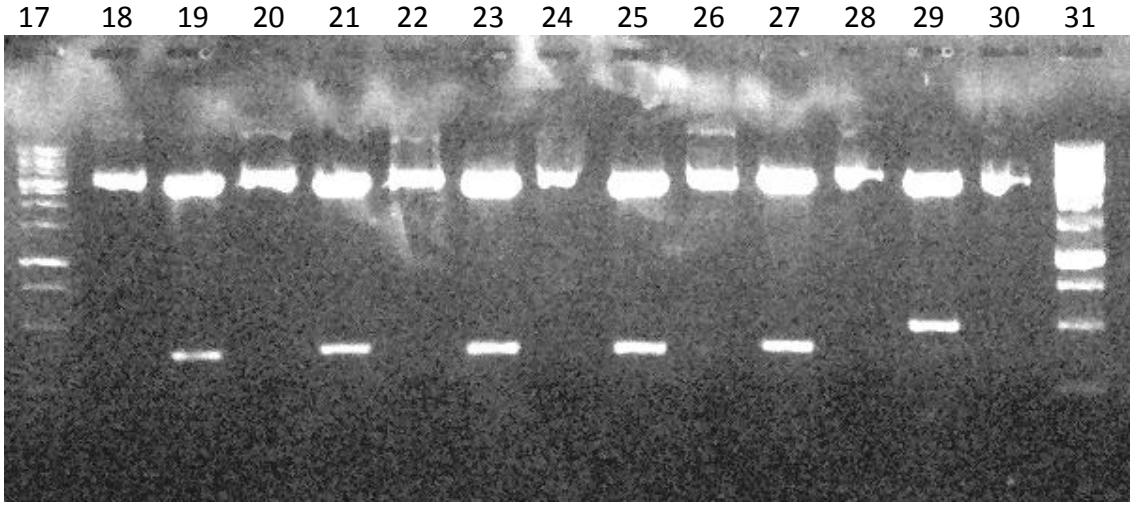


(F)

Figure 19 (A, B, C, D, E, F). Agarose gel electrophoresis of plasmids extracted from transformed bacteria



(A)



(B)

Figure 20 (A, B). Agarose gel electrophoresis of plasmids with cloned insert before and after restriction enzyme digestion

Lanes: 2, 4, 6, 8 - Uncut plasmid with IL-4 insert;

3, 5, 7, 9 - Cut plasmid with release of IL-4 insert;

10, 12, 14, 18 - Uncut plasmid with IL-18 insert;

11, 13, 15, 19 - Cut plasmid with release of IL-18 insert;

20, 22, 24, 26 - Uncut plasmid with TNF-α insert;

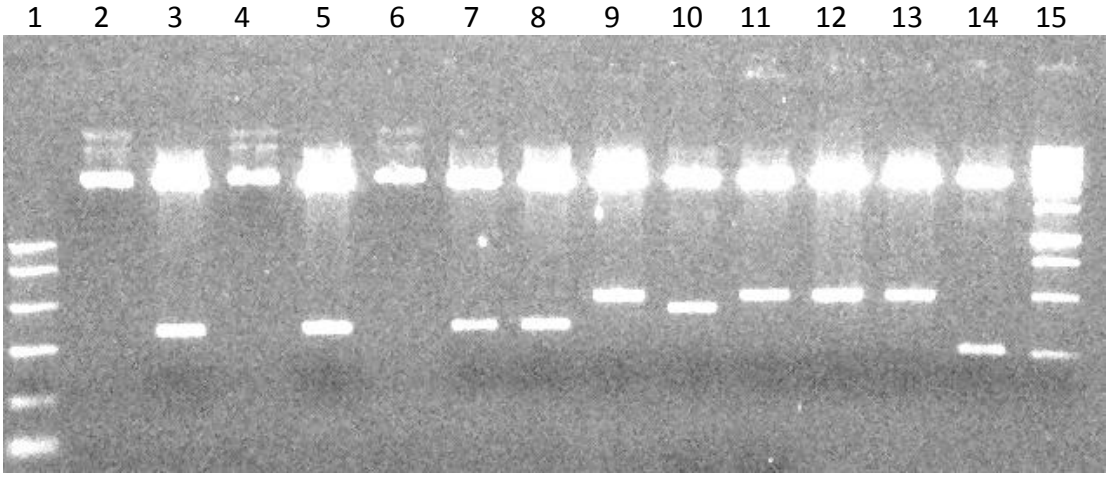
21, 23, 25, 27 - Cut plasmid with release of TNF-α insert;

28 - Uncut plasmid with TLR4 insert;

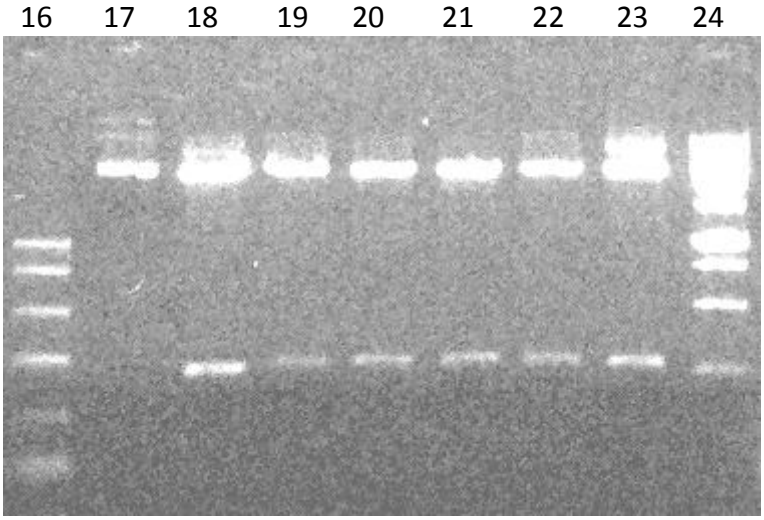
29 - Cut plasmid with release of TLR4 insert;

30 - Uncut plasmid with IL-8 insert;

1, 16, 17, 31 - GenRuler™ 1 kb DNA Ladder, Fermentas



(A)



(B)

Figure 21 (A, B). Agarose gel electrophoresis of plasmids with cloned insert before and after restriction enzyme digestion

Lanes: 2, 4, 6 - Uncut plasmid with IL-2 insert;

3, 5, 7, 8 - Cut plasmid with release of IL-2 insert;

9 - Cut plasmid with release of IL-10 insert;

10 - Cut plasmid with IL-1β insert;

11, 12, 13 - Cut plasmid with release of IL-6 insert;

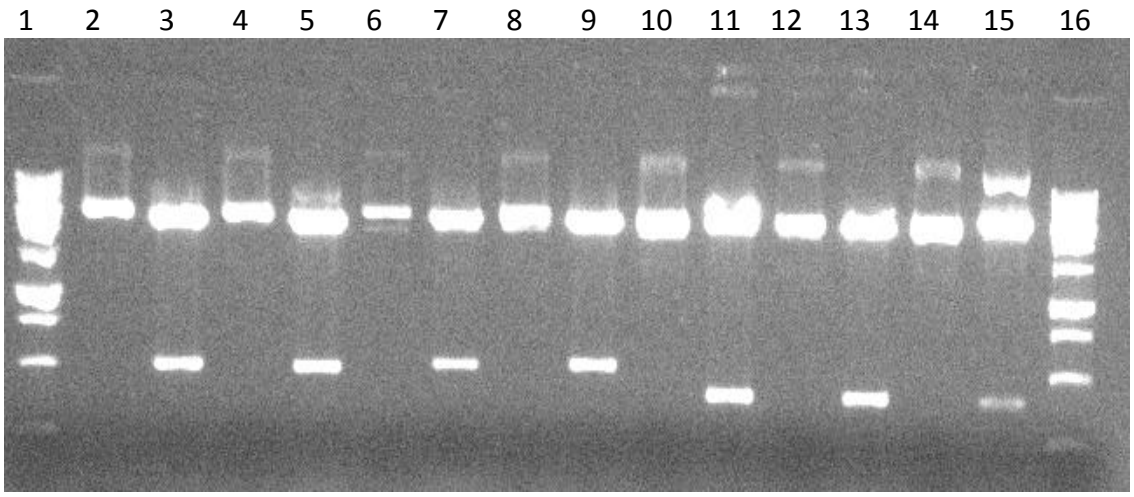
14, 18 - Cut plasmid with release of MCP-1 insert;

17 - Uncut plasmid with MCP-1 insert;

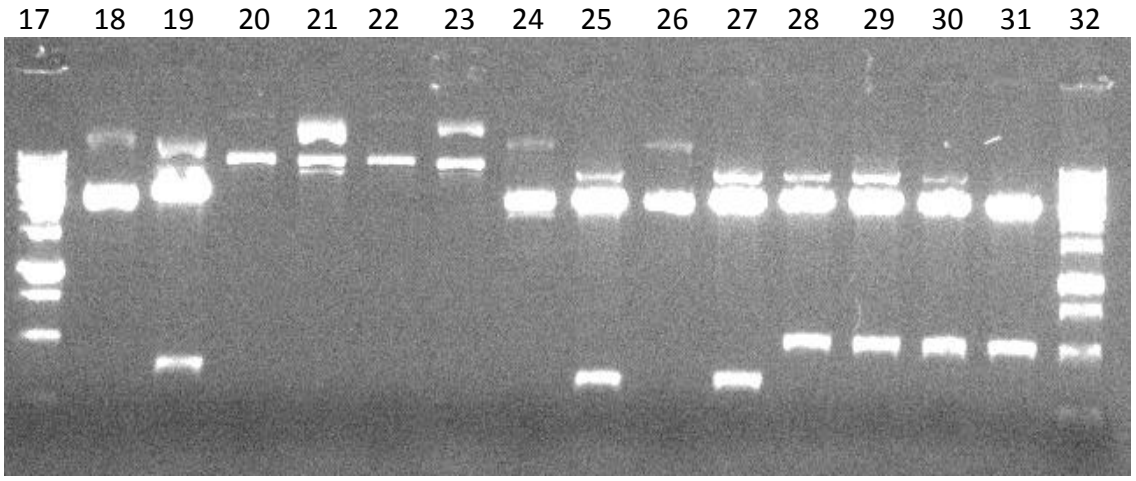
19, 20, 21, 22, 23 - Cut plasmid with release of TGFβ-1 insert;

1, 16 - PCR Markers, Promega;

15, 24 - GenRuler™ 1 kb DNA Ladder, Fermentas



(A)



(B)

Figure 22 (A, B). Agarose gel electrophoresis of plasmids with cloned insert before and after restriction enzyme digestion

Lanes: 2, 4, 6, 8 - Uncut plasmid with IFN-γ insert;

3, 5, 7, 9 - Cut plasmid with release of IFN-γ insert;

10, 12, 14, 18 - Uncut plasmid with TLR9 insert;

11, 13, 15, 19 - Cut plasmid with release of TLR9 insert;

20, 21, 22, 23, 24, 26 - Uncut plasmid with or without IL-10 insert;

25, 27 - Cut plasmid with release of IL-10 insert;

28, 29, 30, 31 - Cut plasmid with release of TLR2 insert;

1, 16, 17, 32 - GenRuler™ 1 kb DNA Ladder, Fermentas

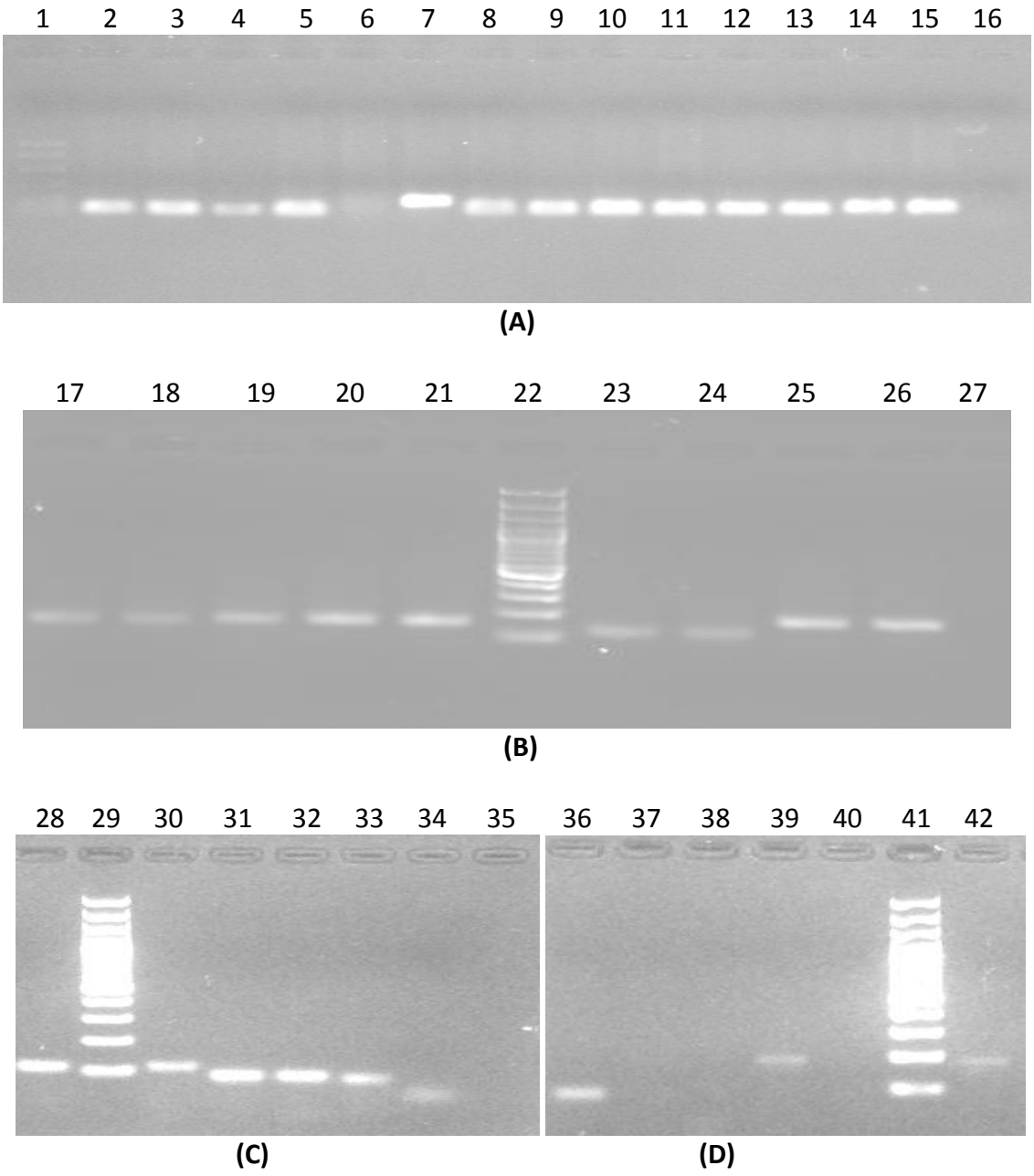
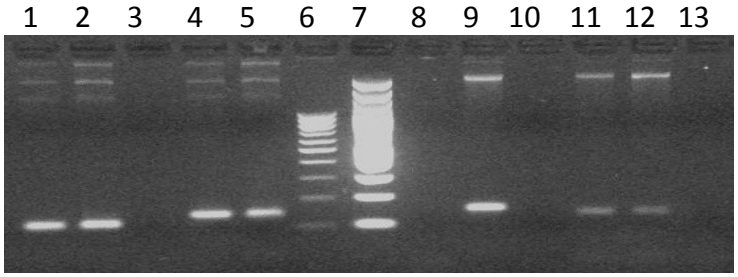
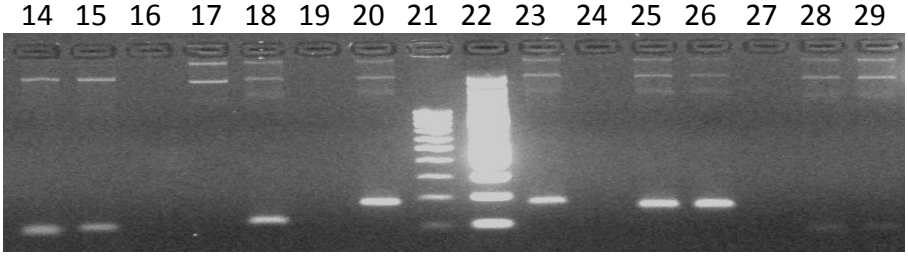


Figure 23 (A, B, C, D). Agarose gel electrophoresis of amplified products to check insert in cloned bacteria

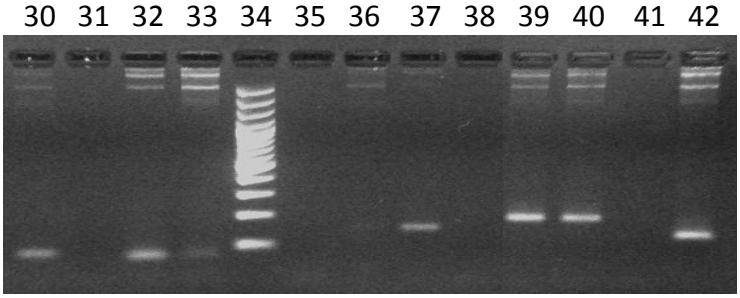
Lanes: 2, 3, 4 - IL-15 (74 bp); 5 -CCL8 (94 bp); 7 - ICAM-1 (156 bp); 8, 9, 10, 11 - CCL5 (82 bp); 12, 13 - CCL3 (82 bp); 14, 15 - Galectin-3 (97 bp); 17, 18 - IL-9 (149 bp); 19, 20, 21 - IFN-αR1 (157 bp); 23, 24 - IL-1α (124 bp); 25, 26 - Perforin (178 bp); 28, 30-IL-27p28 (111 bp); 31, 32- GM-CSF (87 bp); 33 - COX-2 (80 bp); 34, 36 - iNOS (82 bp); 39, 42 - IL-12p40 (186 bp); 16, 27, 35 - Negative controls; 1 - PCR Markers, Promega; 22 - 100 bp DNA Ladder, SibEnzyme; 29, 41 - GeneRuler™ 100 bp Plus DNA Ladder, Fermentas



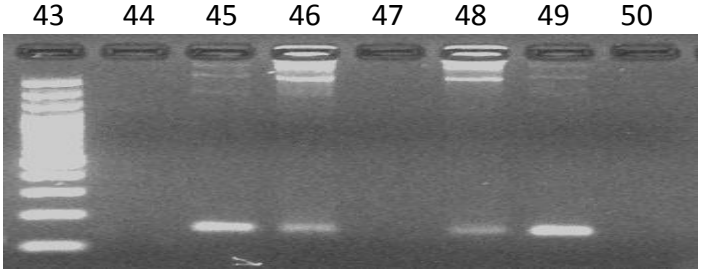
(A)



(B)



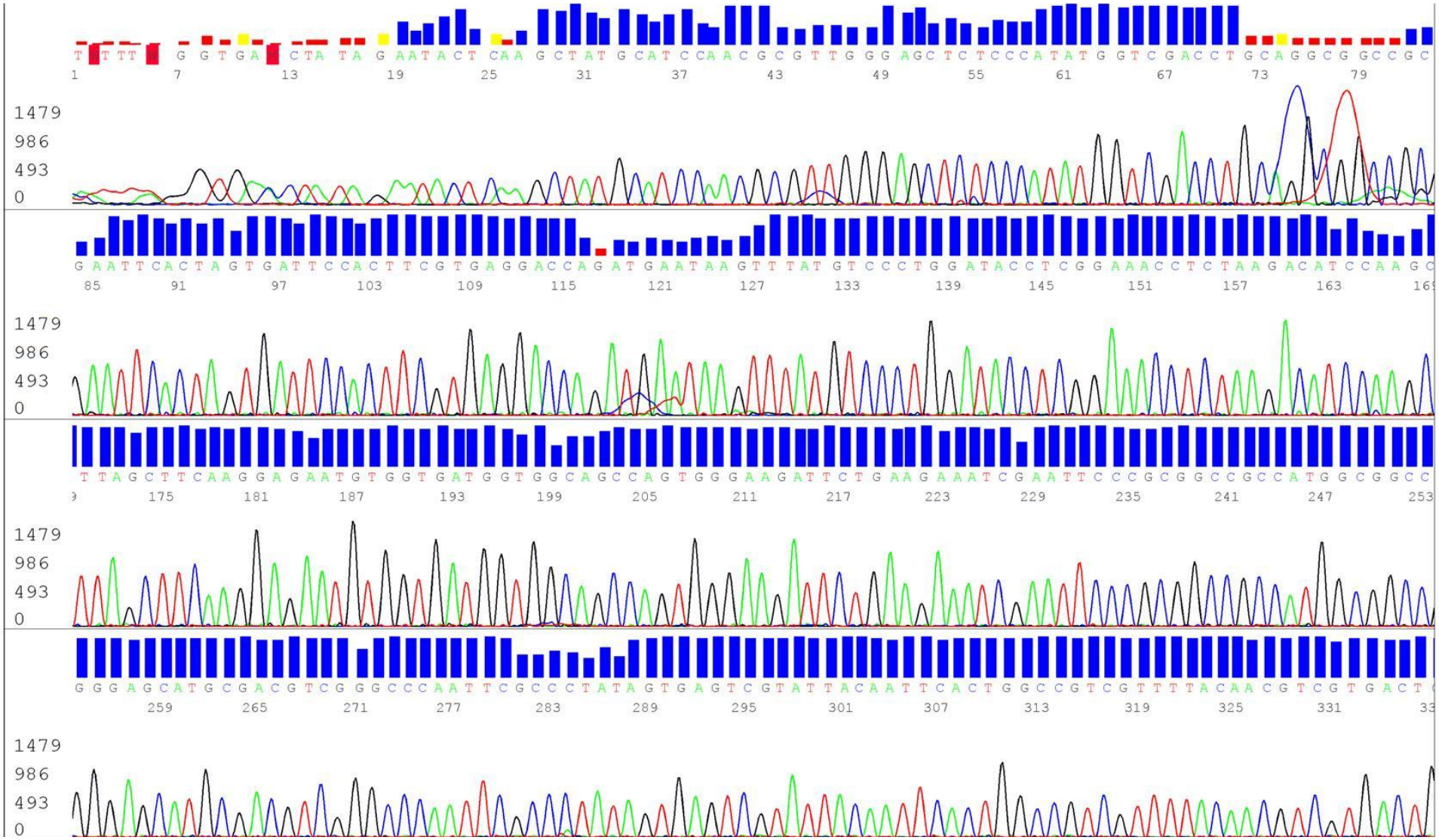
(C)



(D)

Figure 24 (A, B, C, D). Agarose gel electrophoresis of amplified products to check insert in extracted plasmids

Lanes: 1, 2 - IL-13 (111 bp); 4, 5 - IL-17A (138 bp); 9 - IL-20 (149 bp); 11, 12 - IL-21 (132 bp); 14, 15 - IL-25 (100 bp); 17, 18 - IL-27p28 (111 bp); 20, 23 - IL-32 (177 bp); 25, 26 - IL-34 (165 bp); 28, 29 - GM-CSF (87 bp); 30 - iNOS (111 bp); 32, 33 - IL-16 (73 bp); 36, 37 - β-actin (153 bp); 39, 40 - TNF-αSF1 (200 bp); 42 - TGFβ-2 (135 bp); 45, 46 - CD4 (152 bp); 48, 49 - BAD (151 bp); 3, 8, 10, 13, 16, 19, 24, 27, 31, 35, 38, 41, 44, 47, 50 - Negative controls; 6, 21 - 100 bp DNA Ladder, SibEnzyme; 7, 22, 34, 43 - GenRuler™ 100 bp Plus DNA ladder, Fermentas



F-5’- CCA CTT CGT GAG GAC CAG ATG AAT-3’ R-5’- TCT TCA GAA TCT TCC CAC TGG CTG-3’

Plate 1

Bovine IL-1α clone

PDFMD-Sindhu

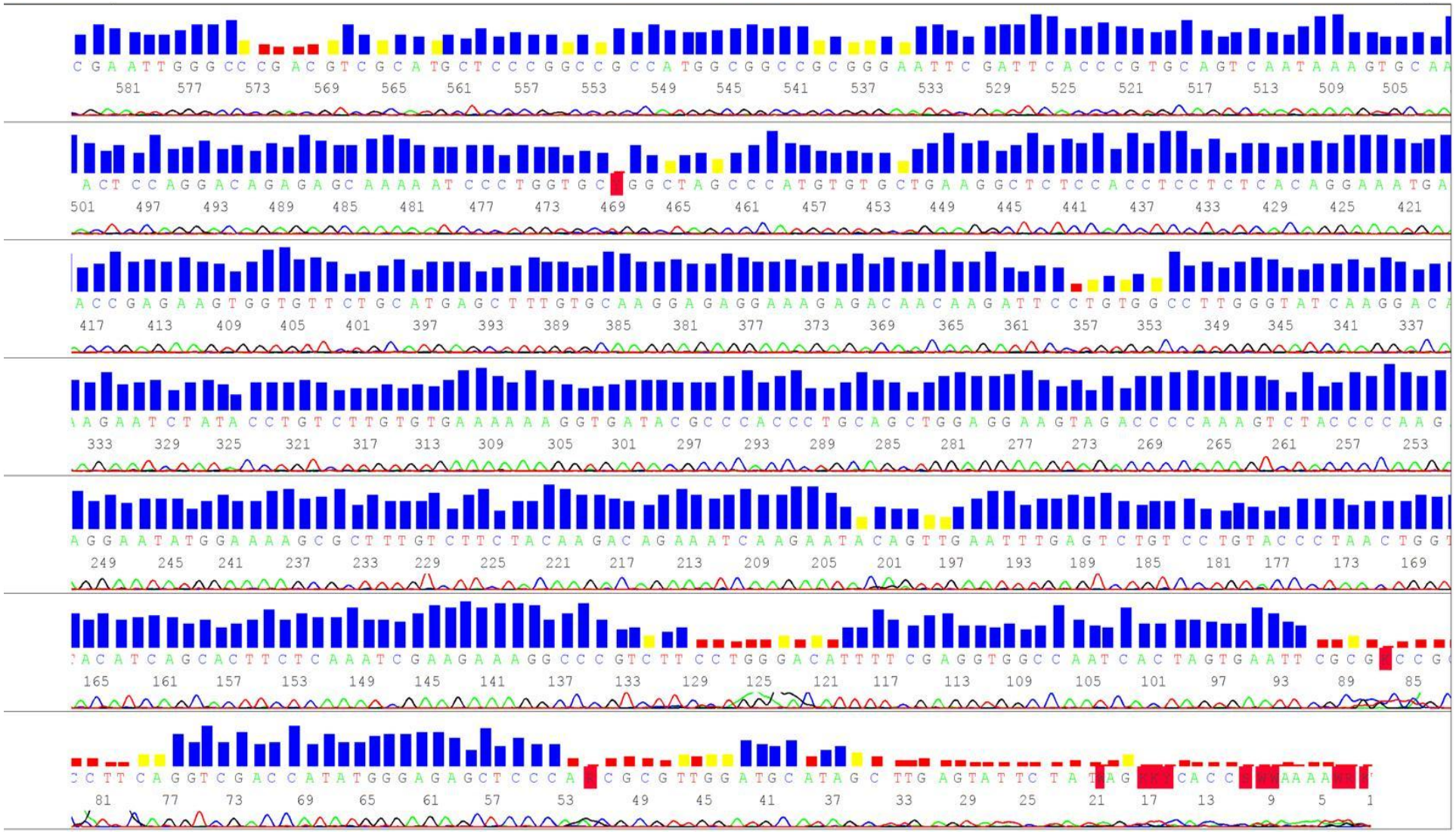


Plate 2 Bovine IL-1β clone PDFMD-Chenab

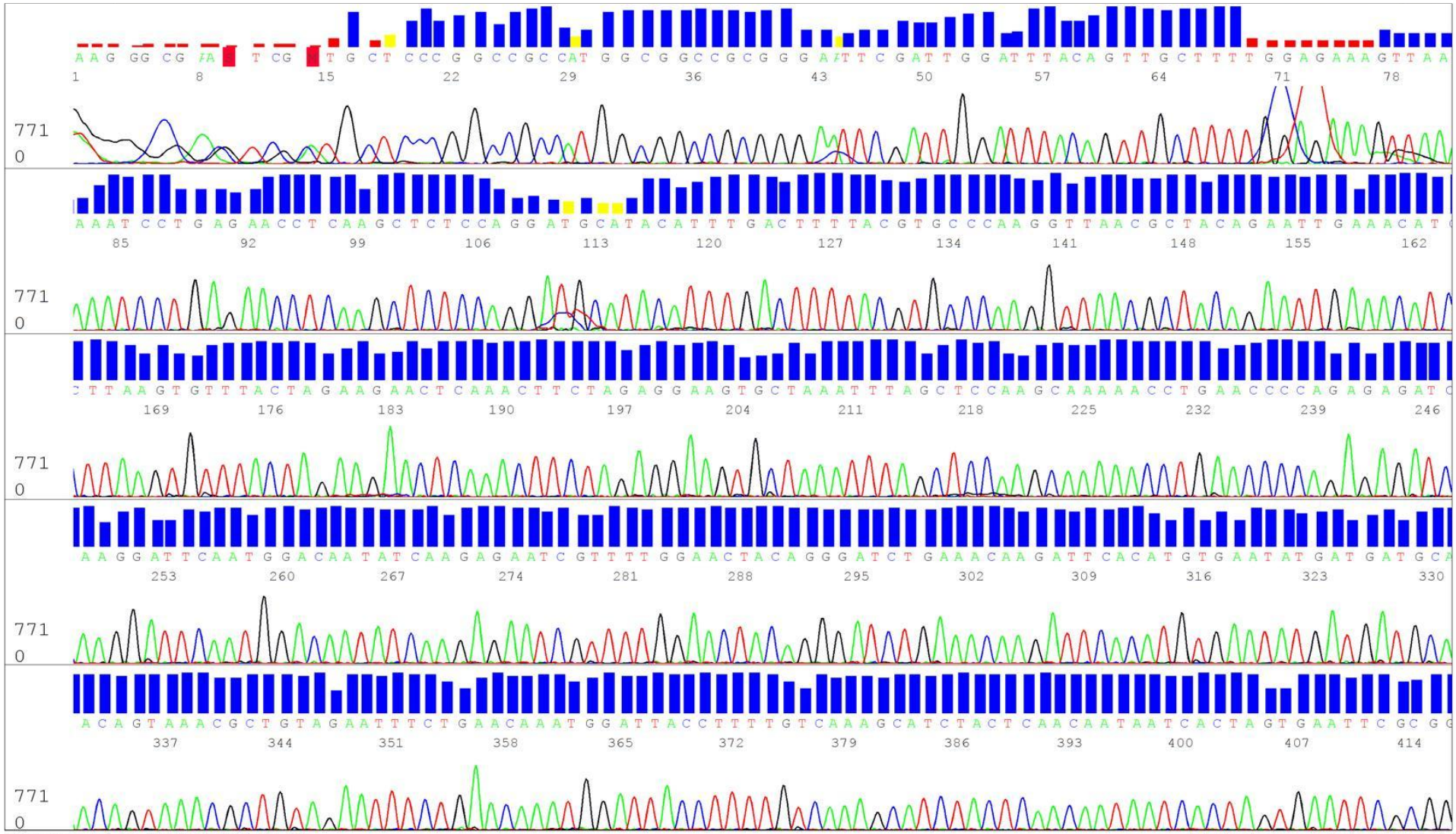
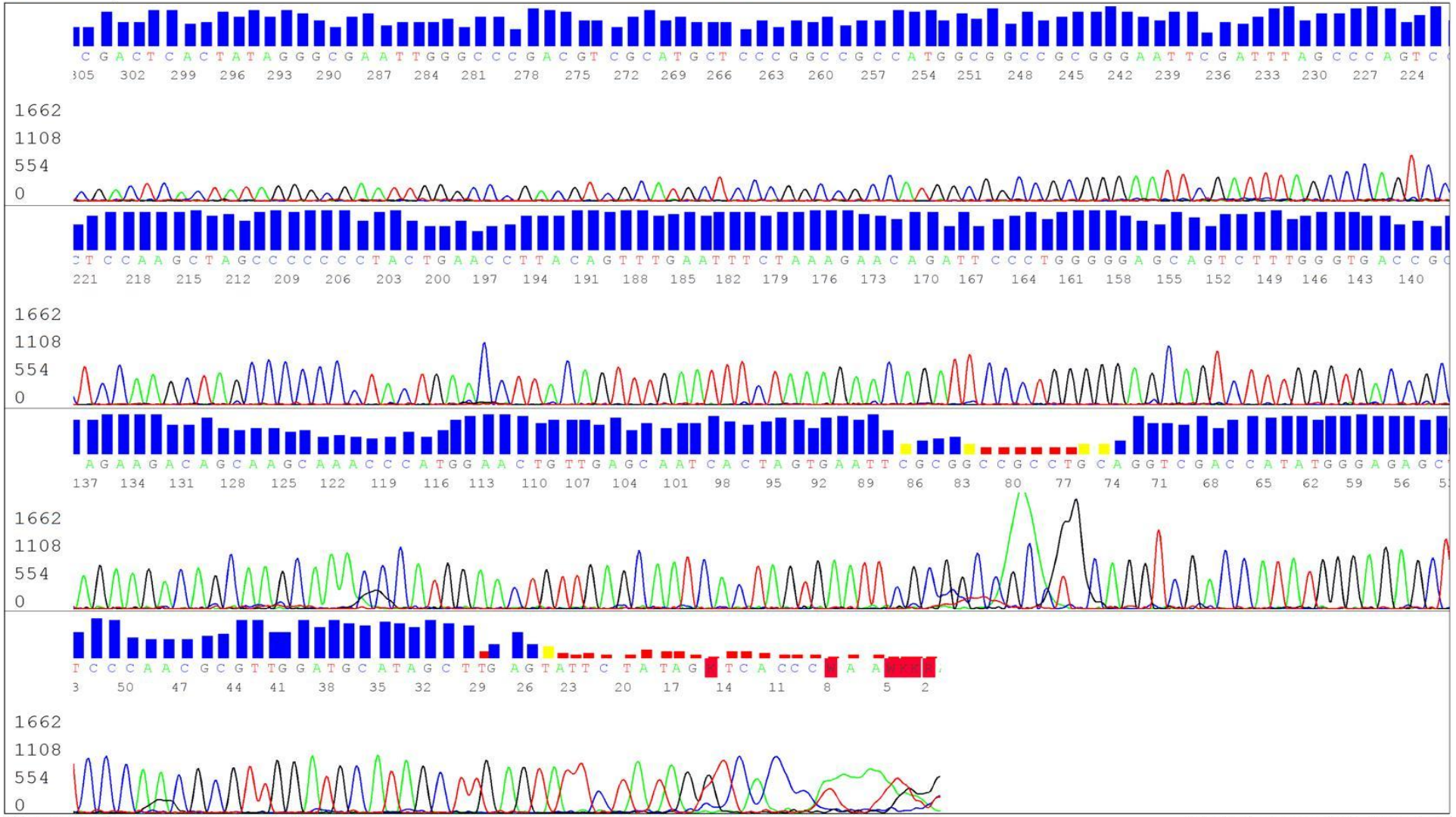


Plate 3 Bovine IL-2 clone PDFMD-Satluj



F-5’-TAG CCC AGT CCT CCA AGC TA-3’ R-5’-GCT CAA CAG TTC CAT GGG TT-3’

Plate 4 **Bovine IL-2Rα clone** PDFMD-Banas

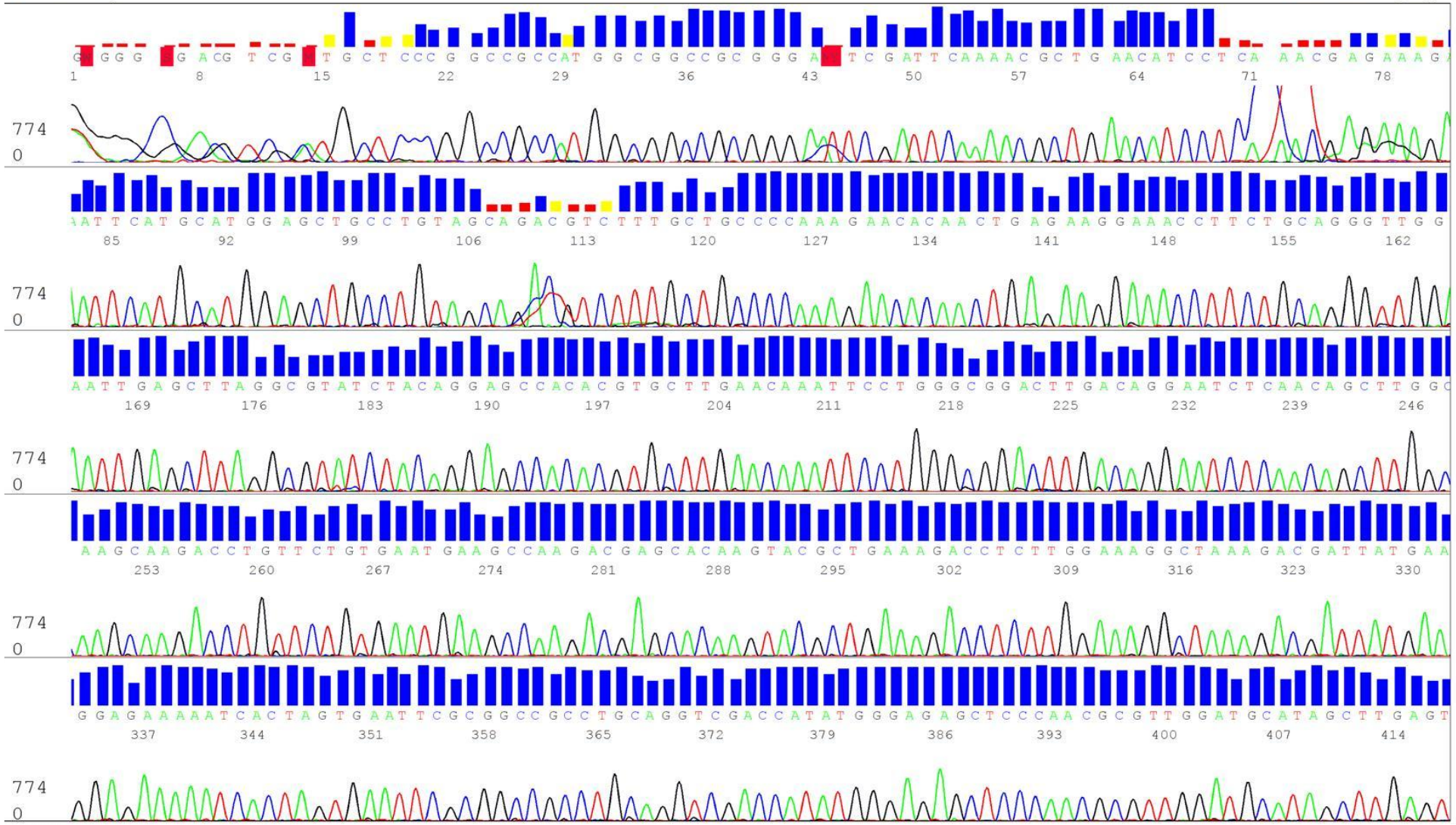


Plate 5 Bovine IL-4 clone PDFMD-Beas

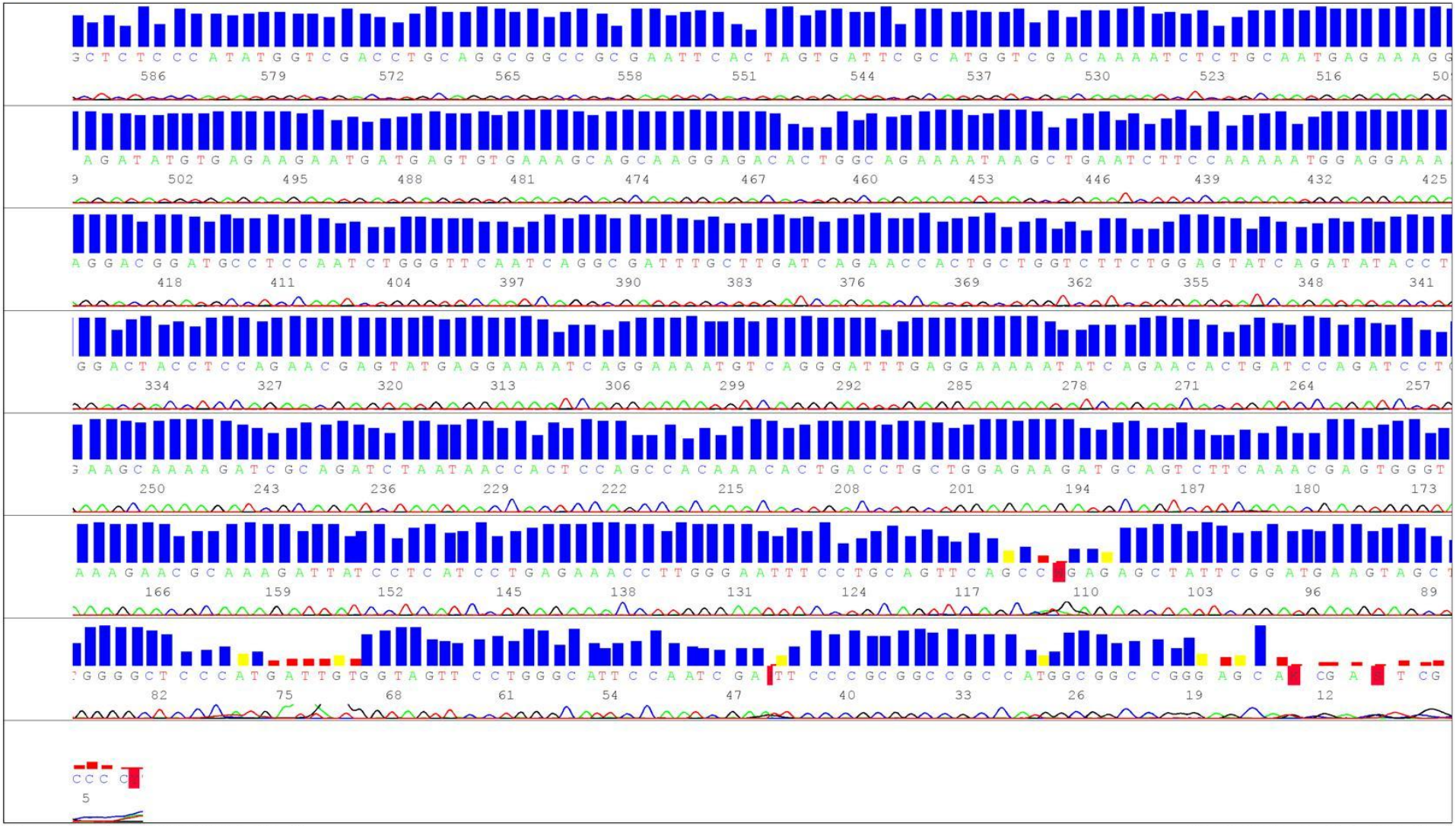


Plate 6 Bovine IL-6 clone PDFMD-Bhagirathi

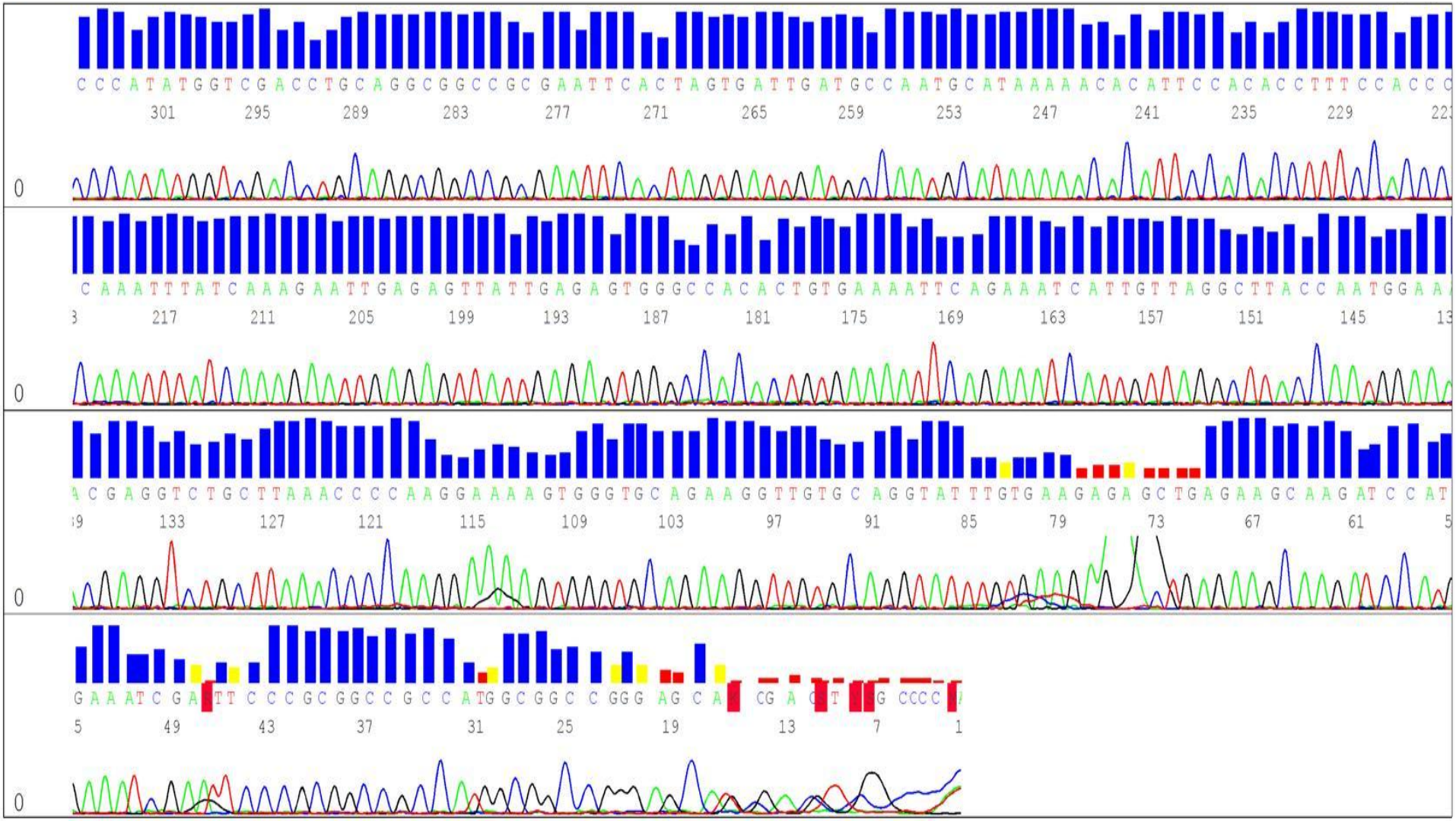
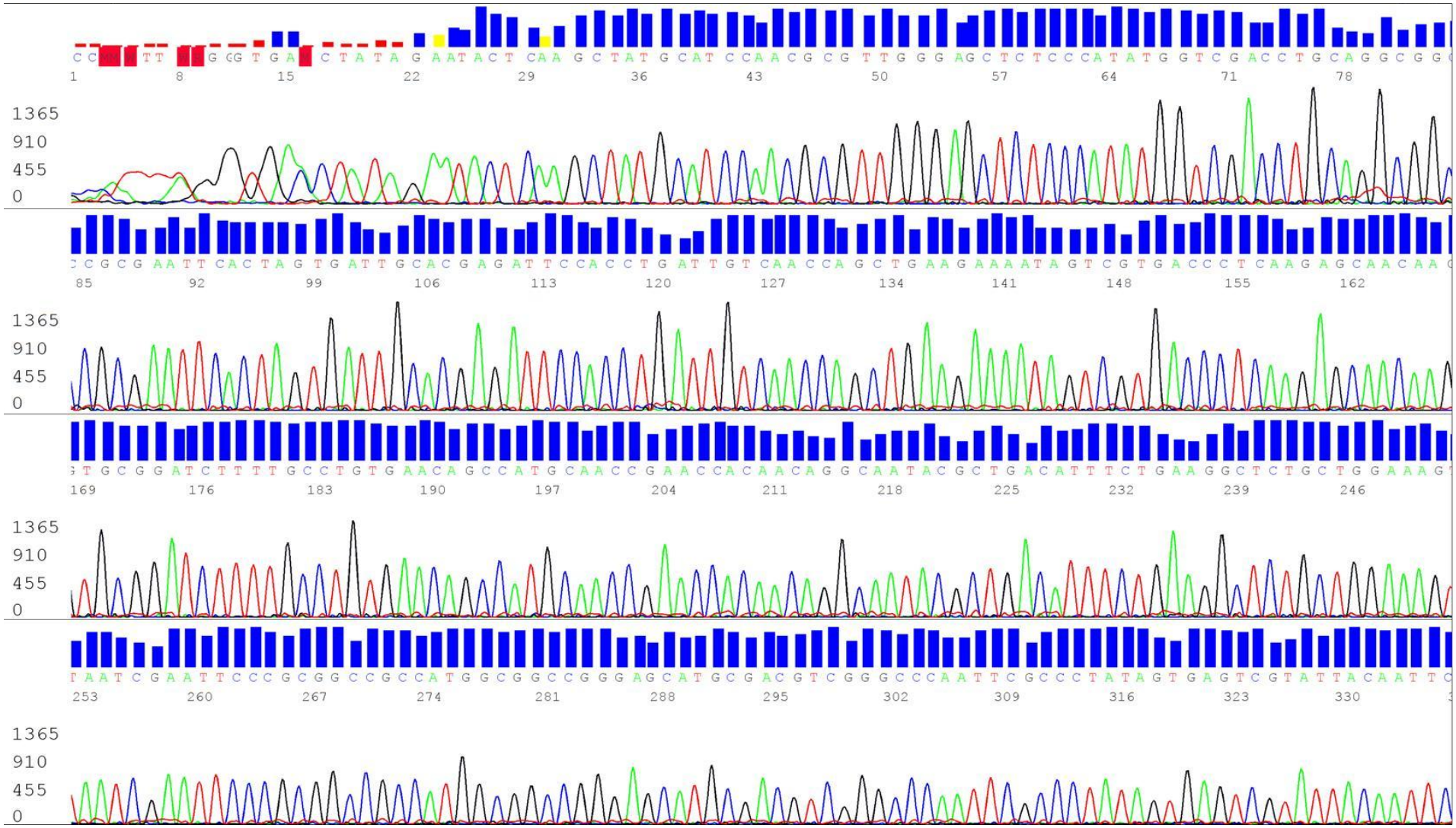


Plate 7 Bovine IL-8 clone PDFMD-Ganga



F- 5’-GCA CGA GAT TCC ACC TGA TT-3’ R - 5’-ACT TTC CAG CAG AGC CTT CA-3’

Plate 8 | Bovine IL-9 clone | PDFMD-Yamuna

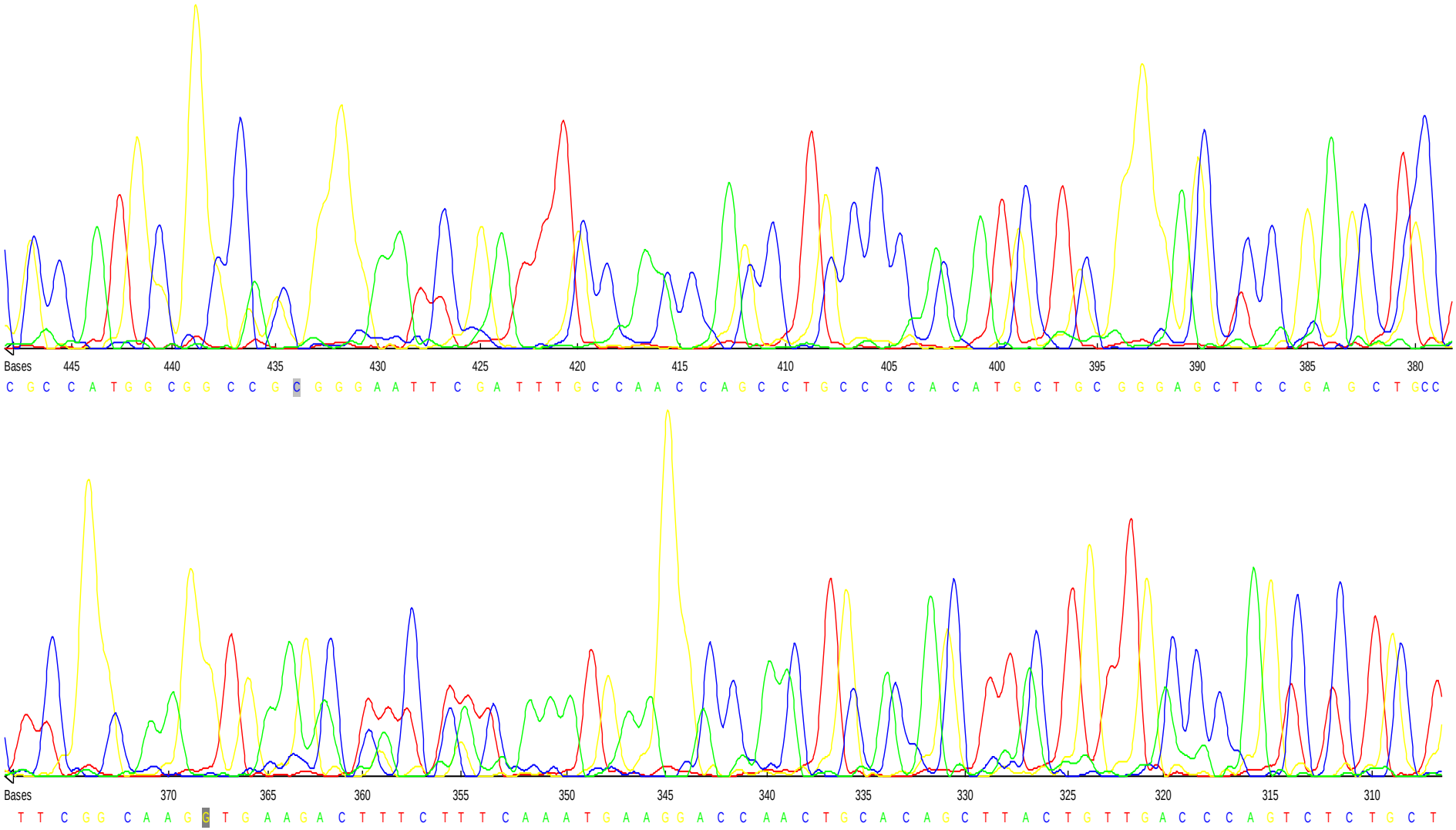


Plate 9a **Bovine IL-10 clone** **PDFMD-Luni (Continuing….**

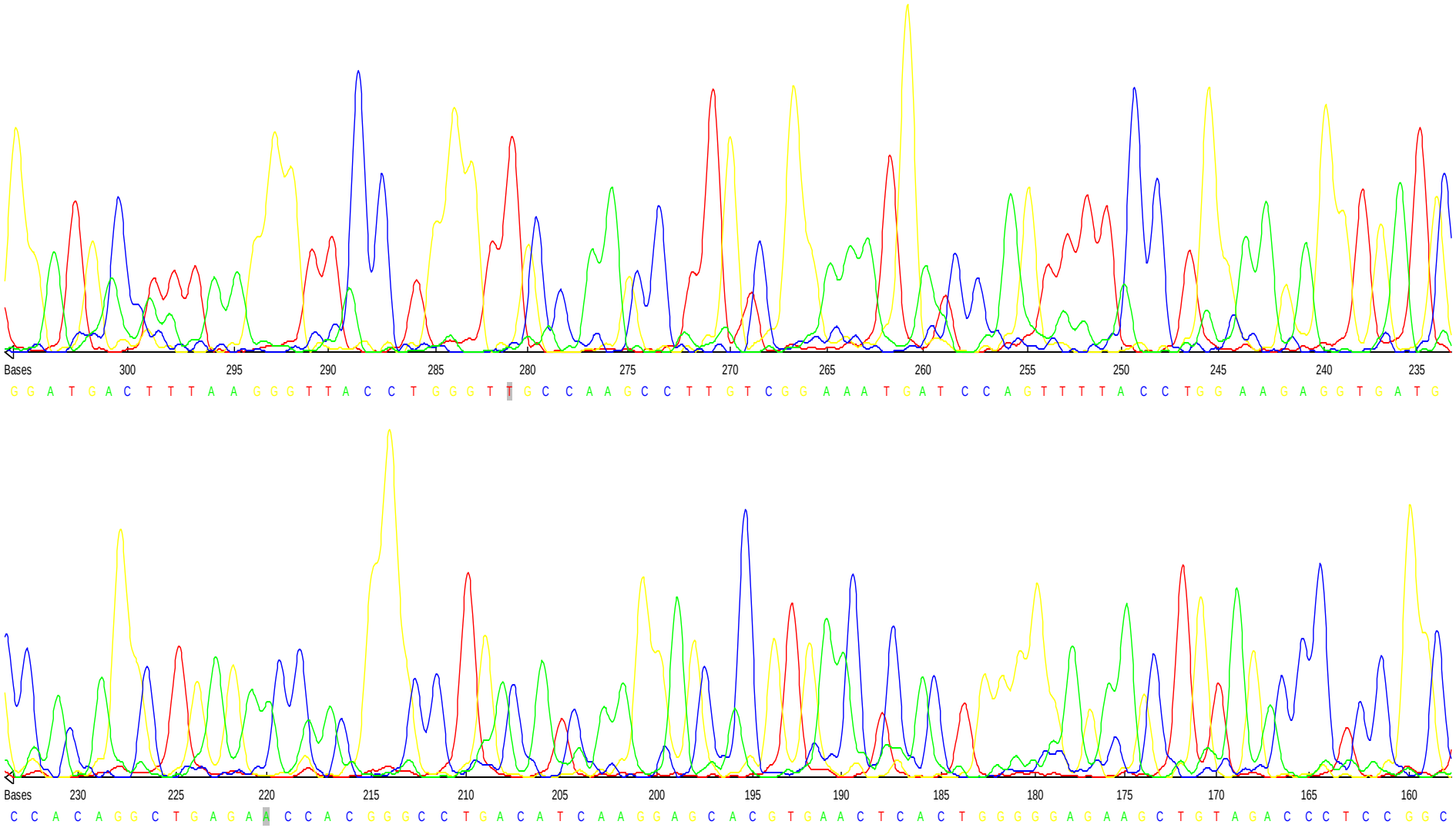


Plate 9b **Bovine IL-10 clone** **PDFMD-Luni (Continuing….**

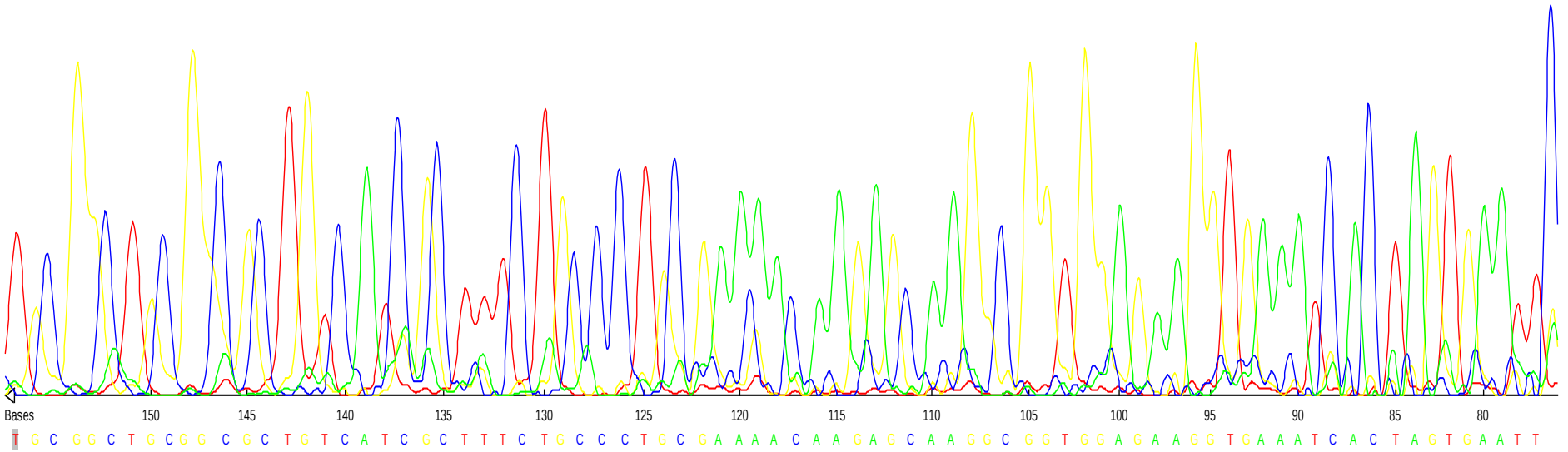
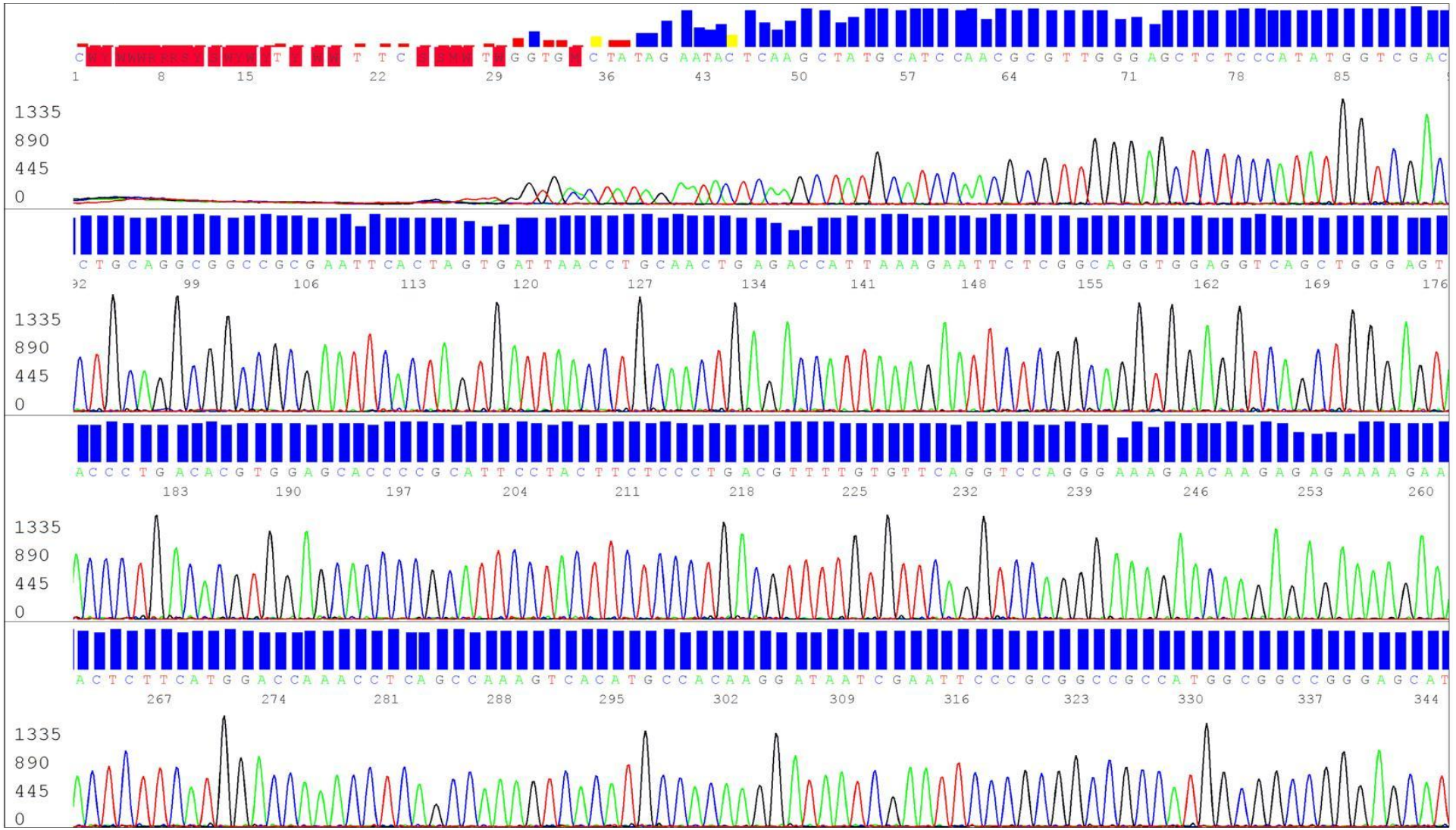
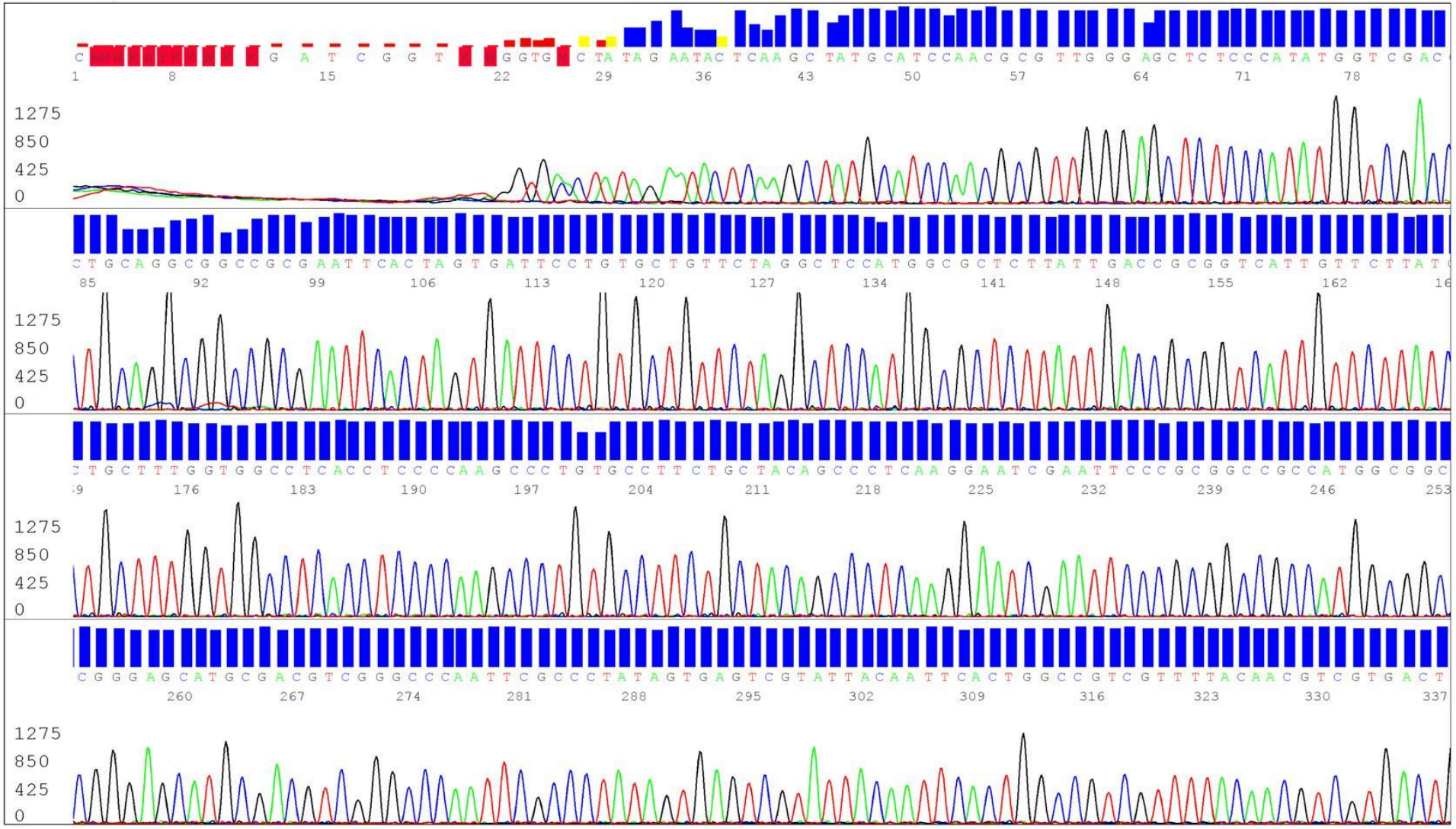


Plate 9c **Bovine IL-10 clone** **PDFMD-Luni**



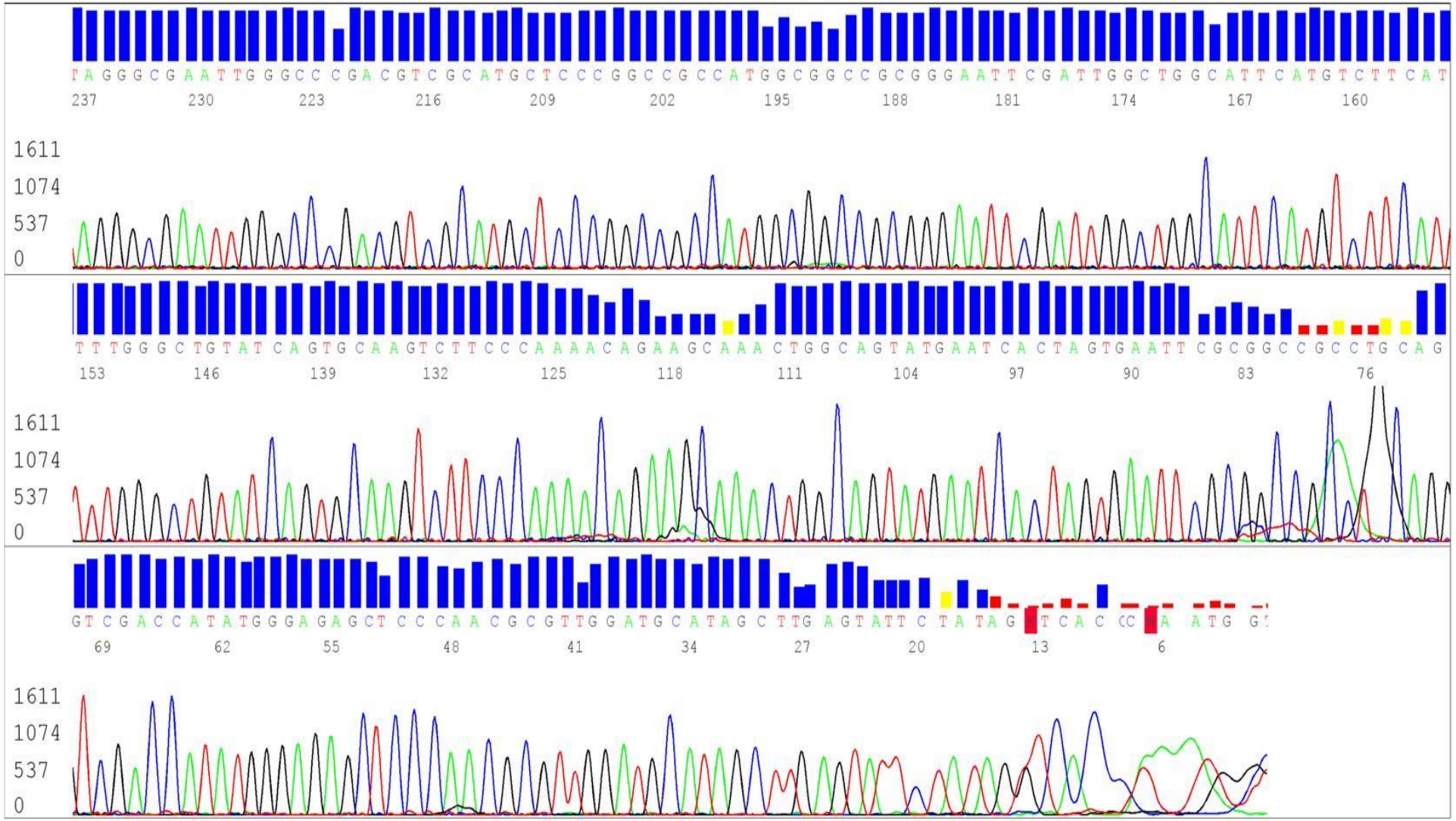
F-5’-AAC CTG CAA CTG AGA CCA TT-3’ R-5’-ATC CTT GTG GCA TGT GAC TT-3’

Plate 10 Bovine/Ovine IL-12p40 clone PDFMD-Mahi



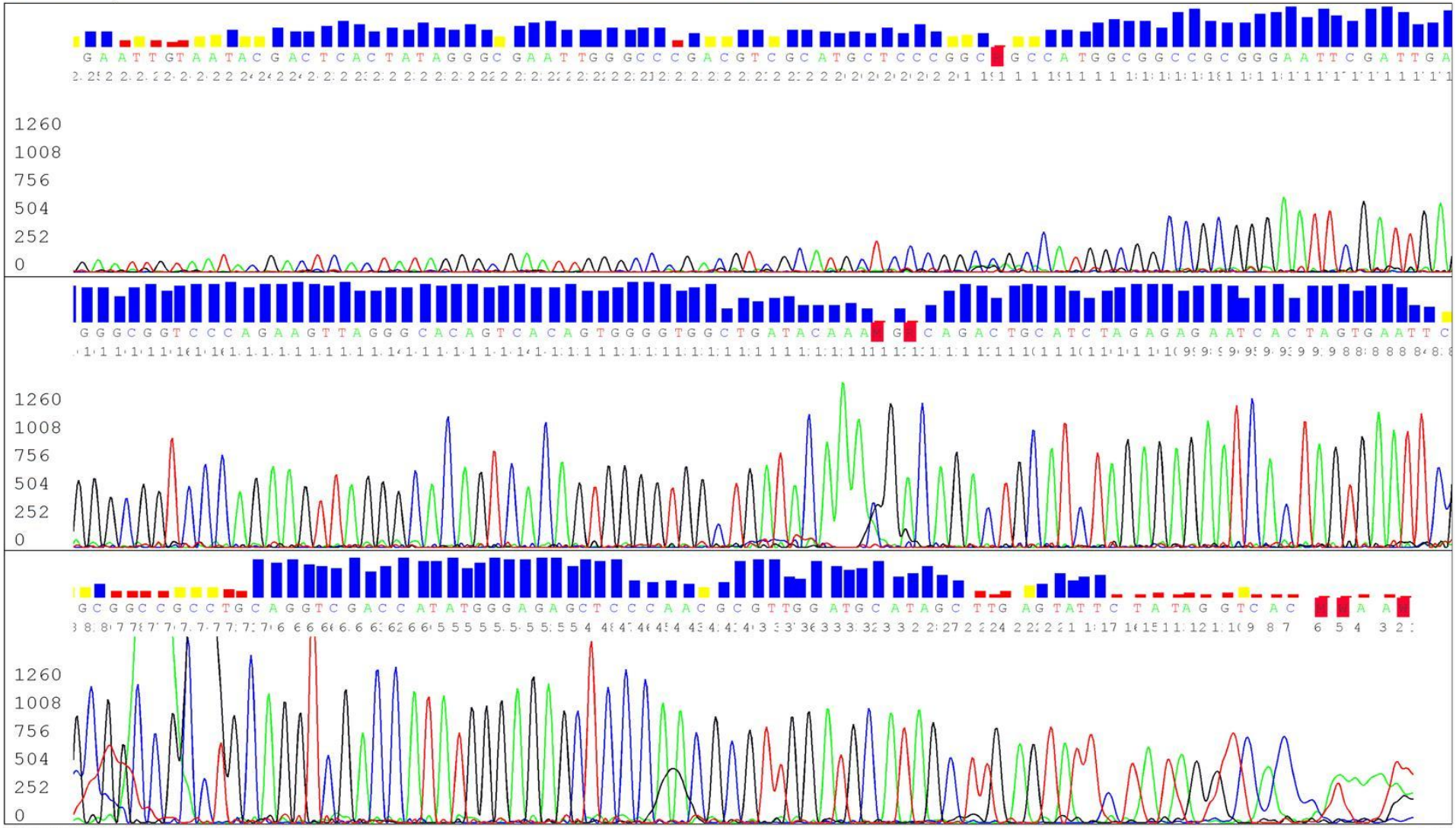
F-5’-CCT GTG CTG TTC TAG GCT CC-3’ R-5’-CCT TGA GGG CTG TAG CAG AA-3’

Plate 11 Bovine IL-13 clone PDFMD-Chambal



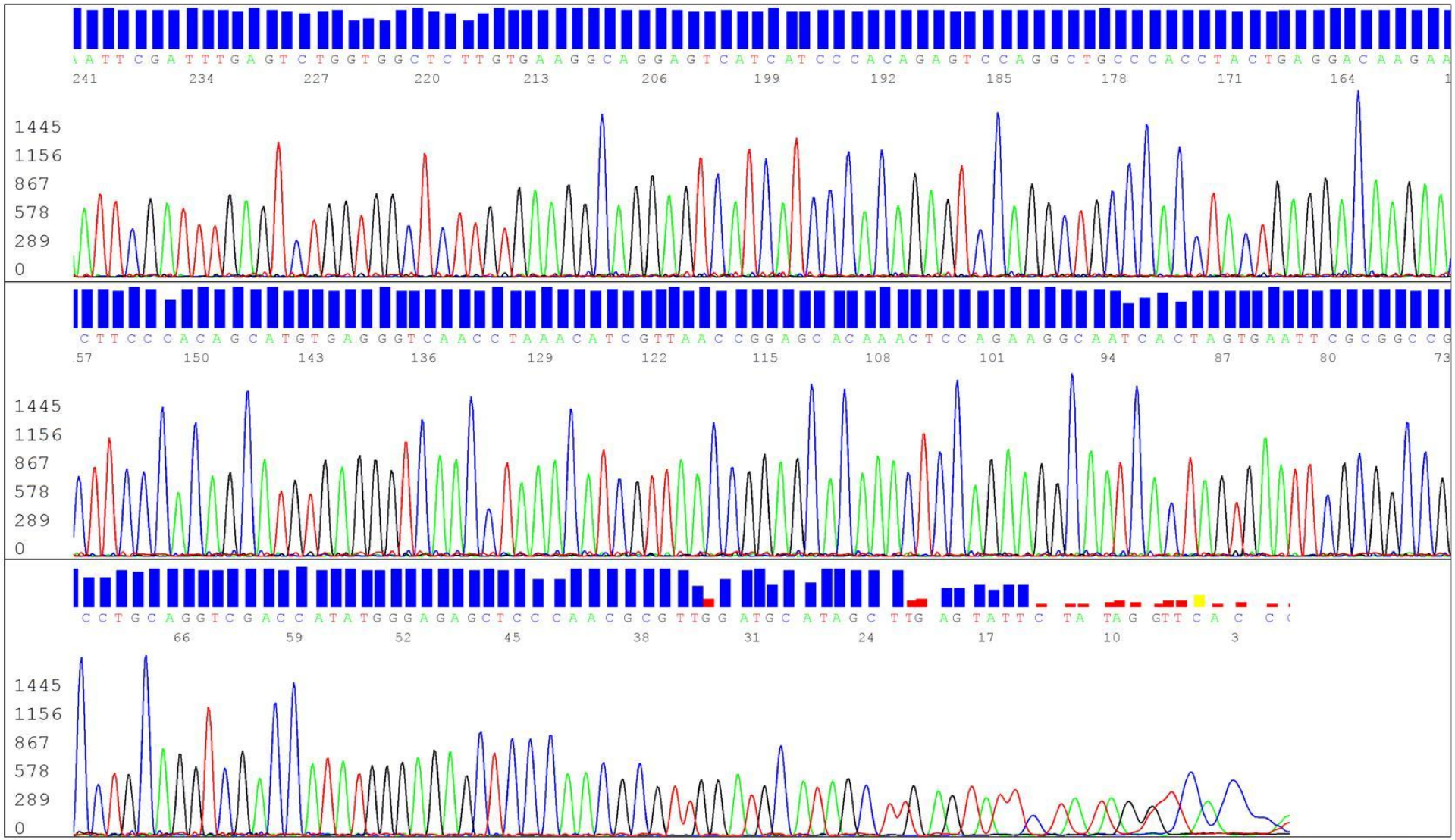
F-5'-GGC TGG CAT TCA TGT CTT CA-3' R-5'-CAT ACT GCC AGT TTG CTT CTG TTT-3'

Plate 12 **Bovine IL-15 clone** PDFMD-Sabarmati



F-5’- GAG GGC GGT CCC AGA AGT-3’ R- 5’- CTC TCT AGA TGC AGT CTG TCG TTT GT- 3’

Plate 13 Bovine IL-16 clone PDFMD-Godavari



F-5’-TGA GTC TGG TGG CTC TTG TG-3’ R-5’-GCC TTC TGG AGT TTG TGC TC-3’

Plate 14

Bovine IL-17A clone

PDFMD-Tapi

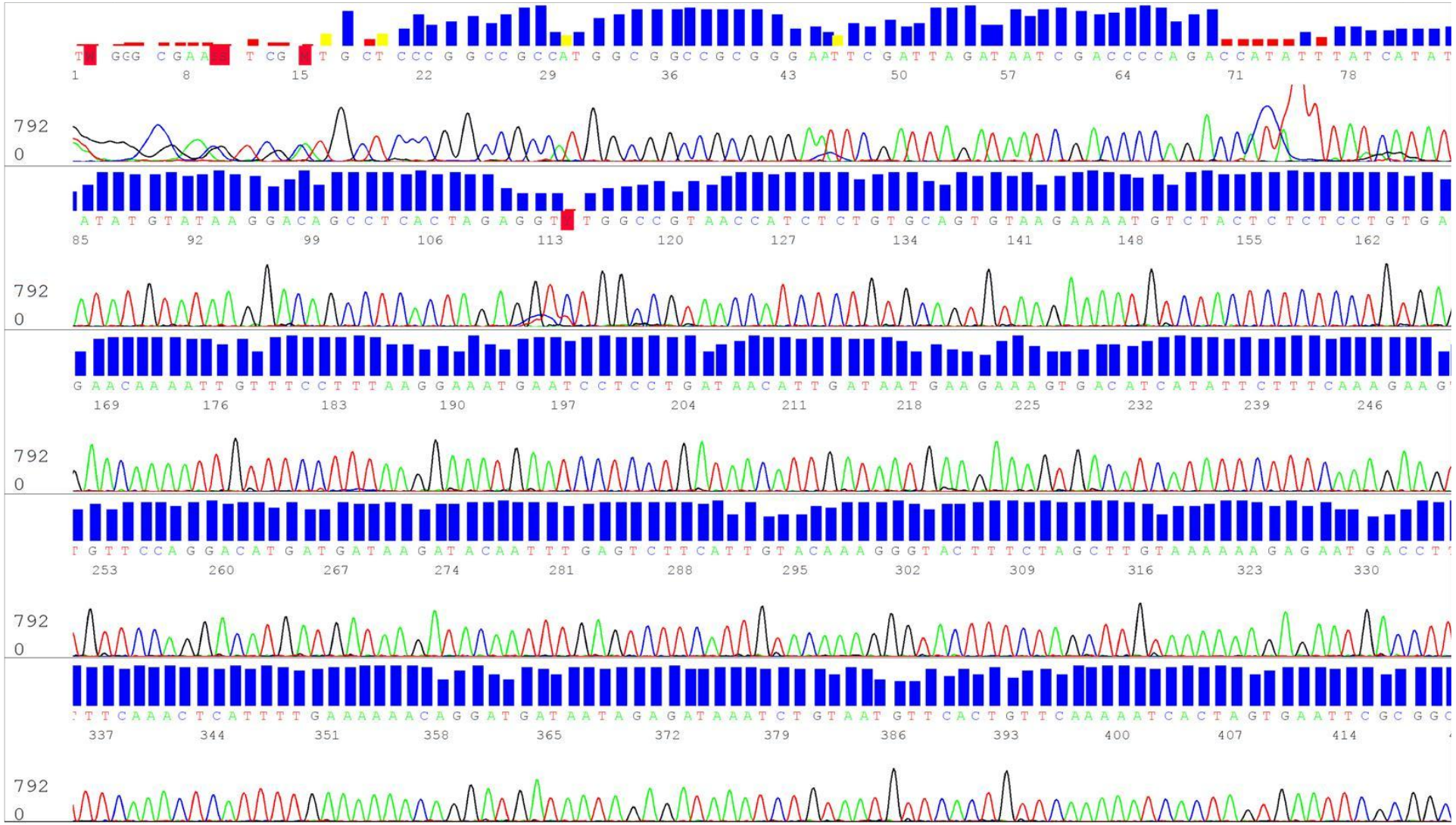
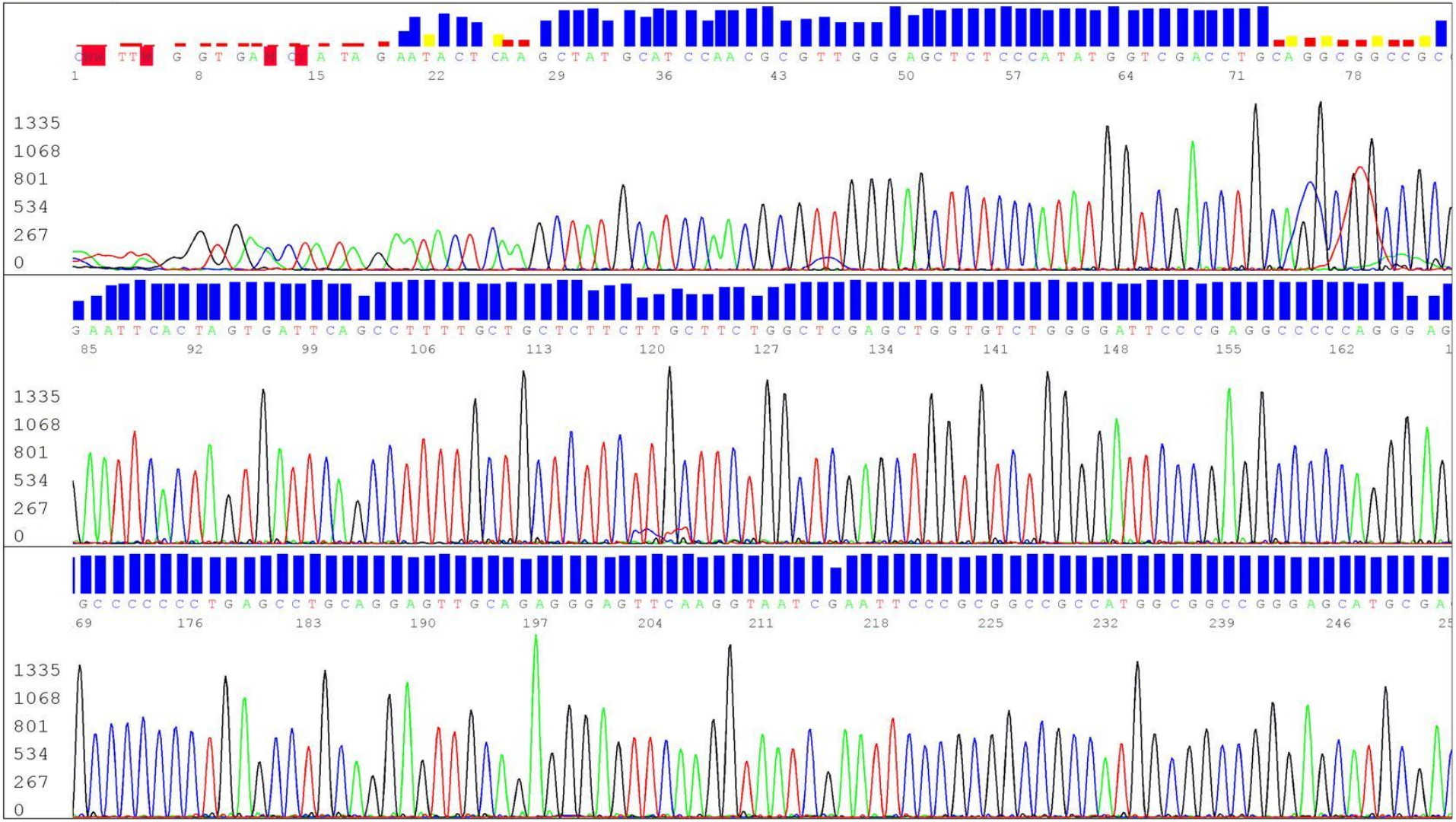
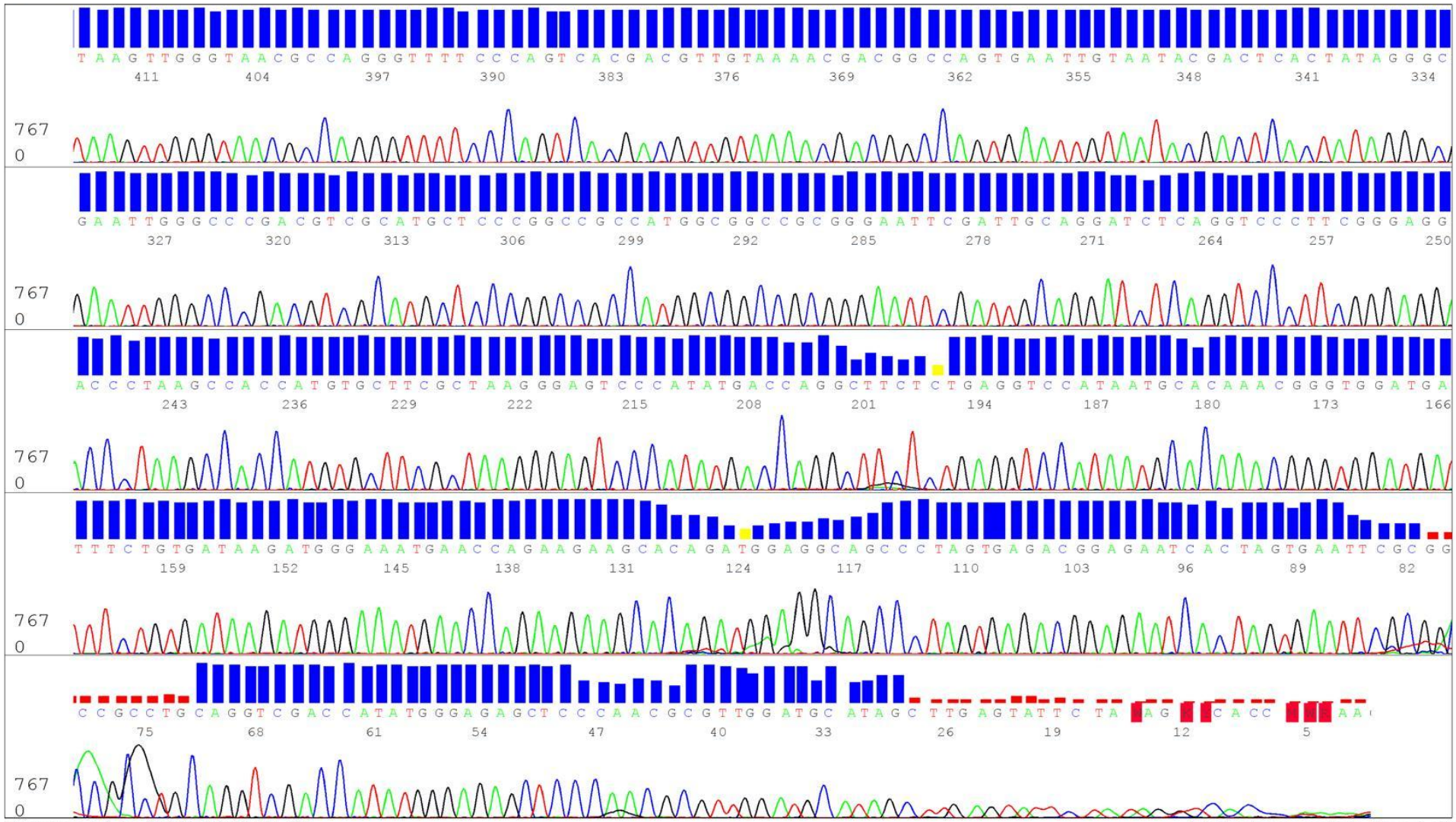


Plate 15 Bovine IL-18 clone PDFMD-Penganga



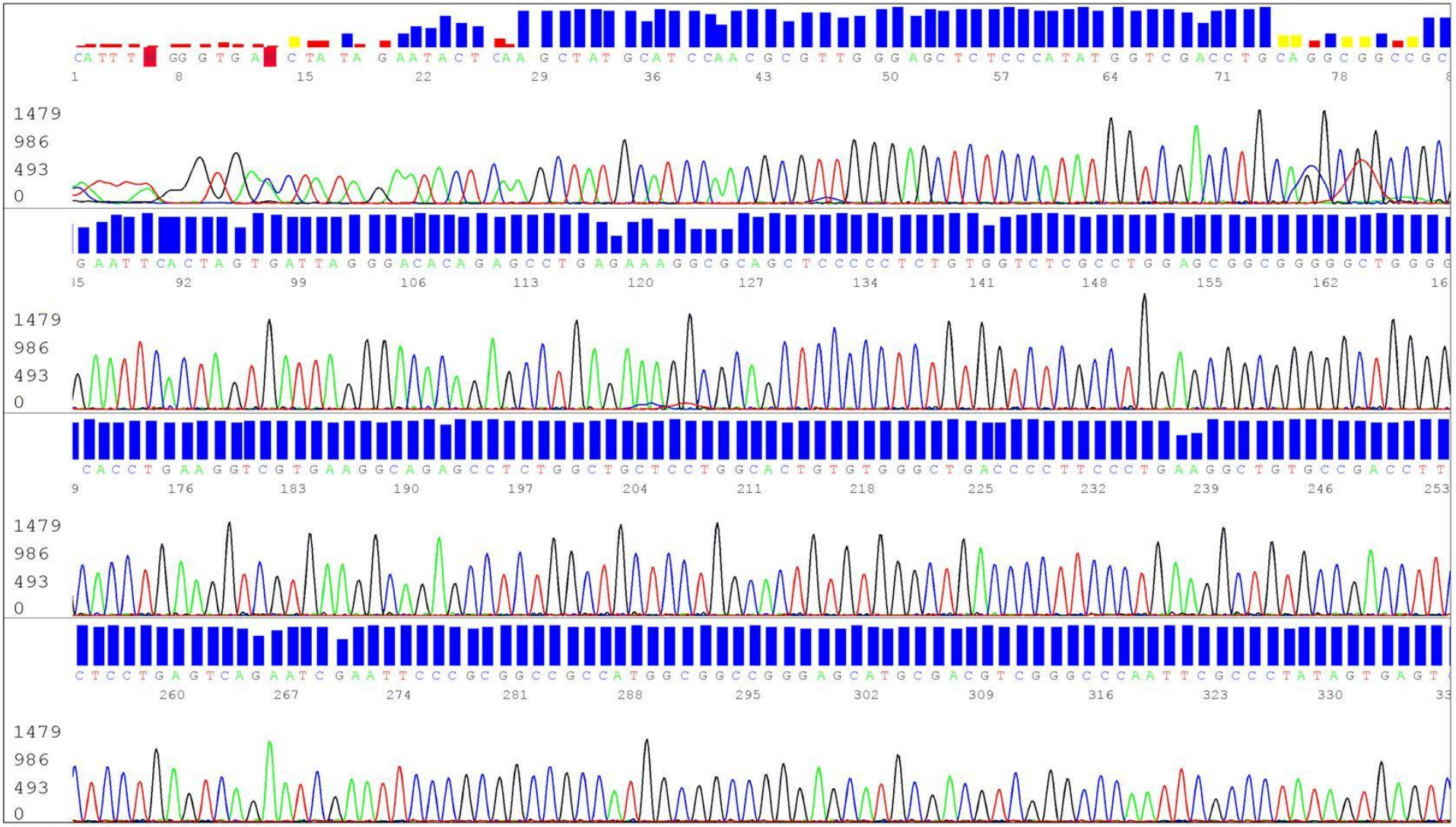
F-5’- CAG CCT TTT GCT GCT CTT CT-3’ R-5’- ACC TTG AAC TCC CTC TGC AA-3’

Plate 16 **Bovine IL-27p28 clone** PDFMD-Narmada



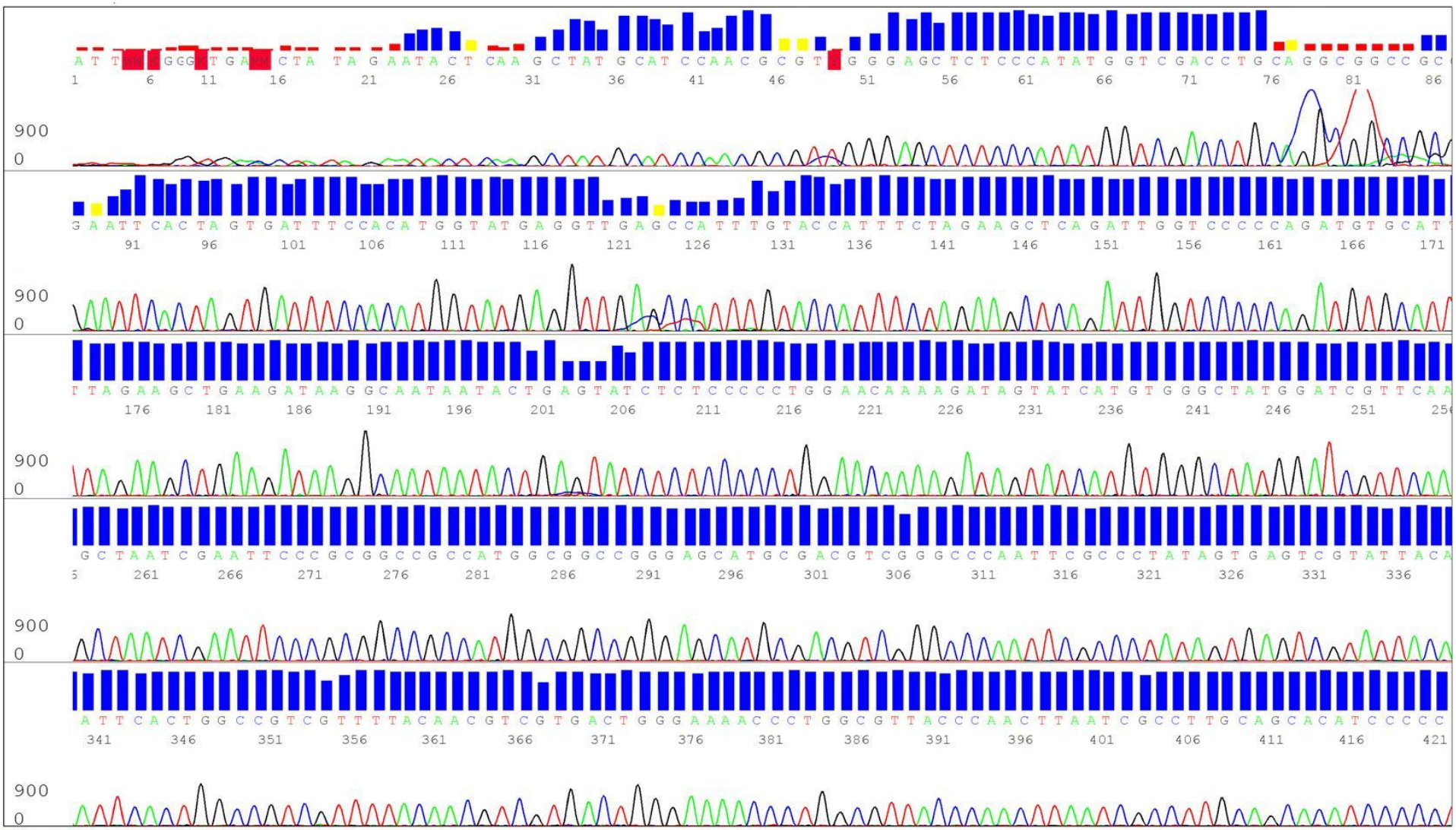
F-5'-GCA GGA TCT CAG GTC CCT T-3' R-5'- CTC CGT CTC ATC TAG GGC TG-3'

Plate 17 Bovine IL-32 clone PDFMD-Krishna



F-5’-AGG GAC ACA GAG CCT GAG AA-3’ R-5’-CTG ACT CAG GAG AAG GTC GG-3’

Plate 18 Bovine IL-34 clone PDFMD-Idukki



F-5’- TCC ACA TGG TAT GAG GTT GAG CCA-3’ R-5’- AGC TTG AAC GAT CCA TAG CCC ACA-3’

Plate 19 Bovine IFN-αR1 clone PDFMD-Periyar

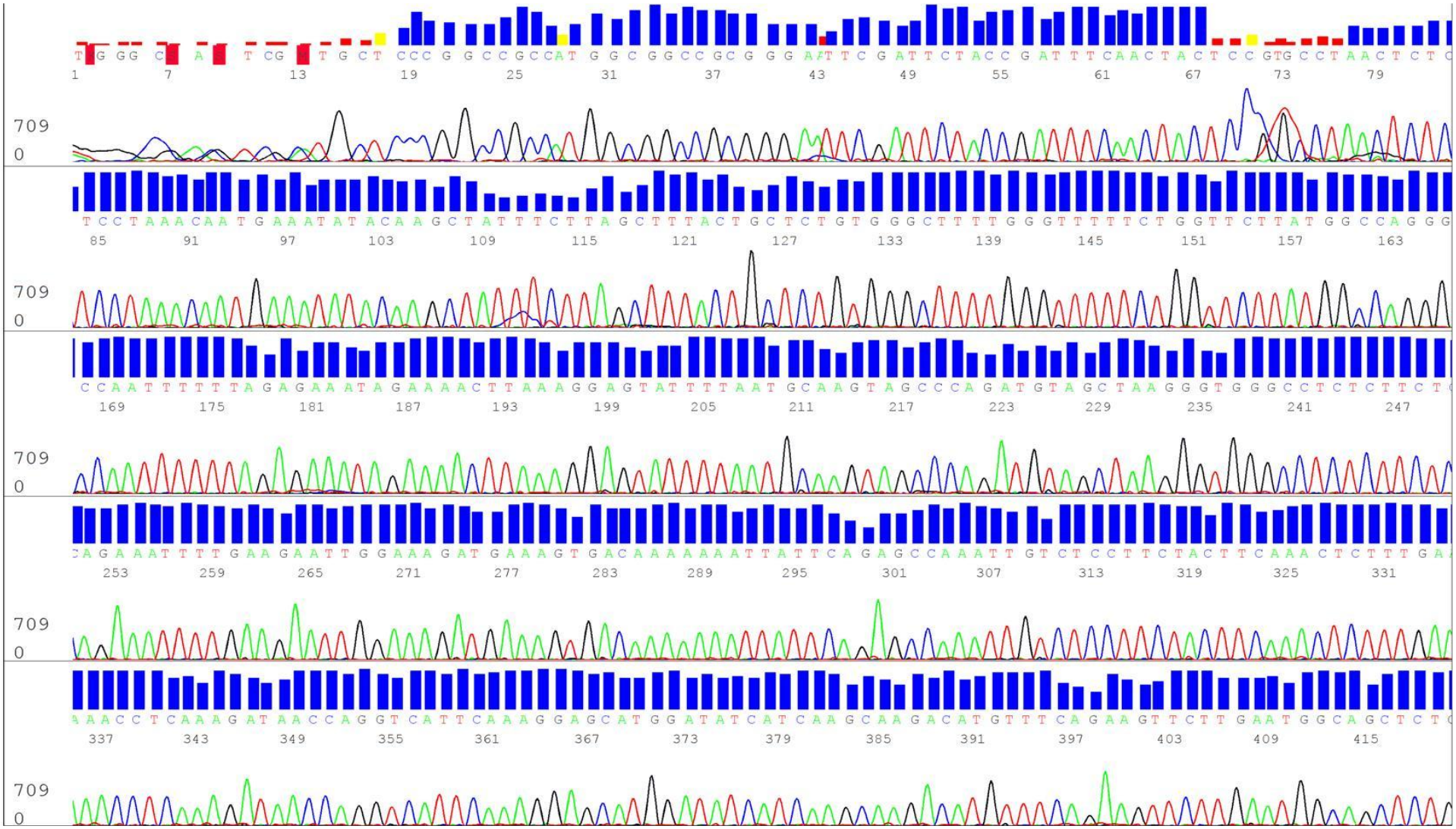


Plate 20 Bovine IFN-γ clone PDFMD-Kaladi

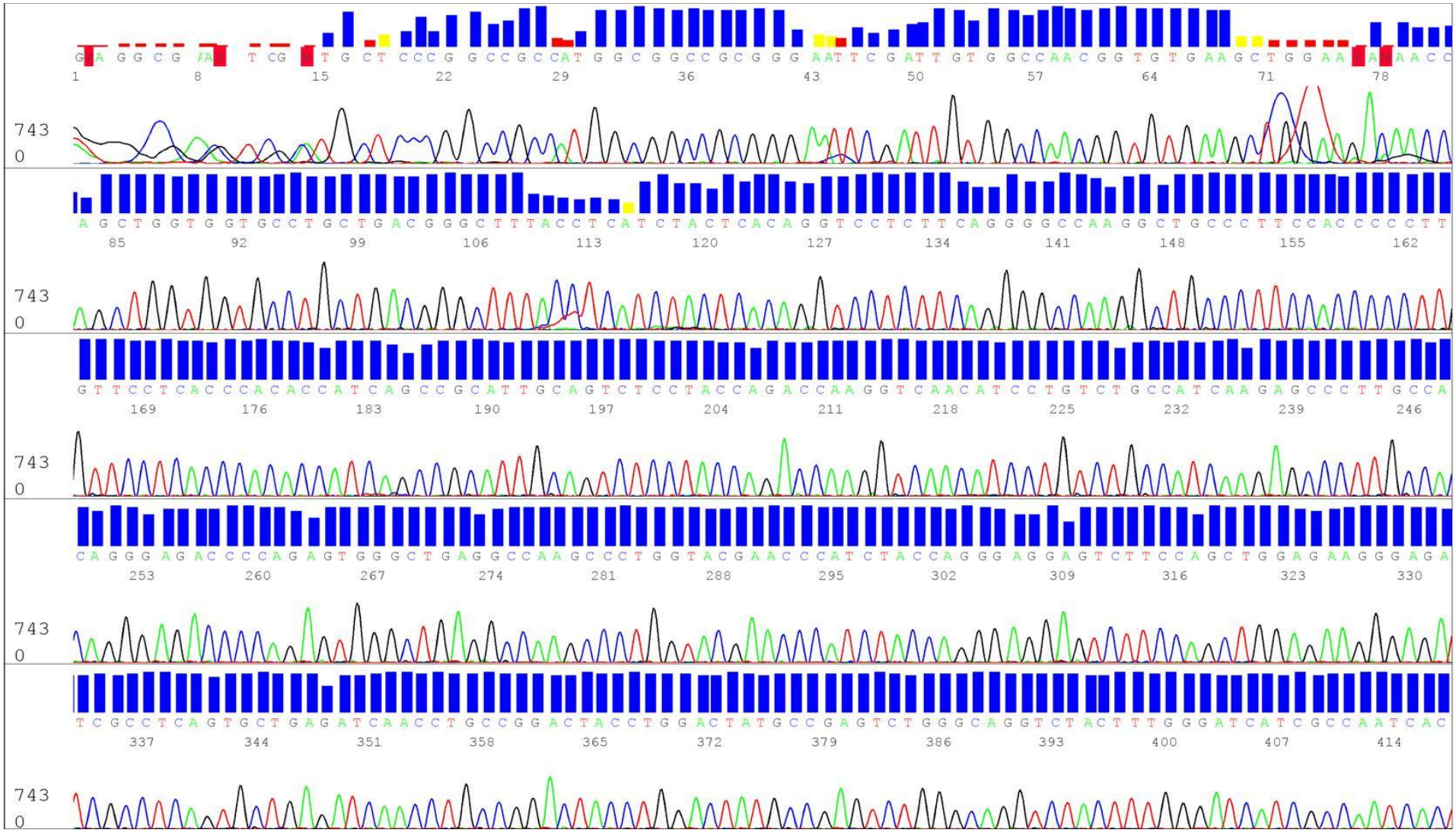
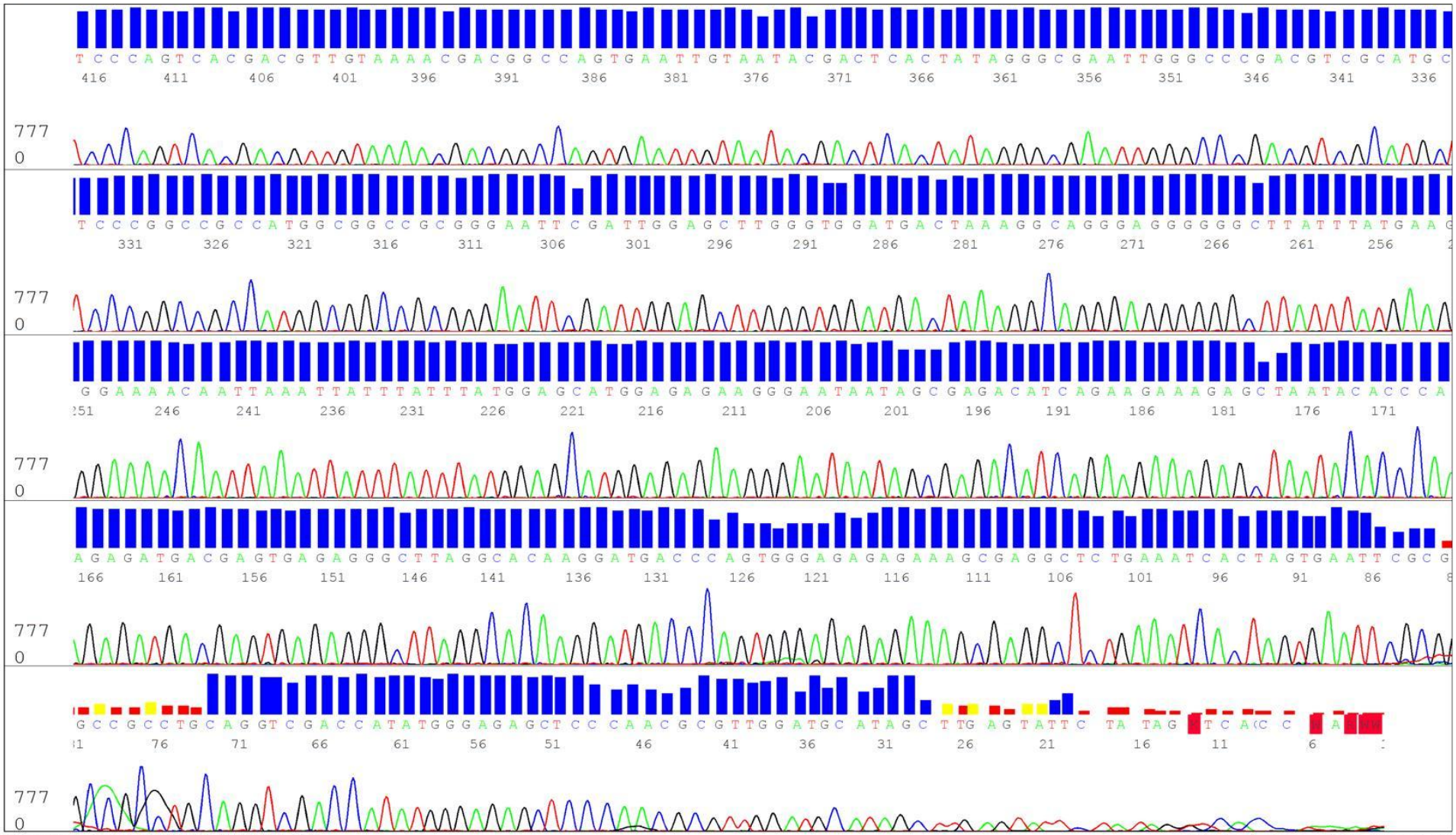


Plate 21 Bovine TNF-α clone PDFMD-Mahanadi



F-5’- GGA GCT TGG GTG GAT GAC TA-3’ R-5’-TCA GAG CCT CGC TTT CTC TC-3’

Plate 22 **Bovine TNF-αSF1 clone** PDFMD-Tungbhadra

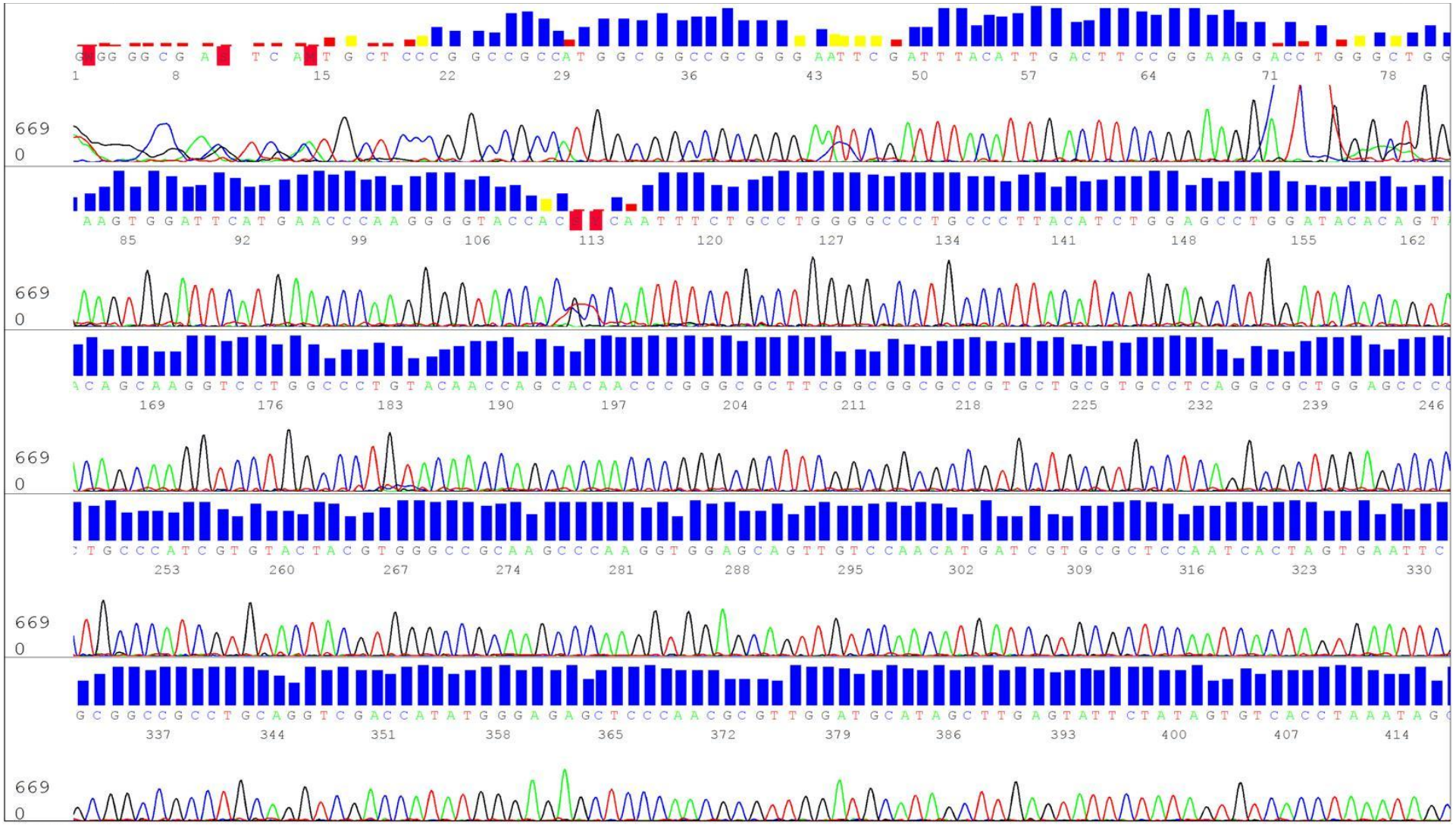
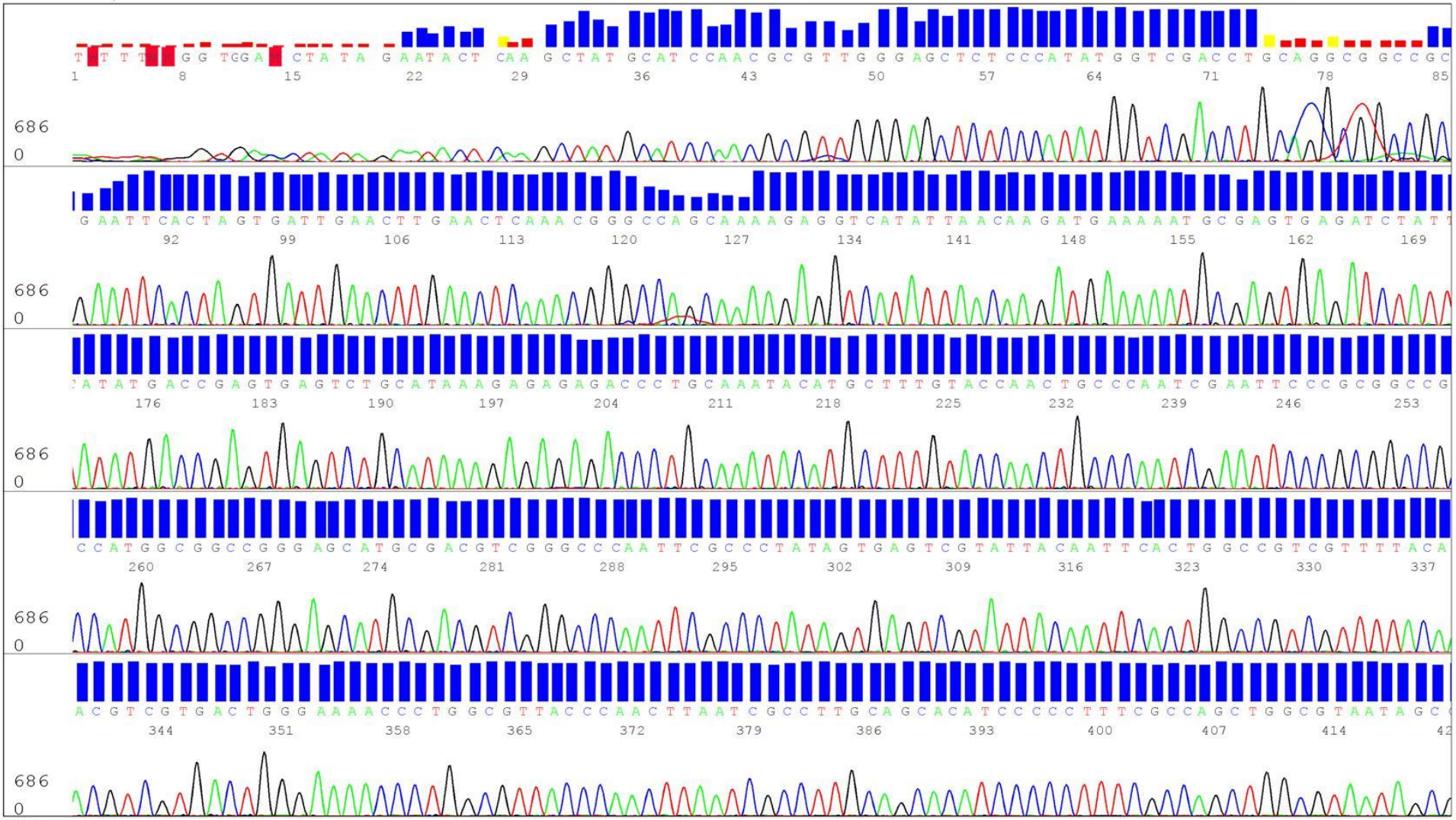


Plate 23 Bovine TGFβ-1 clone PDFMD-Kaveri

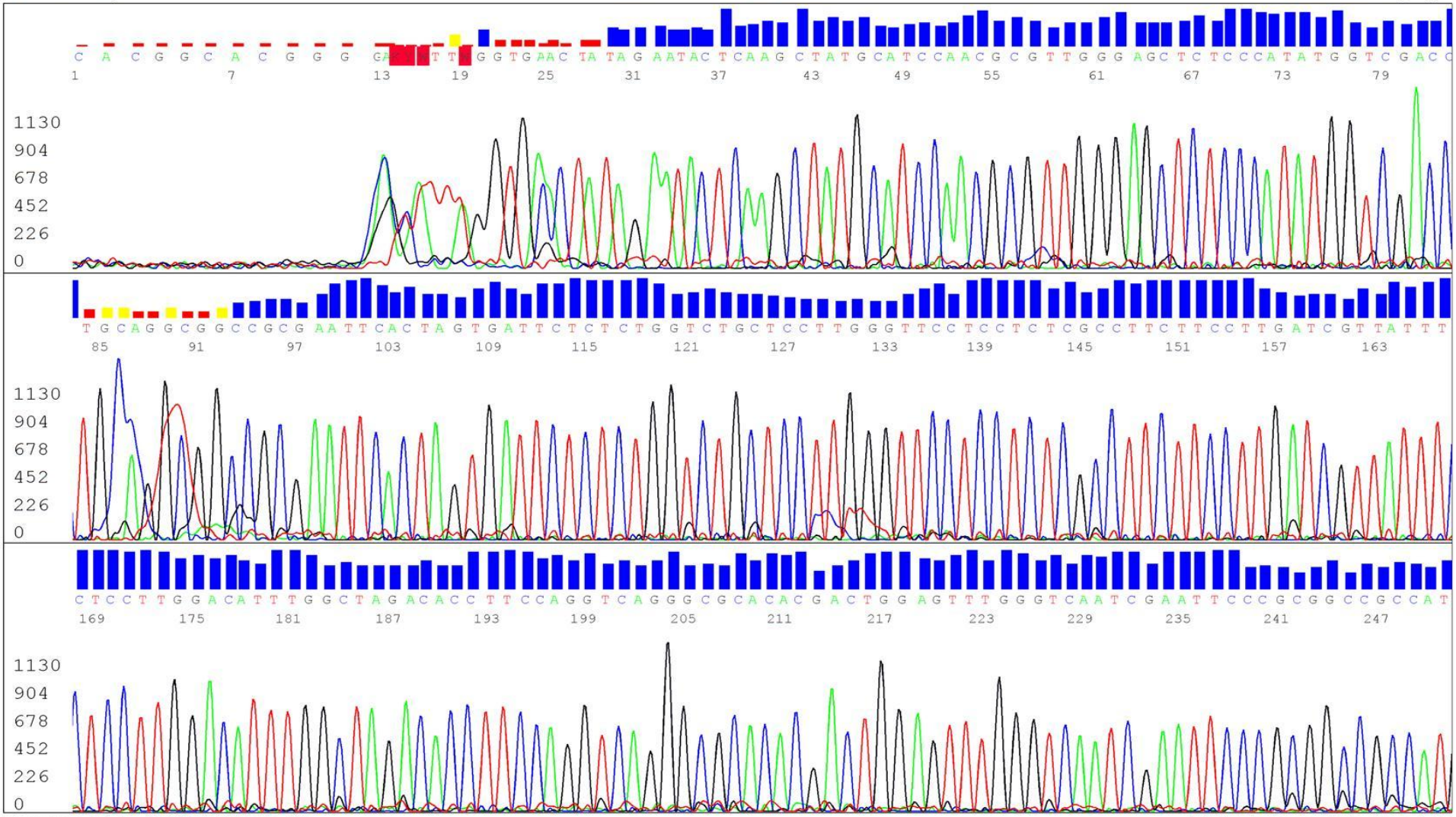


F-5’- GAA CTT GAA CTC AAA CGG GC-3’ R-5’-GGG CAG TTG GTA CAA AGC AT-3’

Plate 24

Bovine TGFβ-2 clone

PDFMD-Damodar

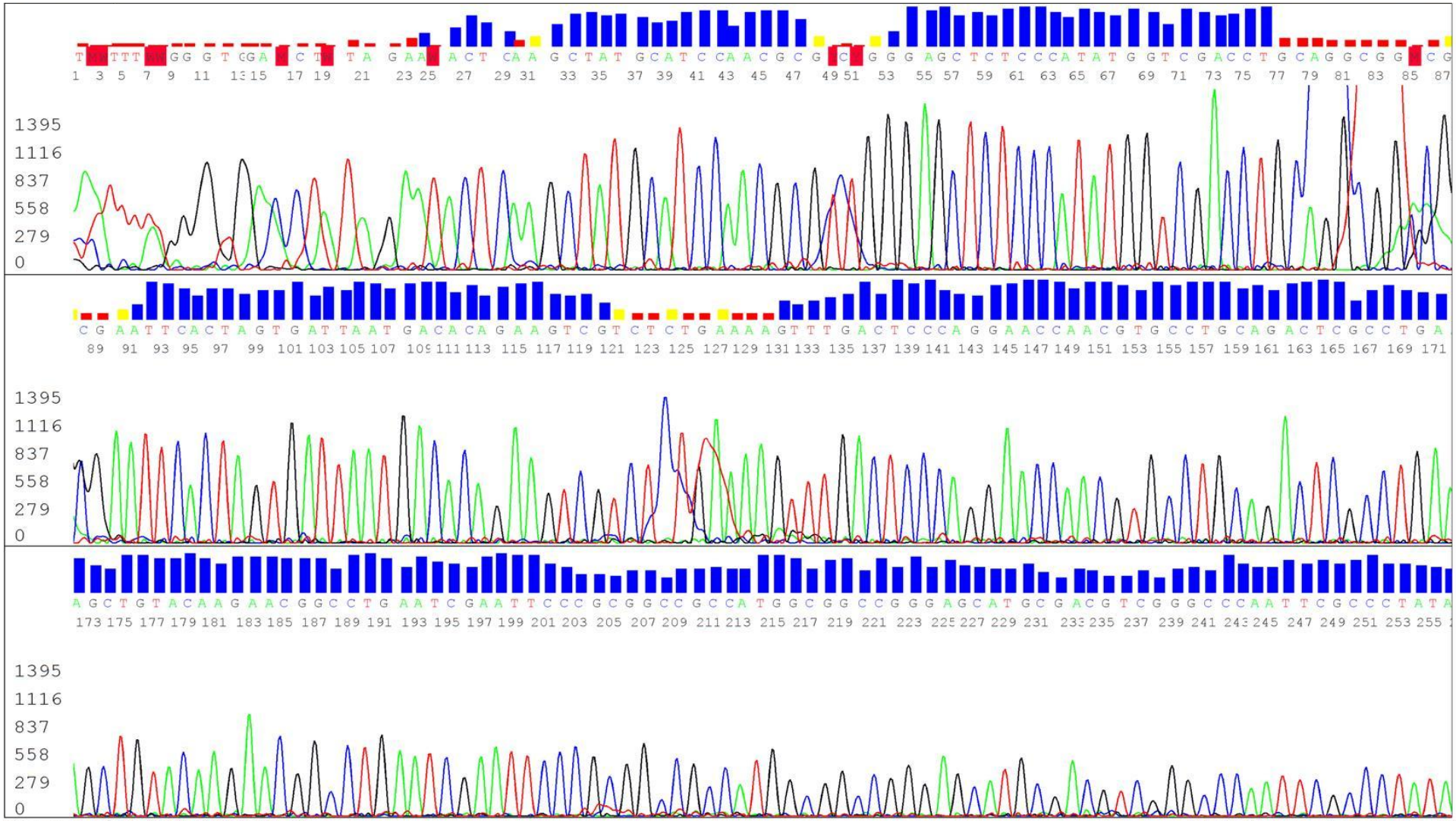


F-5’- CTC TCT GGT CTG CTC CTT GG-3’ R-5’-GAC CCA AAC TCC AGT CGT GT-3’

Plate 25

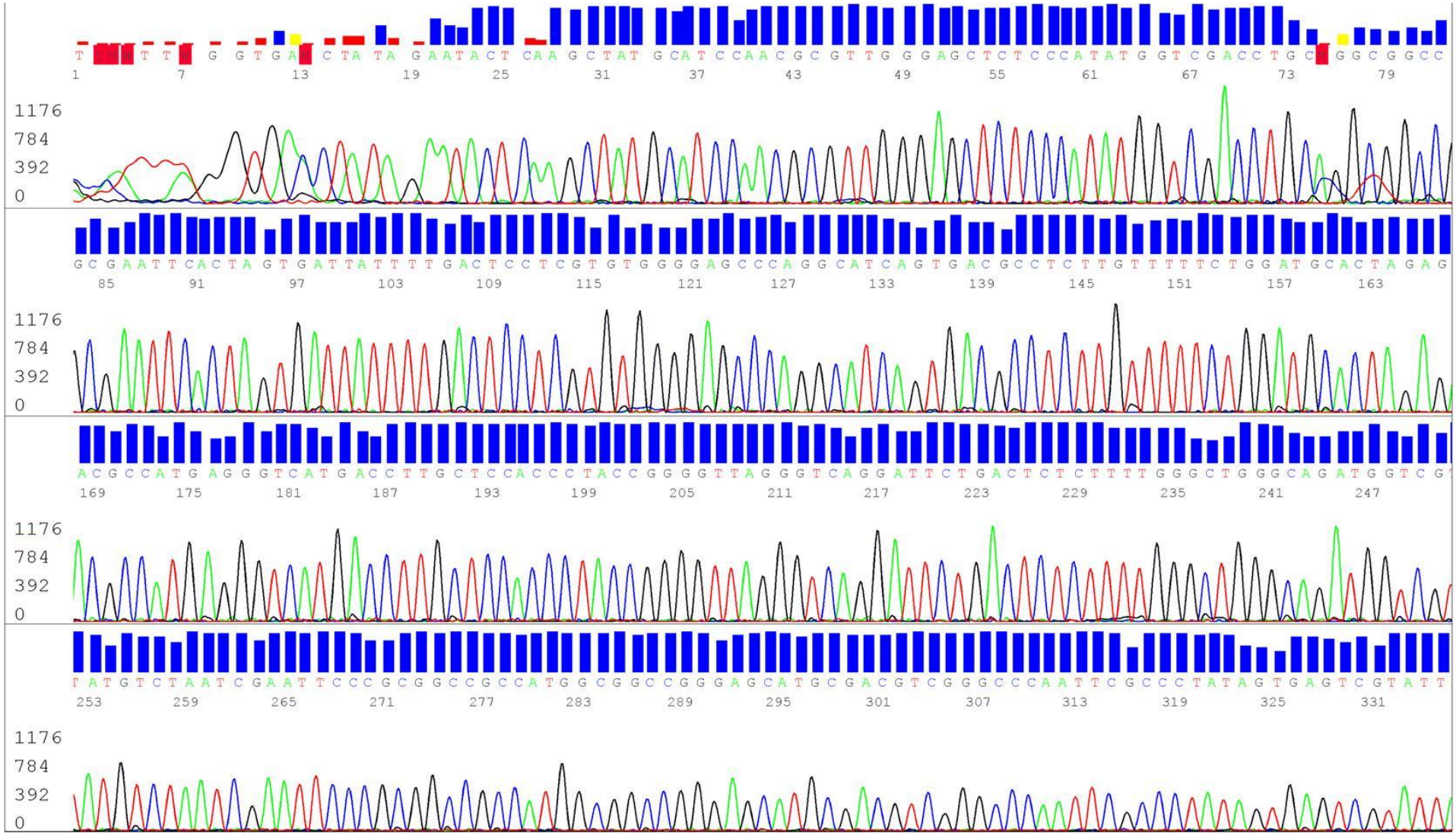
Bovine TGFβ-3 clone

PDFMD-Ghaghra



F-5’- AAT GAC ACA GAA GTC GTC TCT GAA A-3’ R-5’- CAG GCC GTT CTT GTA CAG CTT-3’

Plate 26 Bovine GM-CSF clone PDFMD-Wardha



F-5’-ATT TTG ACT CCT CGT GTG GG-3’ R-5’-AGA CAT ACG ACC ATC TGC CC-3’

Plate 27 Bovine CSF clone PDFMD-Penneru

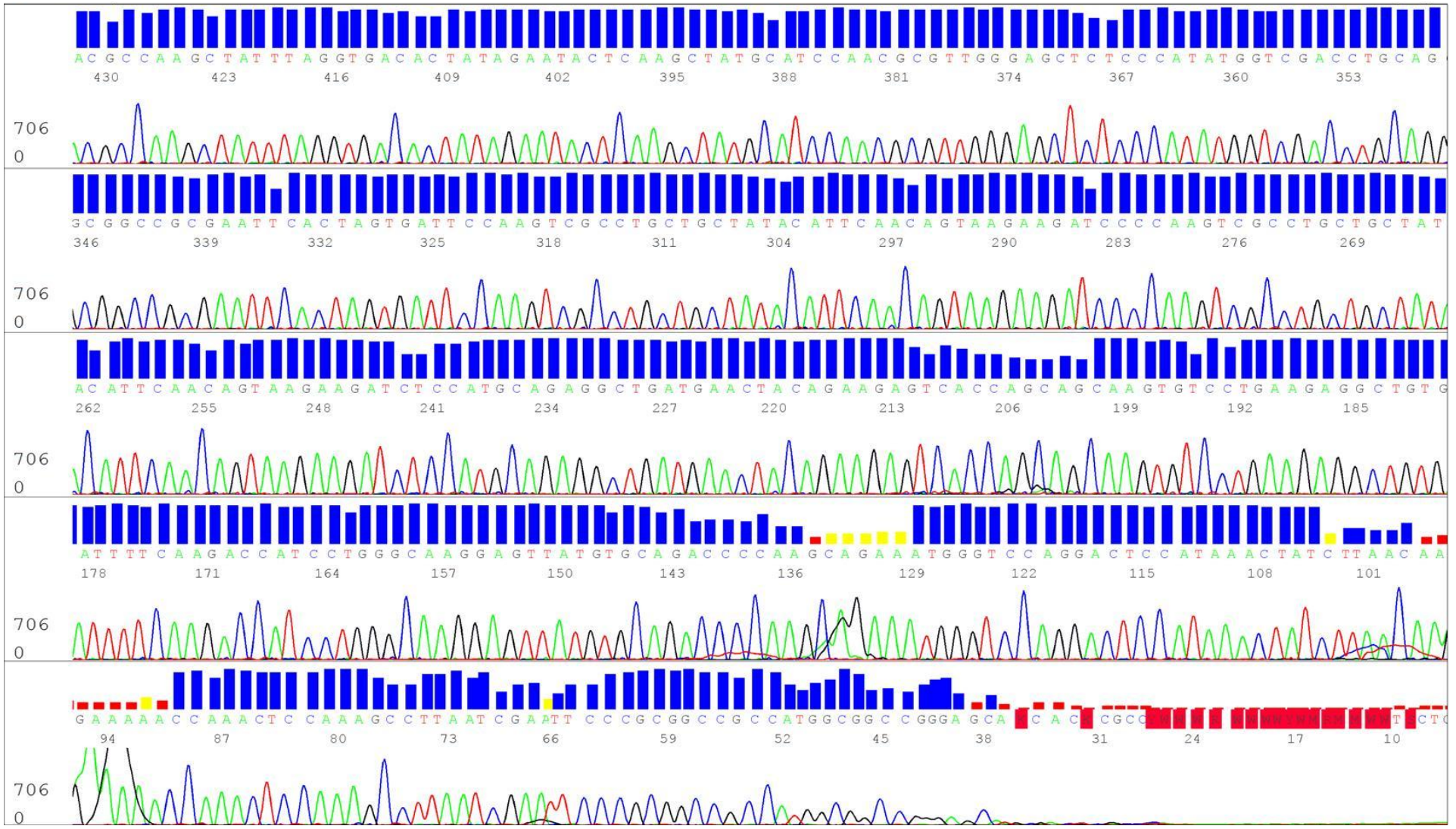
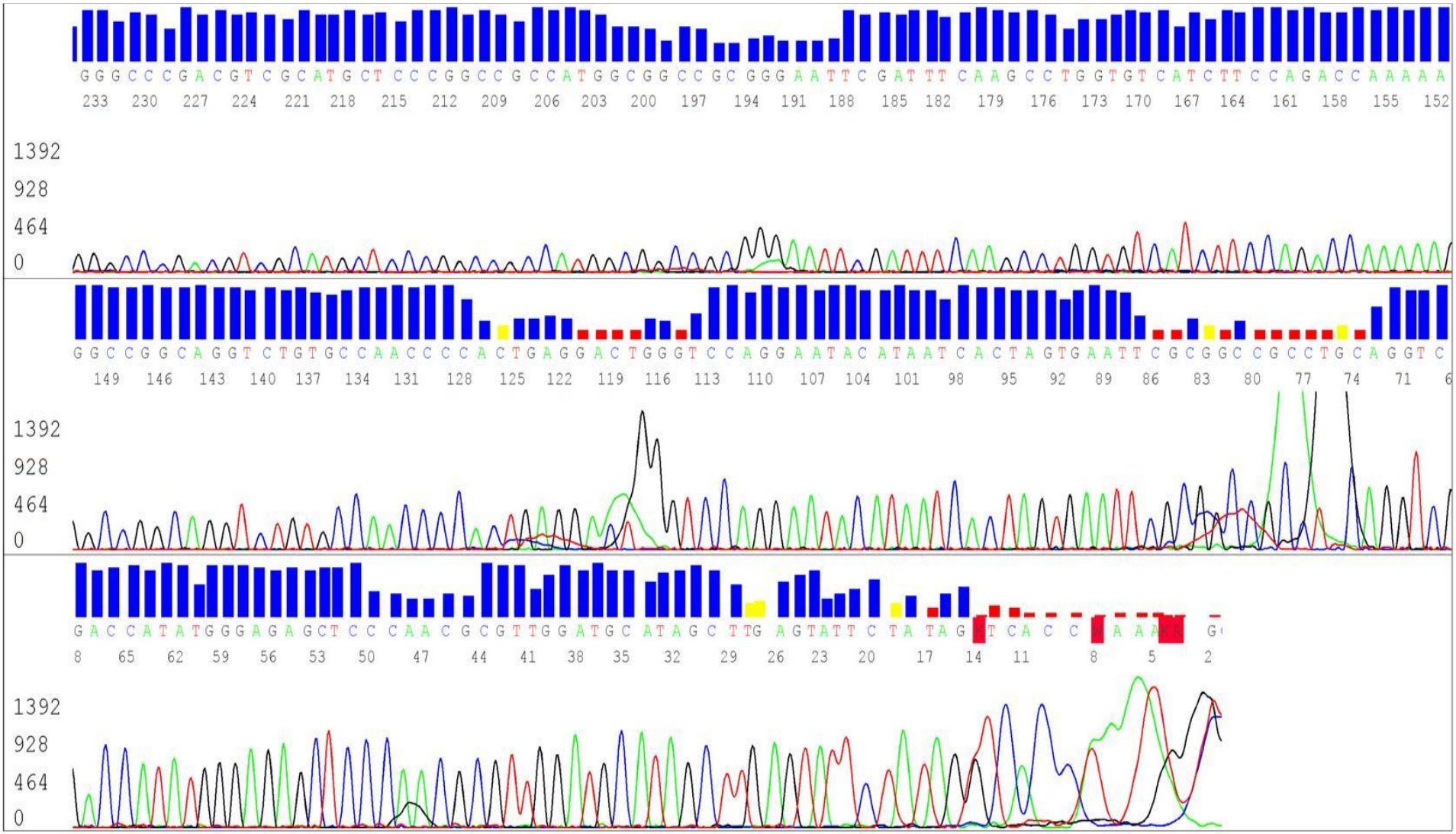
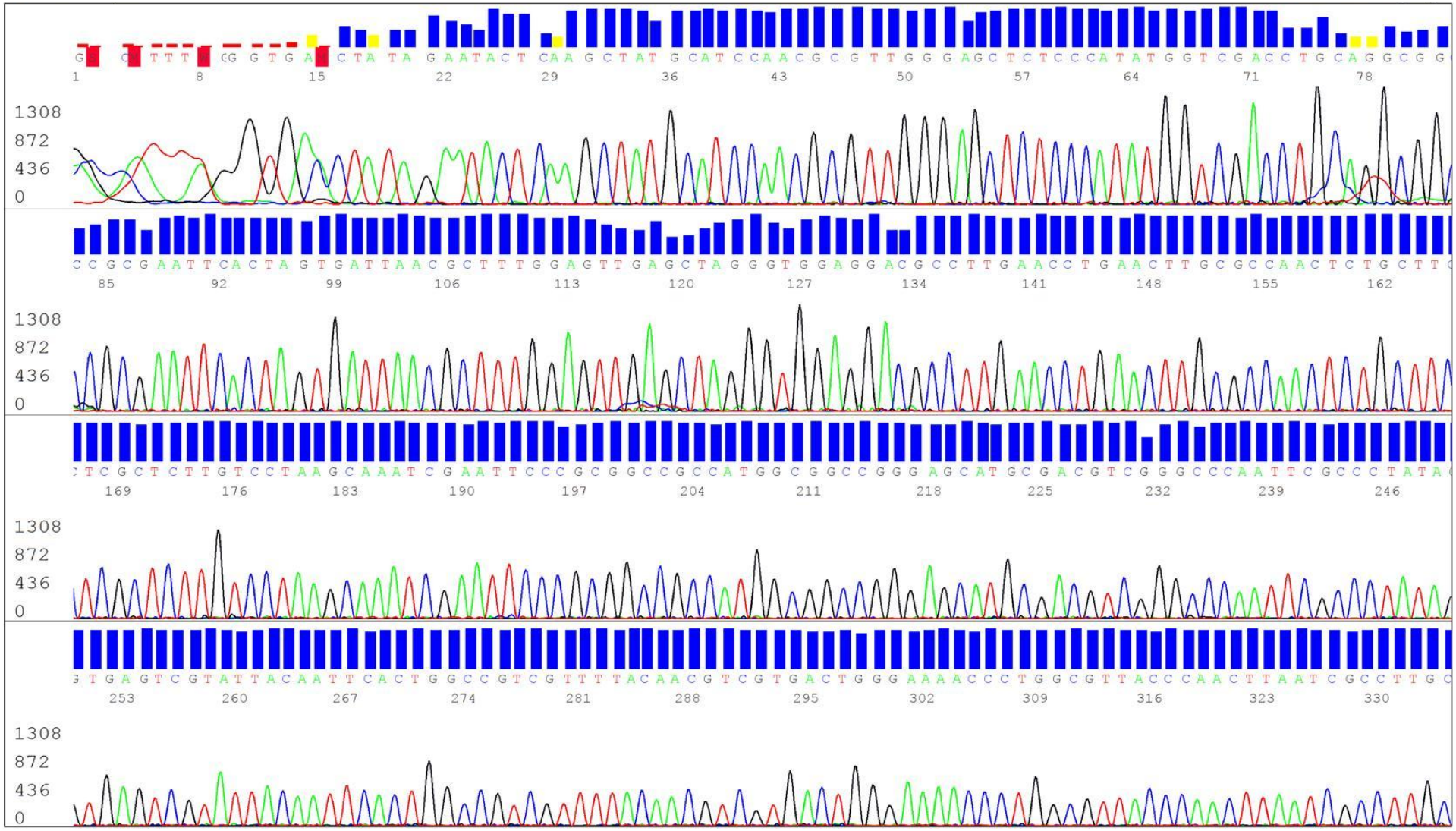


Plate 28 Bovine MCP-1 clone PDFMD-Hooghly



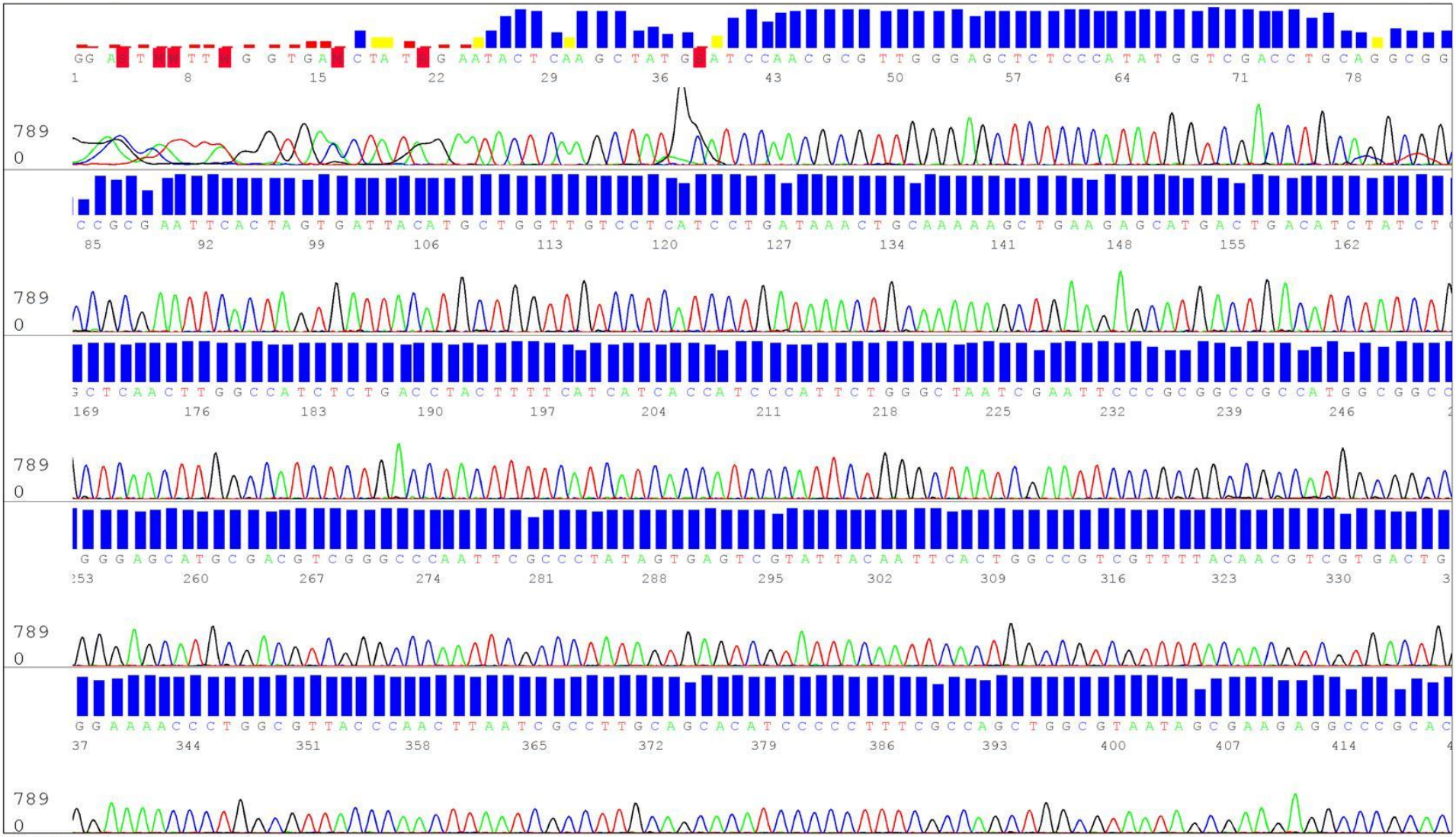
F-5’-TCA AGC CTG GTG TCA TCT TCC AGA-3’ R-5’-ATG TAT TCC CTG GAC CCA GTC CTC A-3’

Plate 29 Bovine CCL3 clone PDFMD-Jhelum



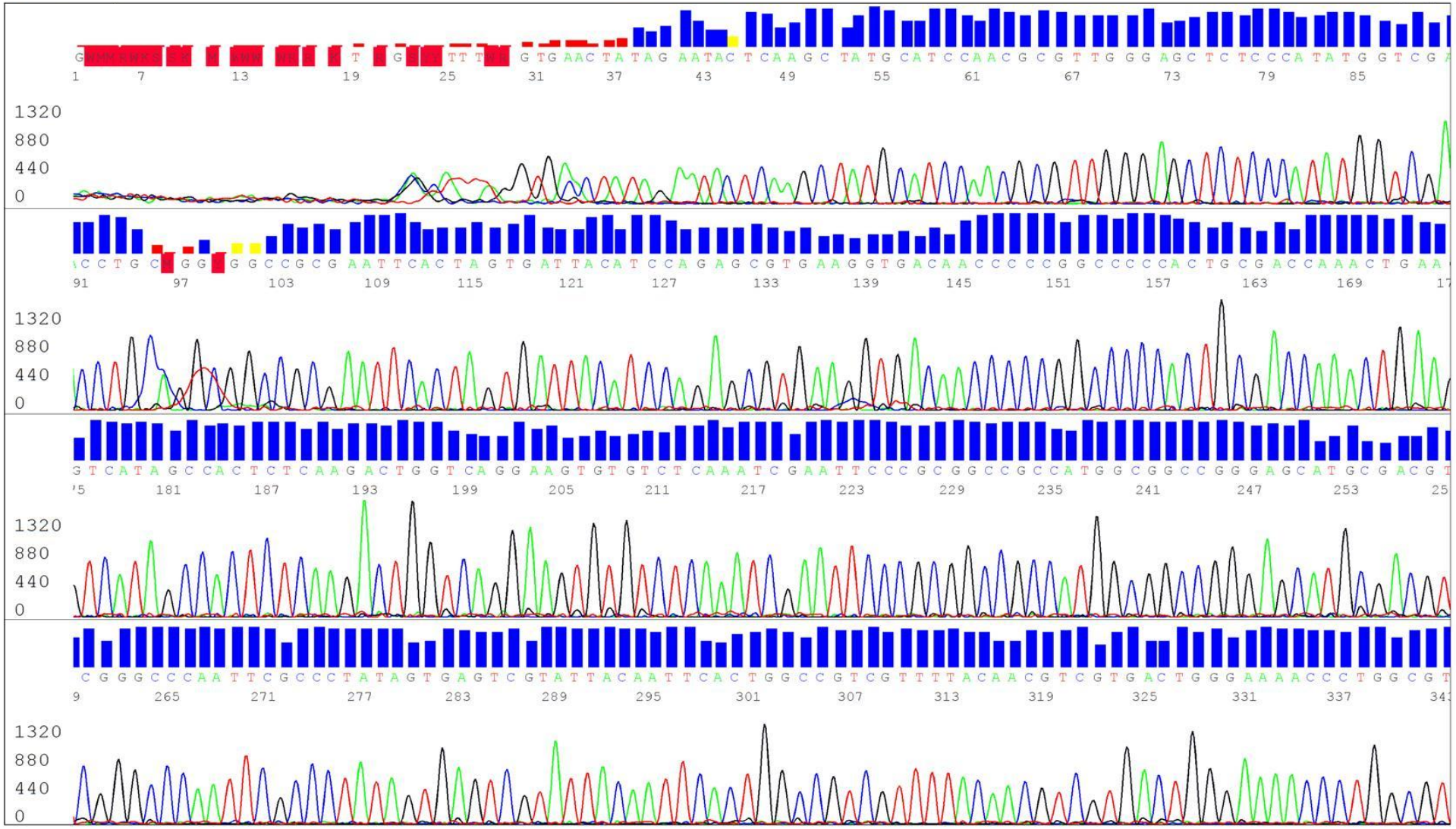
F-5’-AAC GCT TTG GAG TTG AGC TAG GGT-3’ R-5’-TGC TTA GGA CAA GAG CGA GAA GCA-3’

Plate 30 Bovine CCL5 clone PDFMD-Sharda



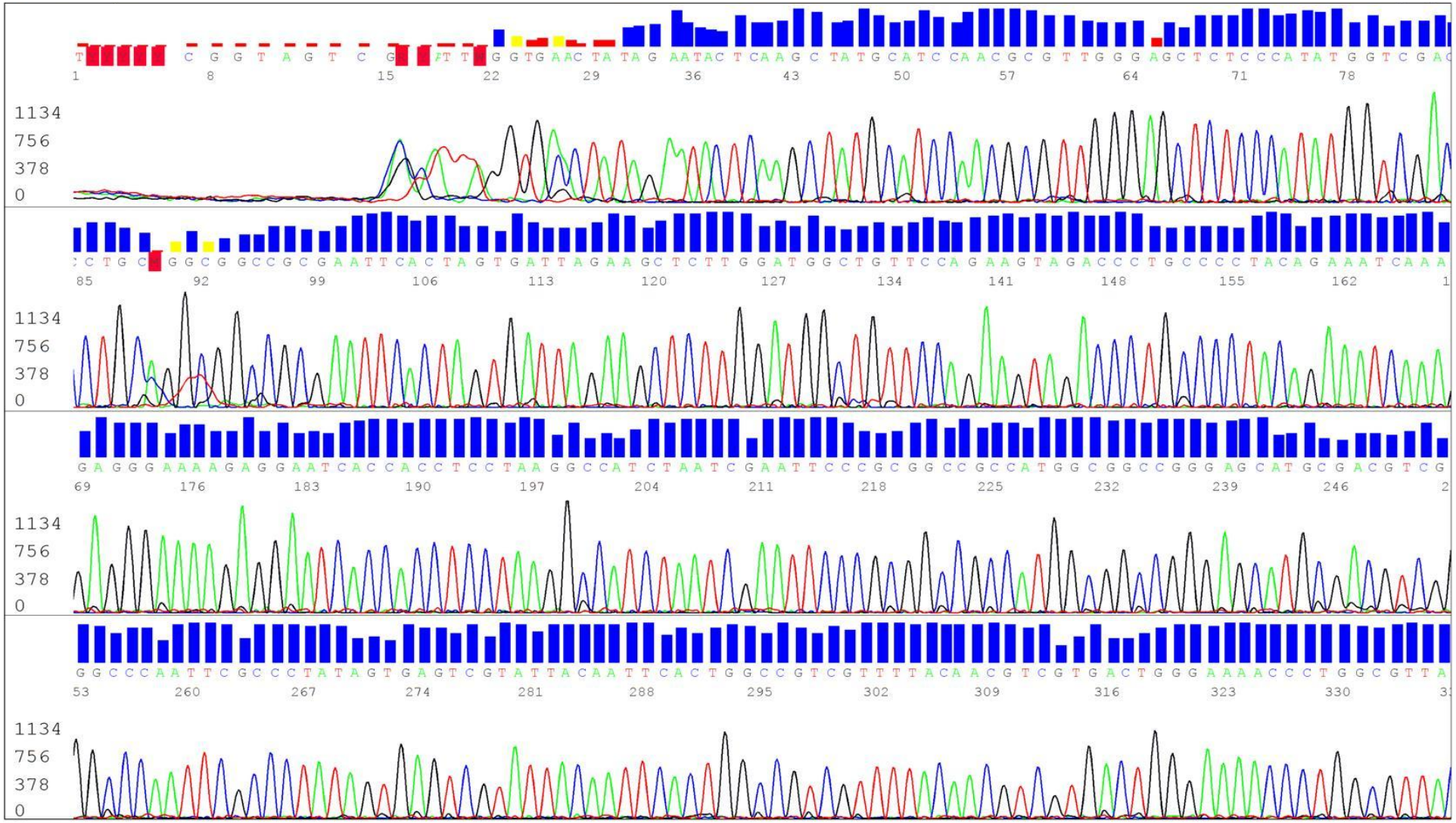
F-5’-ACA TGC TGG TTG TCC TCA TCC TGA-3’ R-5’-AGC CCA GAA TGG GAT GGT GAT GAT-3’

Plate 31 Bovine CCR5 clone PDFMD-Manjari



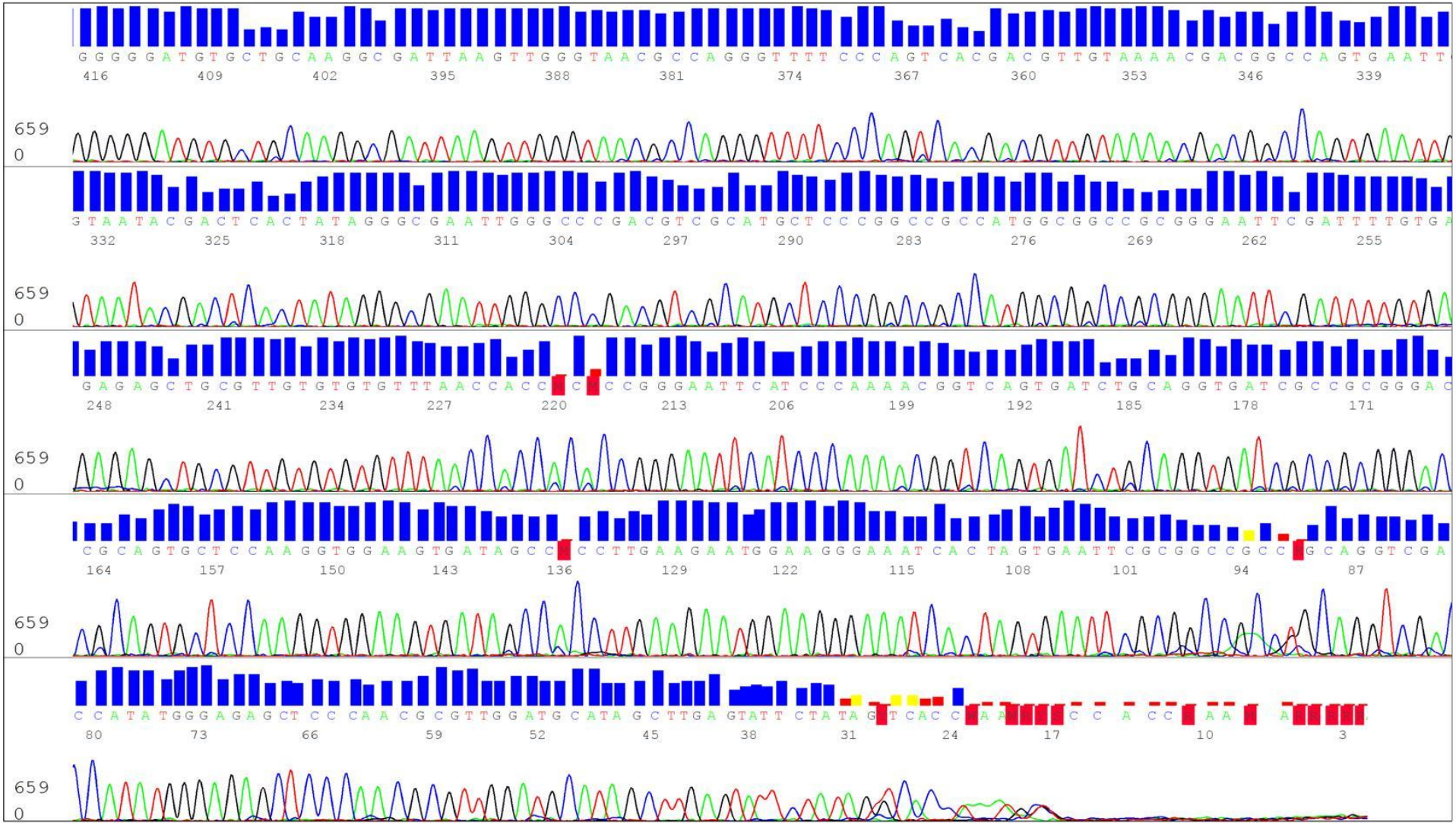
F-5’-ACA TCC AGA GCG TGA AGG TGA CAA-3’ R-5’-TGA GAC ACA CTT CCT GAC CAG TCT-3’

Plate 32 Bovine CXCL1 clone PDFMD-Sabari



F-5’-AGA AGC TCT TGG ATG GCT GTT CCA-3’ R-5’-AGA TGG CCT TAG GAG GTG GTG ATT-3’

Plate 33 **Bovine CXCL2 clone** PDFMD-Ghaggar

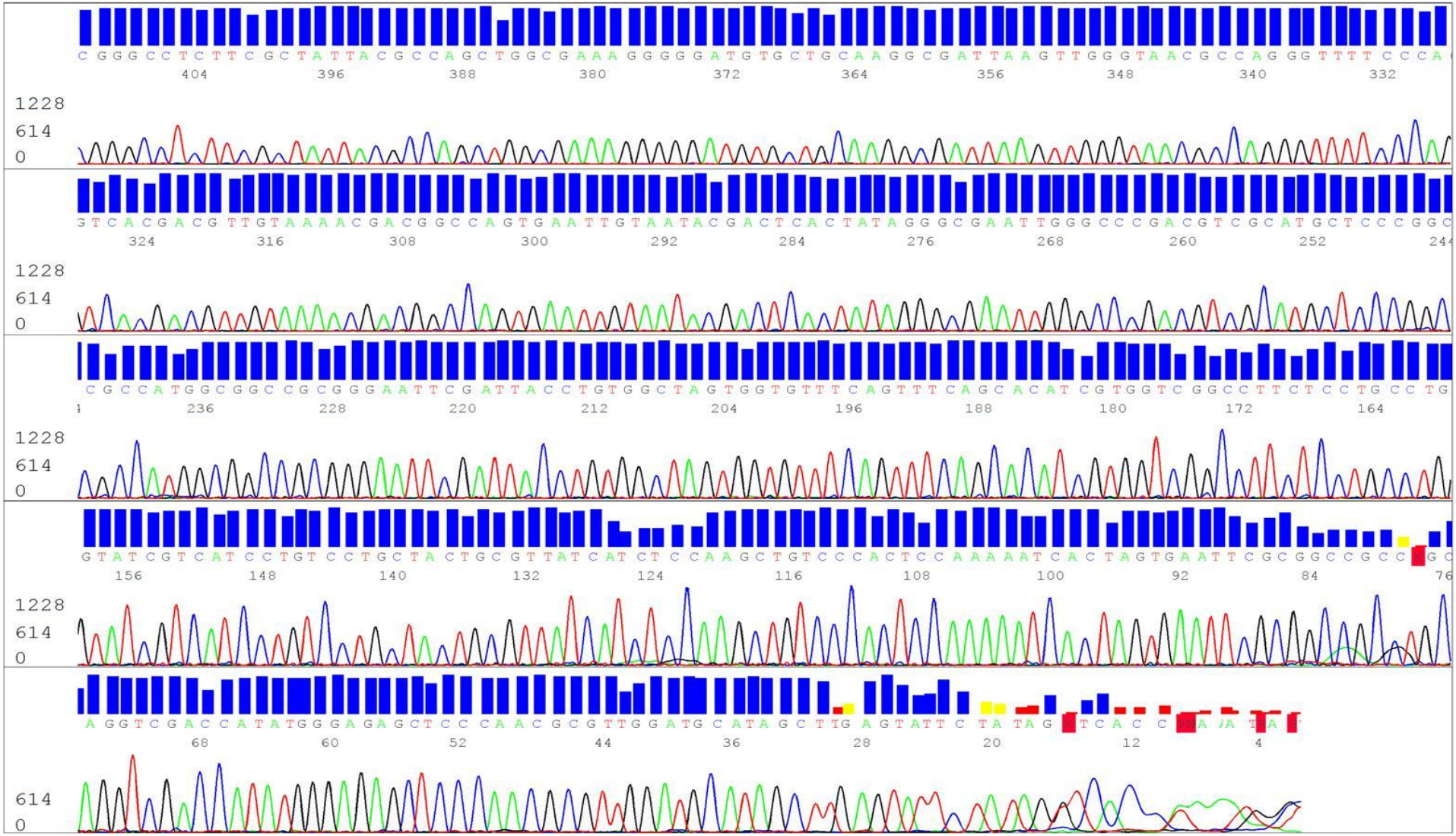


F-5’-TTG TGA GAG AGC TGC GTT GTG TGT-3’ R-5’-TCC CTT CCA TTC TTC AAG GTG GCT-3’

Plate 34

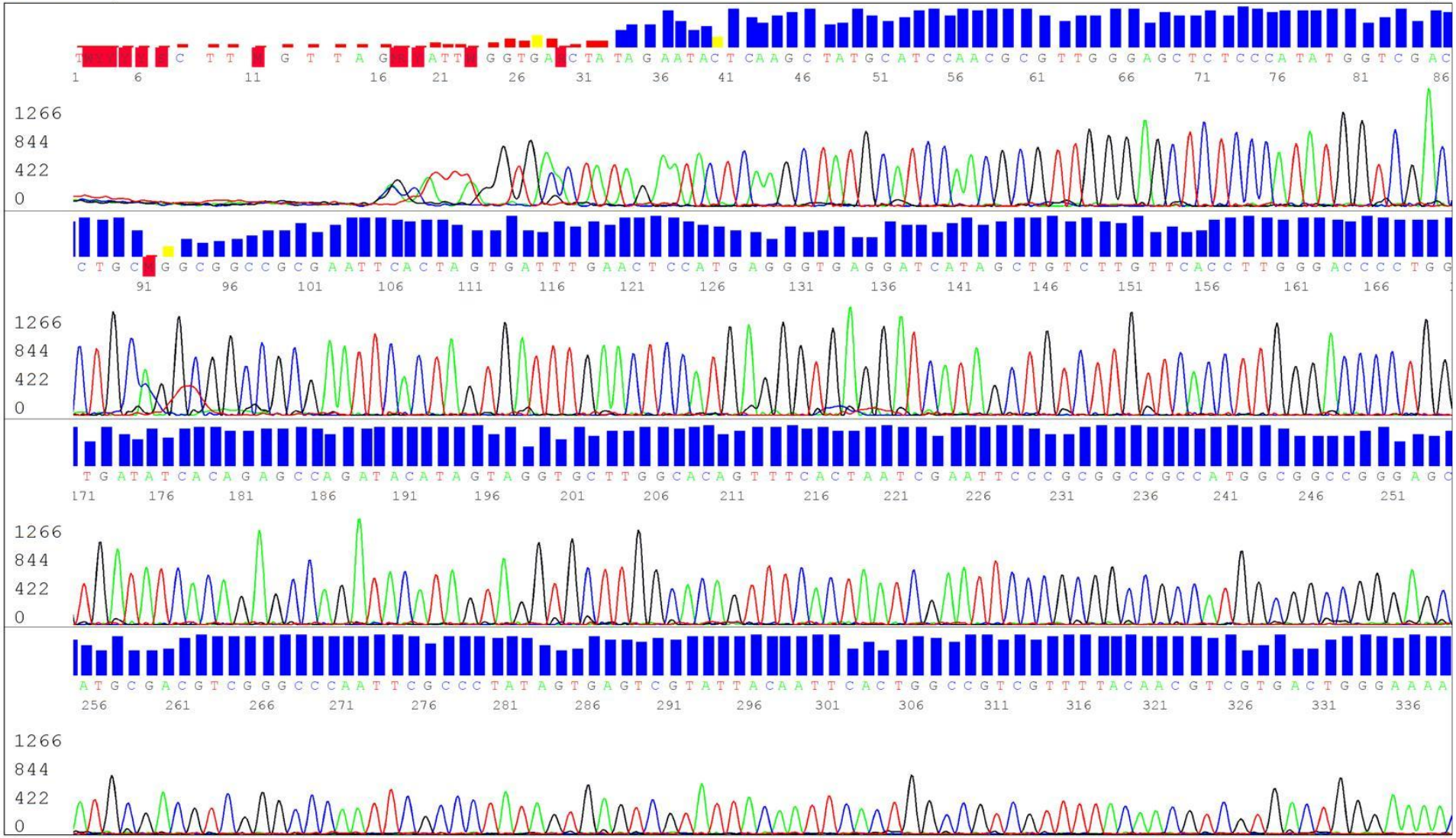
Bovine CXCL6 clone

PDFMD-Gandak



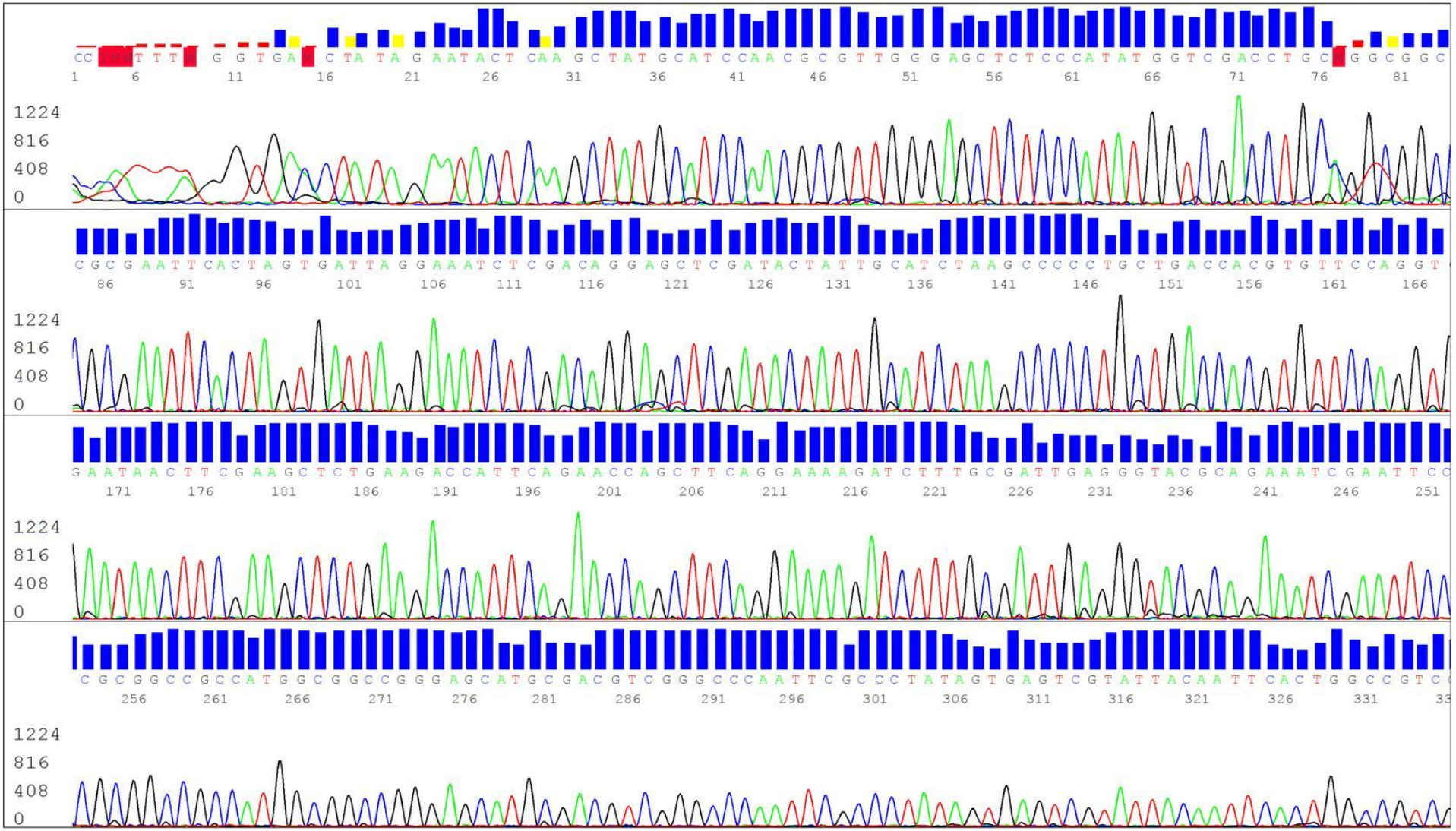
F-5’-ACC TGT GGC TAG TGG TGT TTC AGT-3’ R-5’-TTT GGA GTG GGA CAG CTT GGA GAT-3’

Plate 35 Bovine CXCR4 clone PDFMD-Wainganga



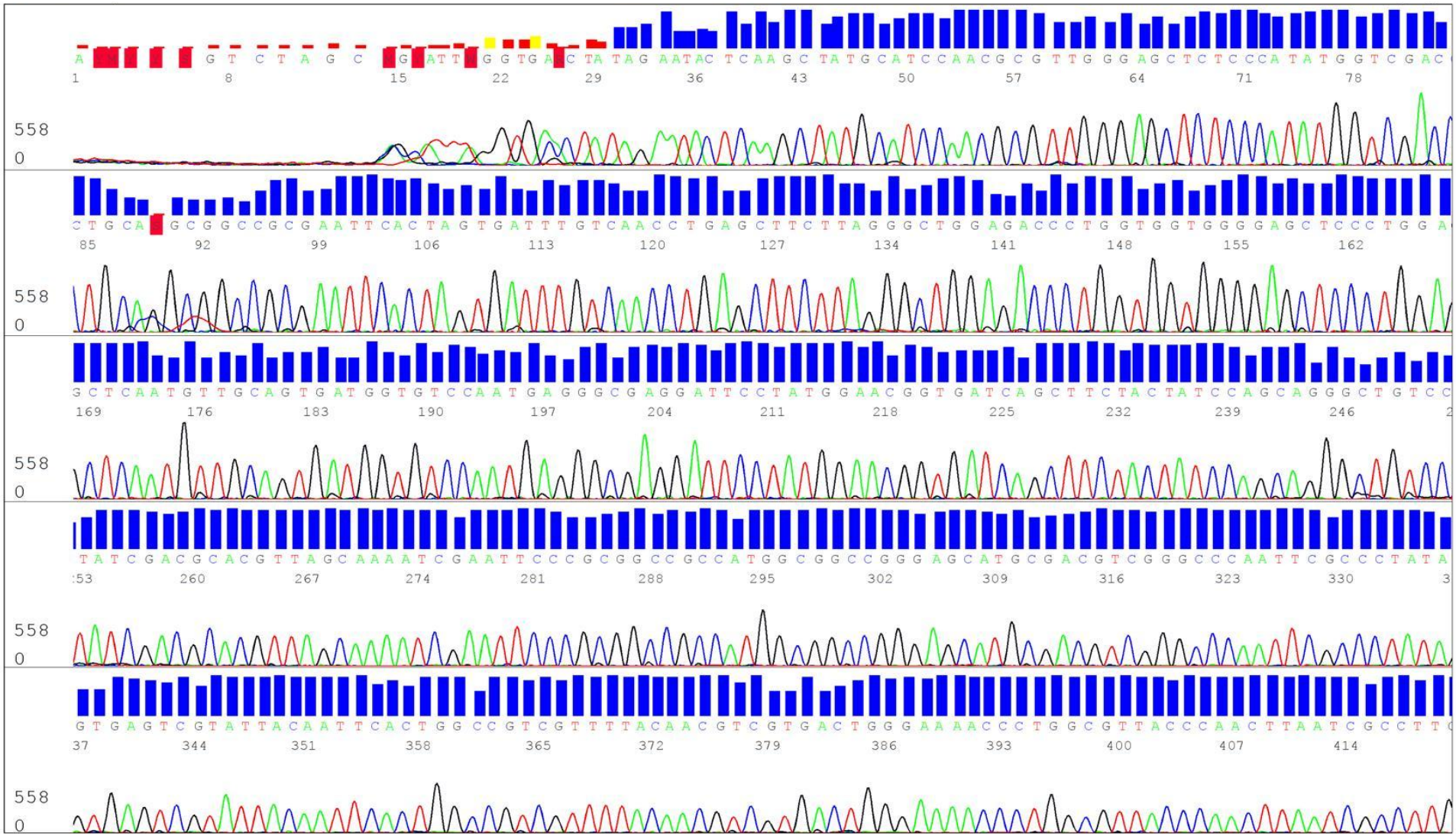
F-5’-TGA ACT CCA TGA GGG TGA GGA TCA-3’ R-5’-AGT GAA ACT GTG CCA AGC ACC TAC-3’

Plate 36 Bovine CD11a clone PDFMD-Chitravati



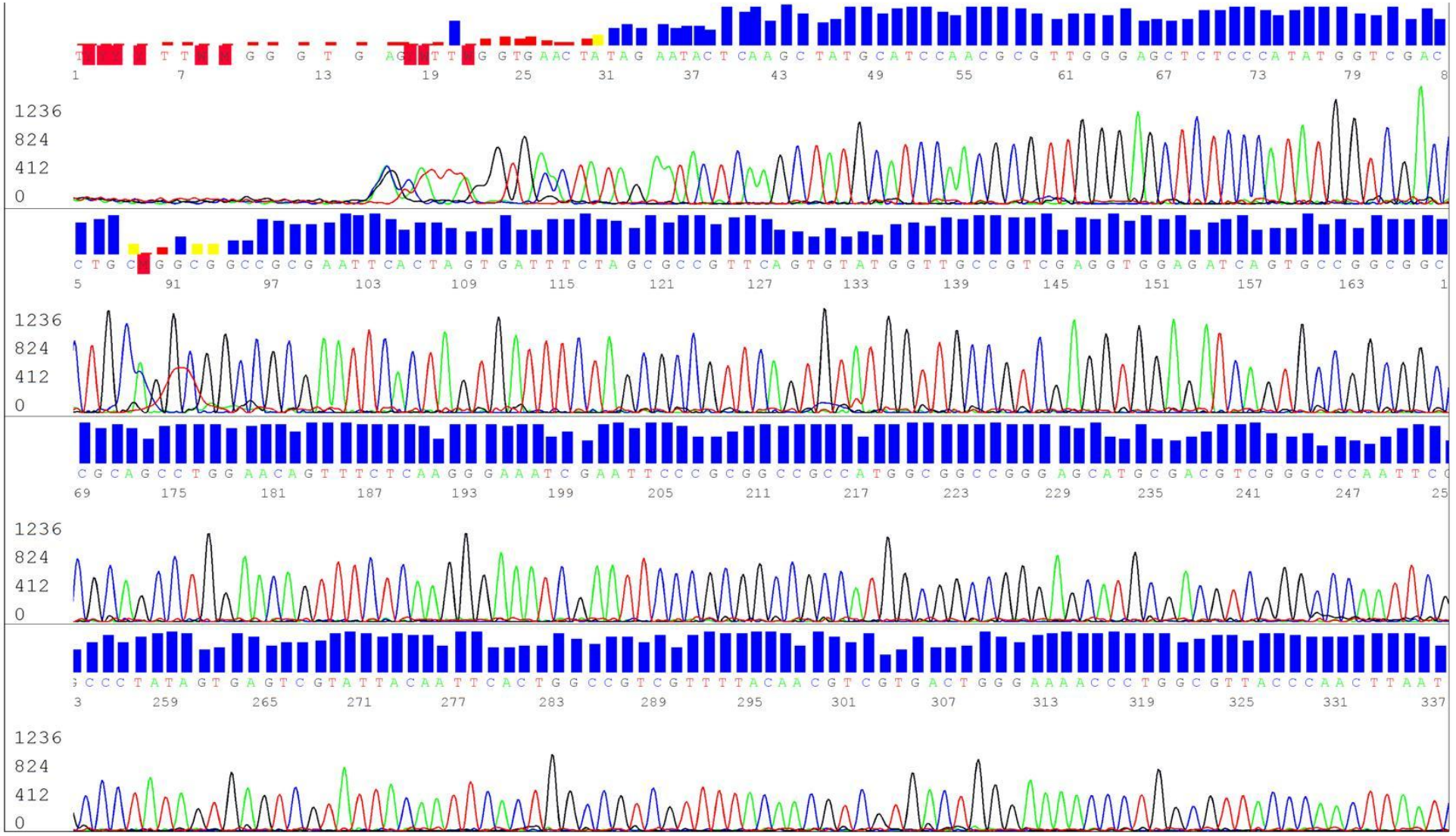
F-5’-AGG AAA TCT CGA CAG GAG CTC GAT-3’ R-5’-TCT GCG TAC CCT CAA TCG CAA AGA-3’

Plate 37 **Bovine CD11b clone** PDFMD-Vaigai



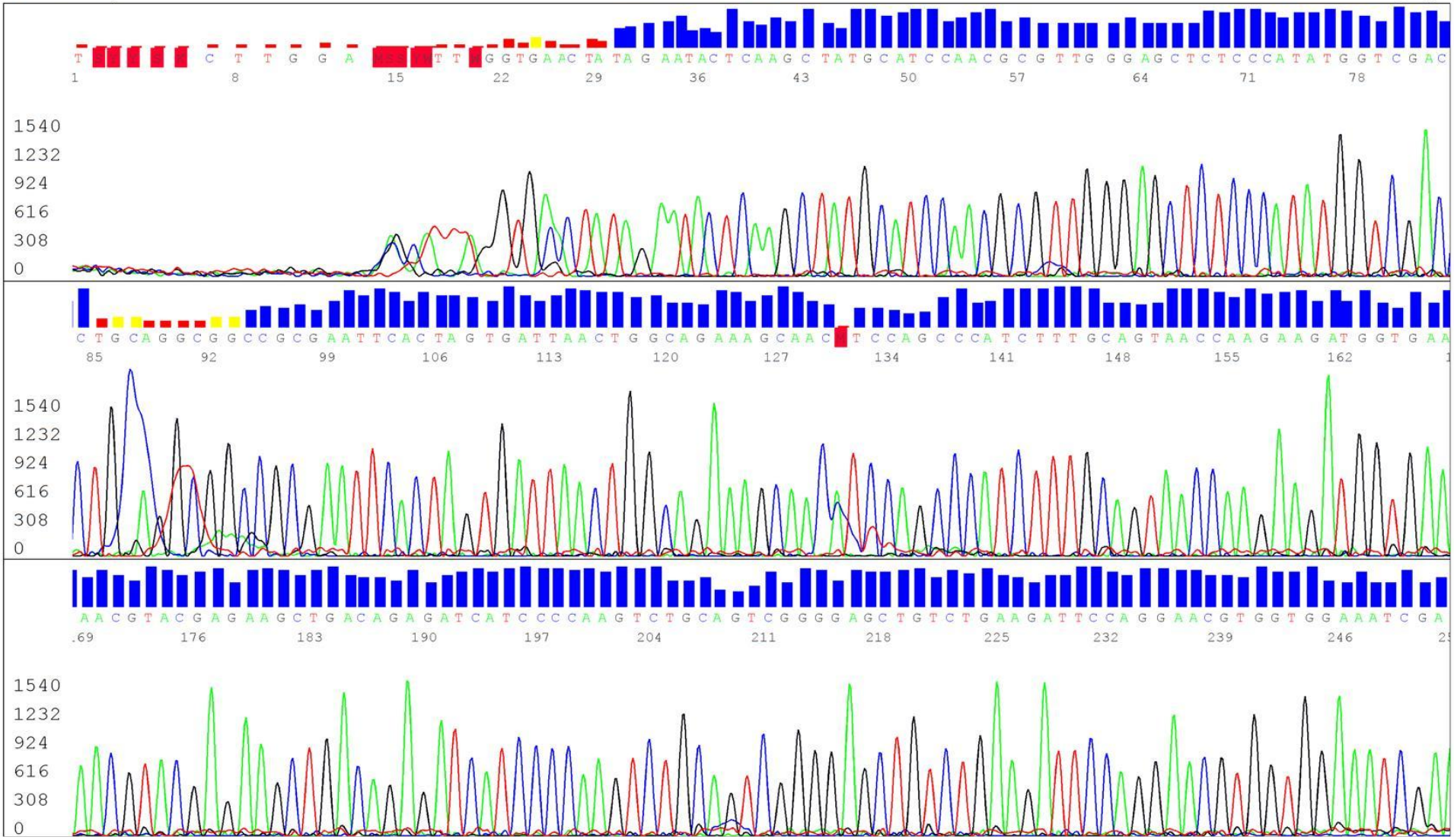
F-5’-TGT CAA CCT GAG CTT CTT AGG GCT-3’ R-5’-TTG CTA ACG TGC GTC GAT AGG ACA-3’

Plate 38 Bovine CD11c clone PDFMD-Kollidam



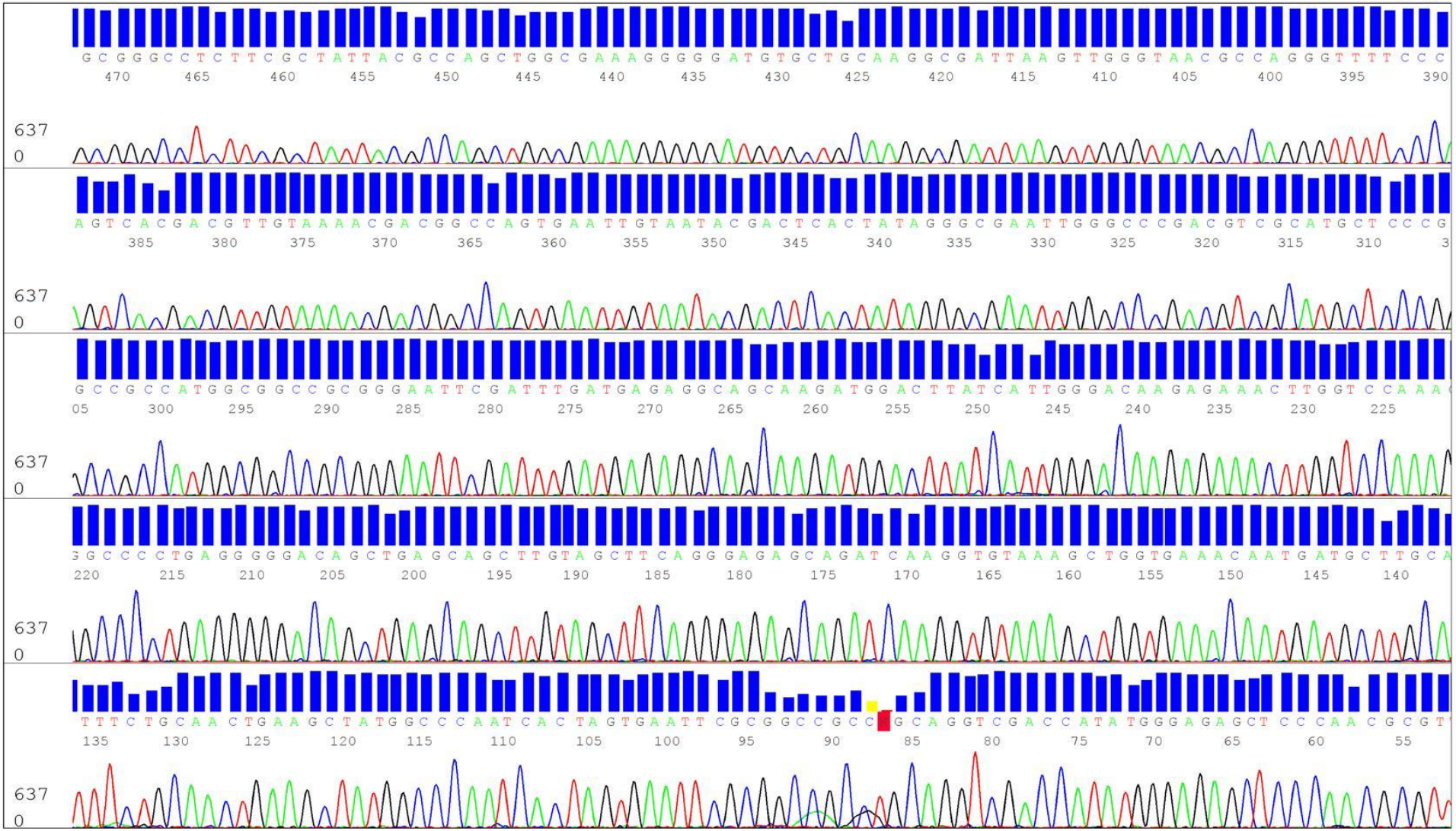
F-5’-TCT AGC GCC GTT CAG TGT ATG GTT-3’ R-5’-TCC CTT GAG AAA CTG TTC CAG GCT-3’

Plate 39 Bovine CD14 clone PDFMD-Manas



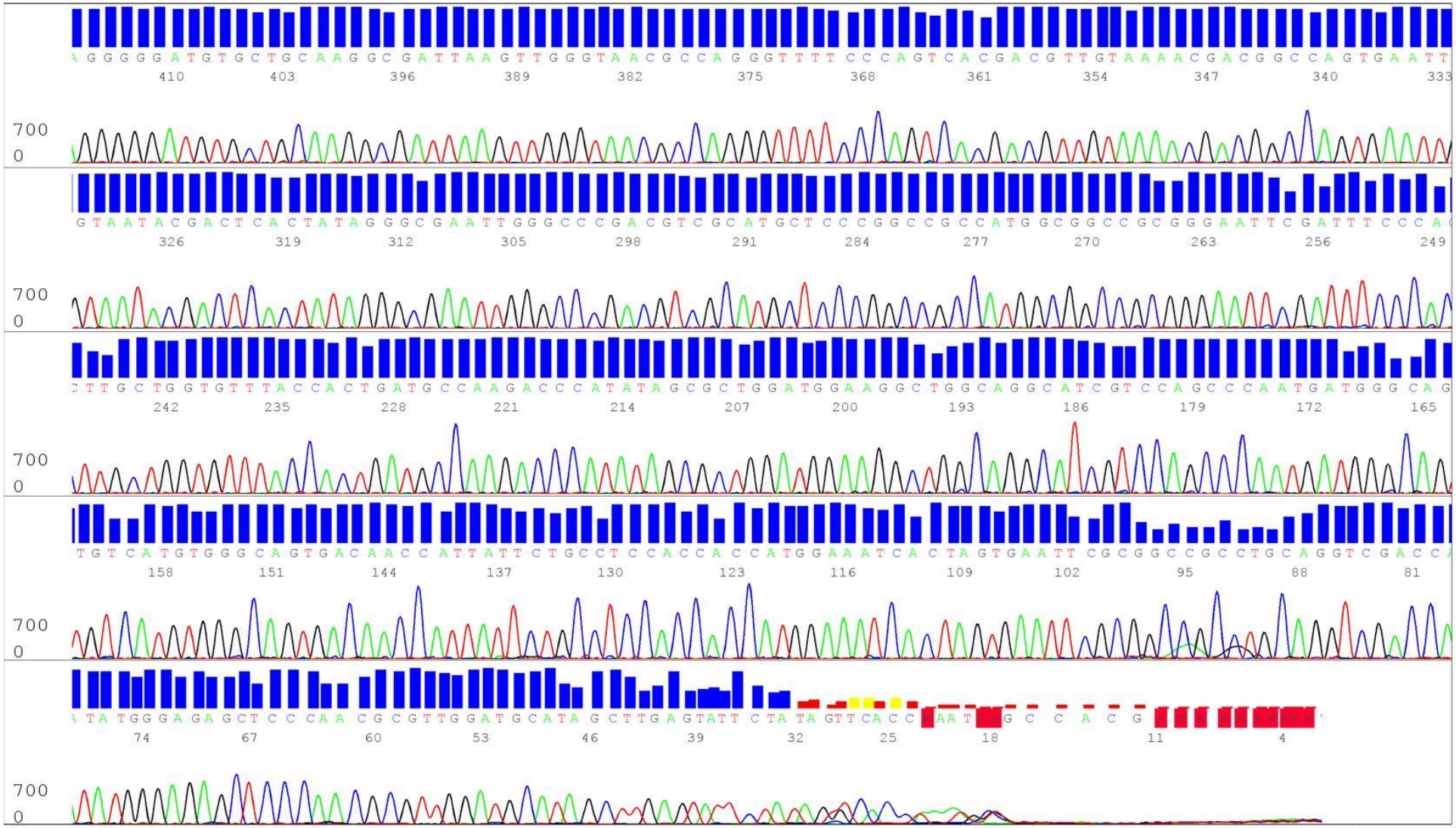
F-5’-AAC TGG CAG AAA GCA ACA TCC AGC-3’ R-5’-TCC ACC ACG TTC CTG GAA TCT TCA-3’

Plate 40 Bovine CD18 clone PDFMD-Warna



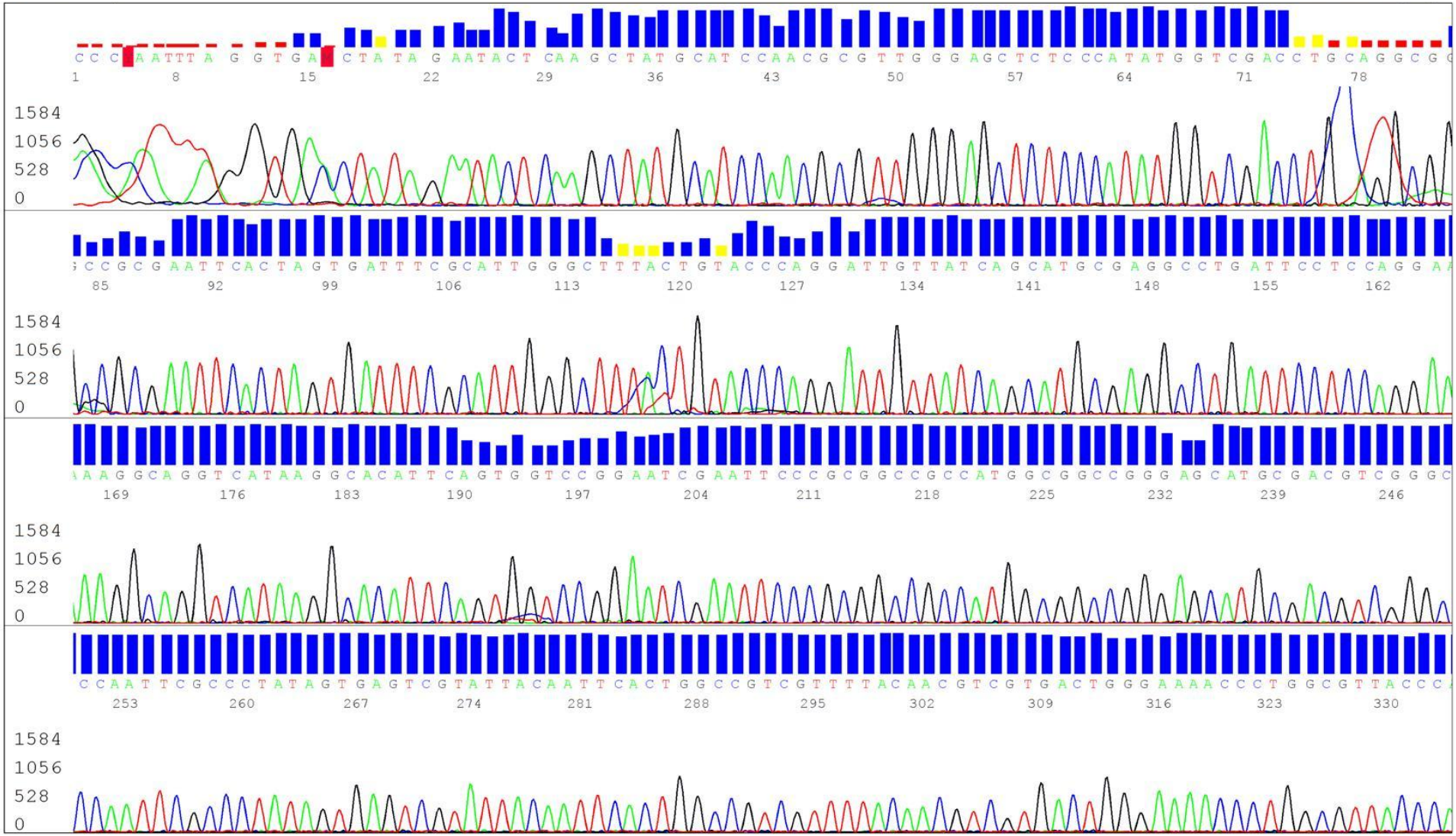
F-5’-TGA TGA GAG GCA GCA AGA TGG ACT-3’ R-5’-GGG CCA TAG CTT CAG TTG CAG AAA-3’

Plate 41 Bovine CD68 clone PDFMD-Girna



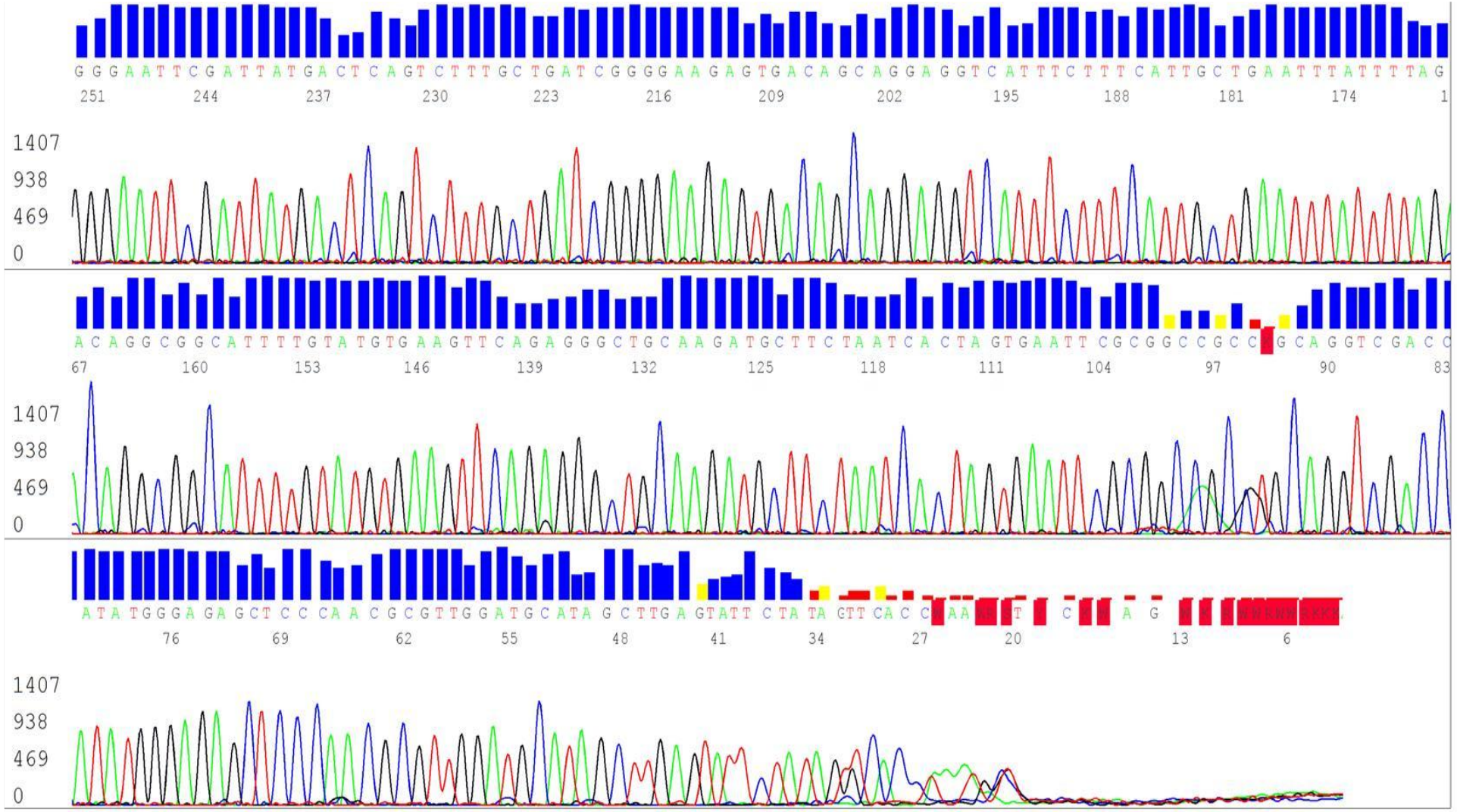
F-5’-TCC CAC TTG CTG GTG TTT ACC ACT-3’ R-5’- TCC ATG GTG GTG GAG GCA GAA TAA-3’

Plate 42 Bovine Integrin β3 clone PDFMD-Palar



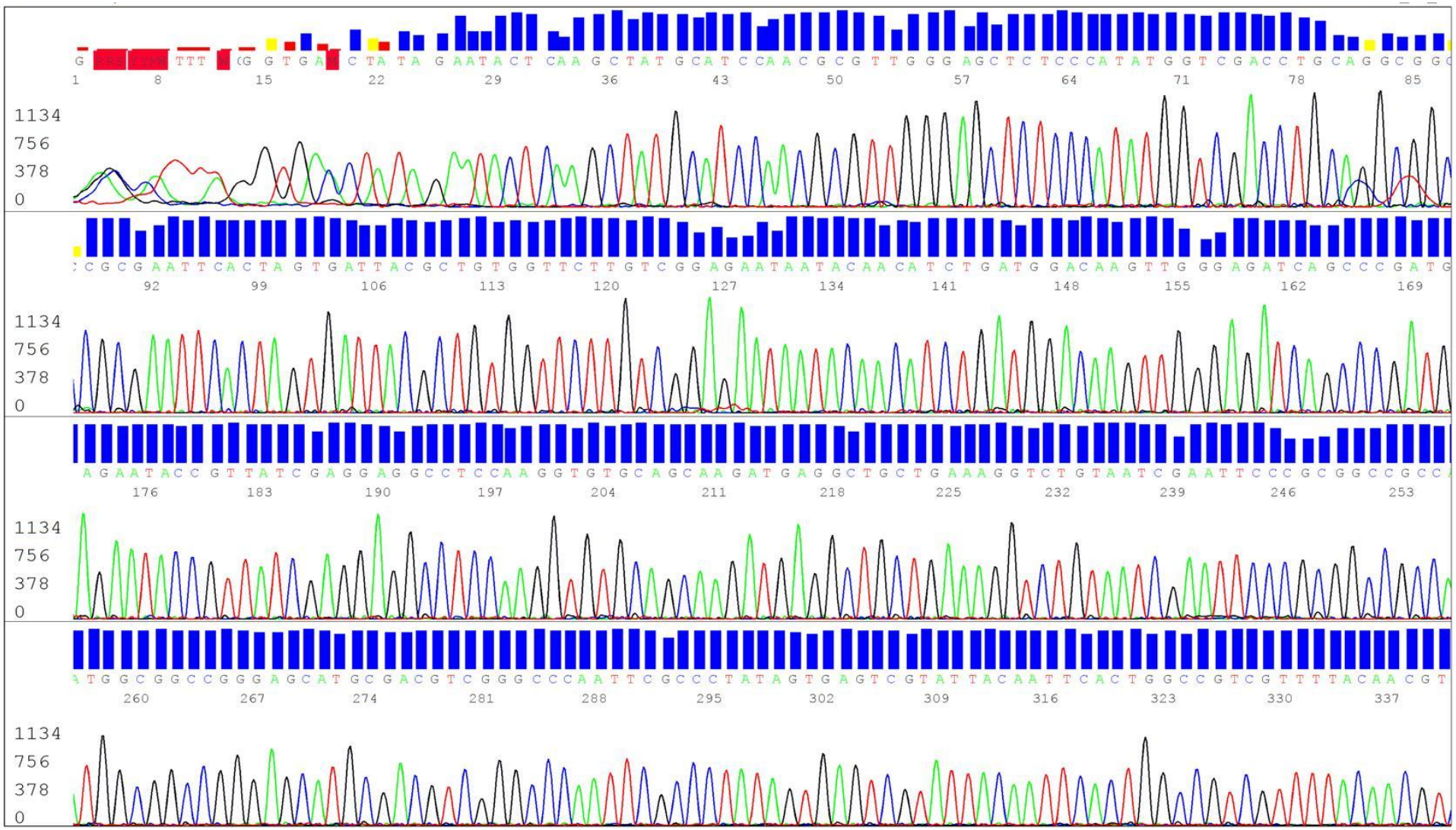
F-5’- TCG CAT TGG GCT TTA CTG TAC CCA-3’ R-5’- TCC GGA CCA CTG AAT GTG CCT TAT-3’

Plate 43 Bovine Galectin-3 clone PDFMD-Ramganga



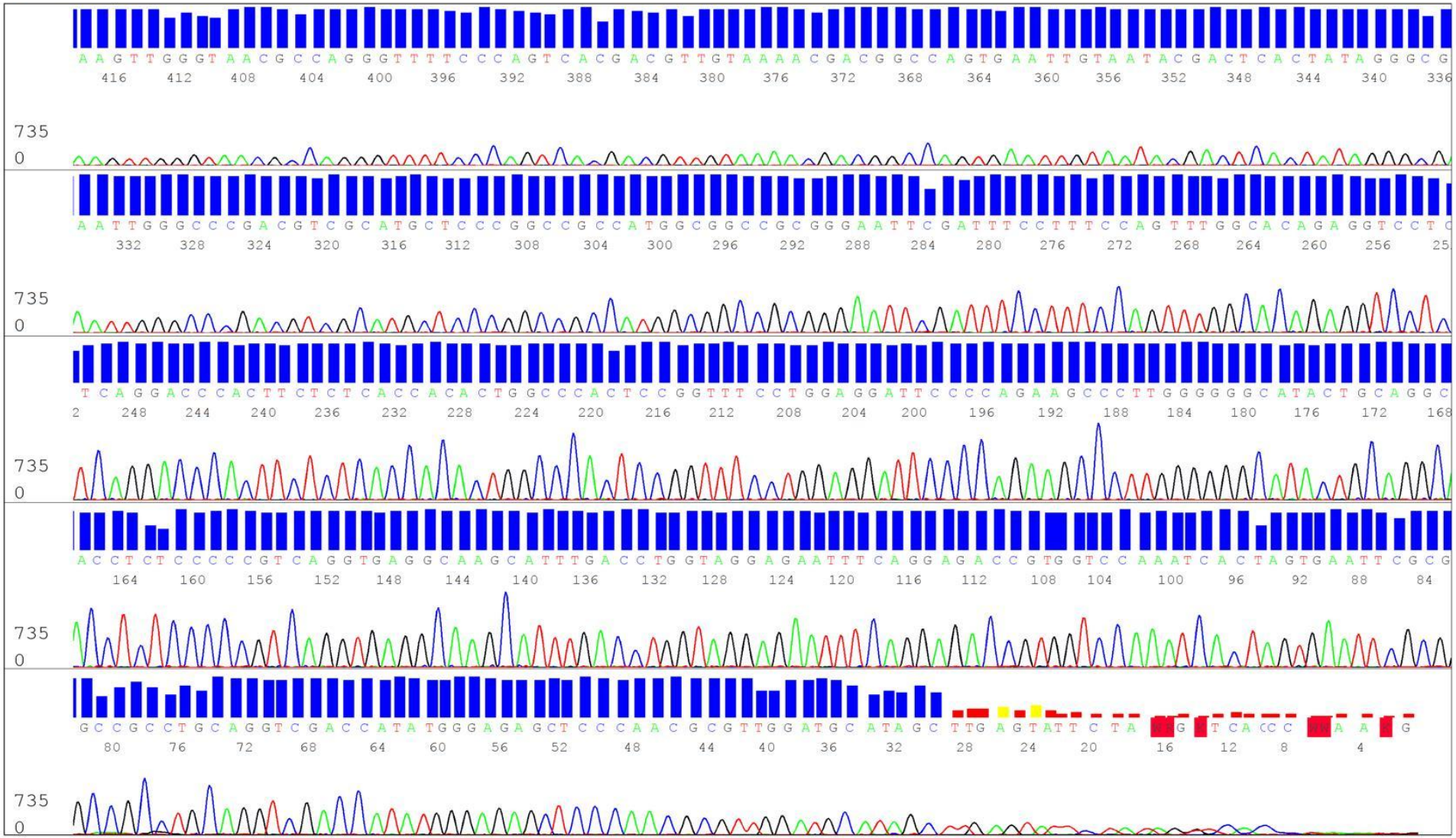
F-5'- ATG ACT CAG TCT TTG CTG ATC GGG-3' R-5'- AGA AGC ATC TTG CAG CCC TCT GAA-3'

Plate 44 **Bovine Granzyme A clone** PDFMD-Vaitarni



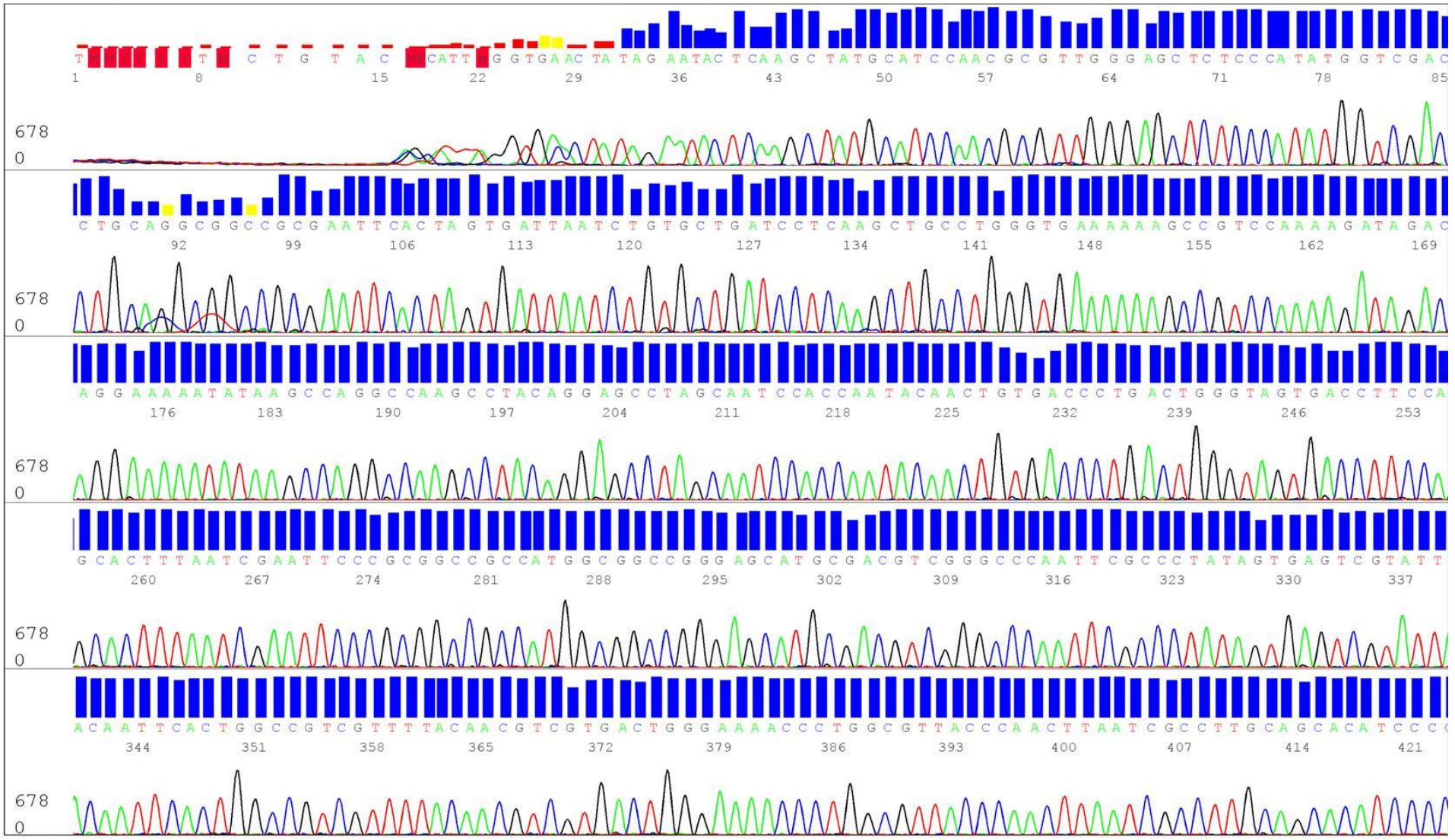
F-5’- ACG CTG TGG TTC TTG TCG GAG AAT-3’ R-5’- ACA GAC CTT TCA GCA GCC TCA TCT-3’

Plate 45 Bovine Granulysin clone PDFMD-Manipur



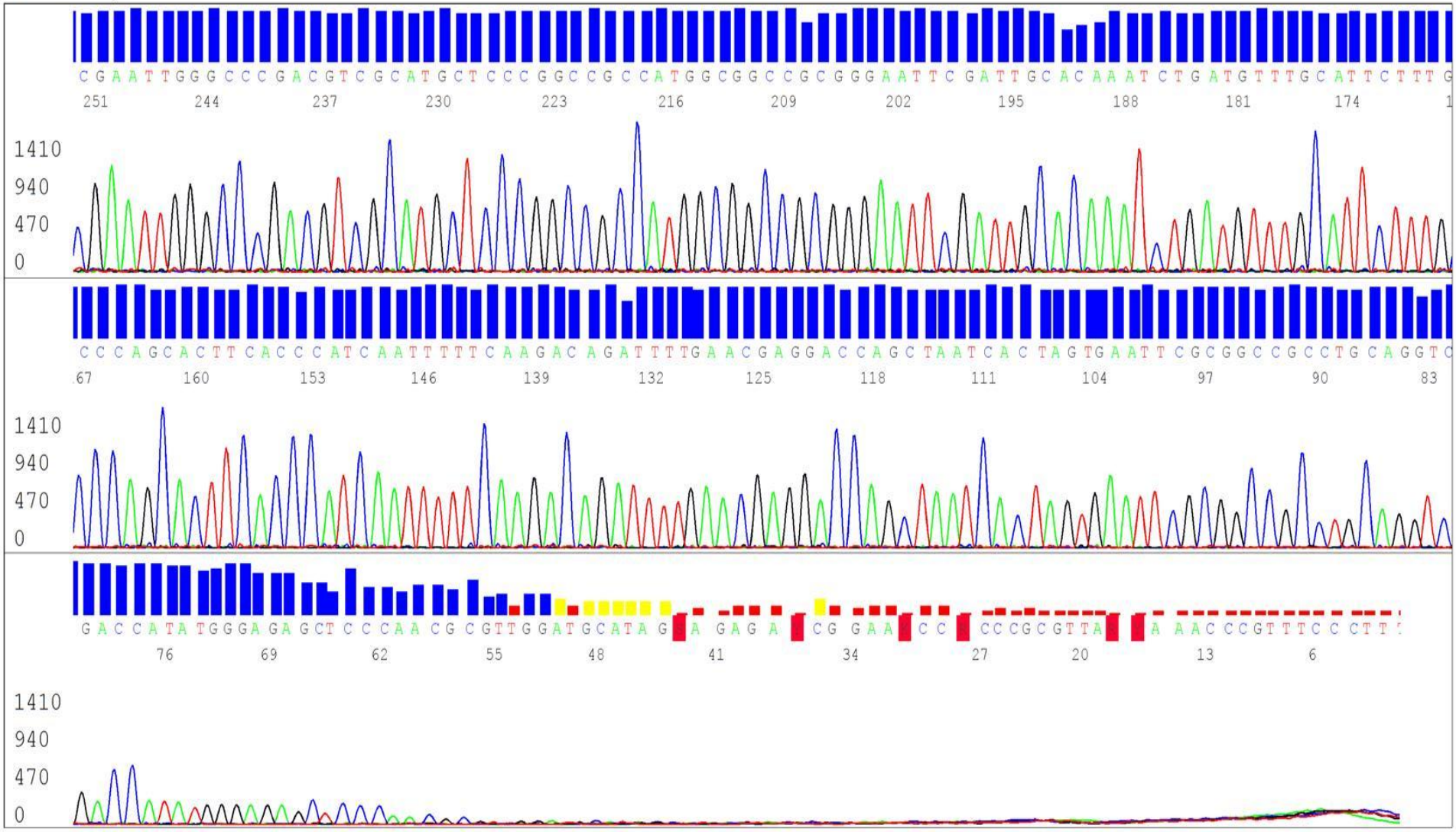
F-5’- TCC TTT CCA GTT TGG CAC AGA GGT-3’ R-5’- TGG ACC ACG GTC TCC TGA AAT TCT-3’

Plate 46 **Bovine Perforin clone** PDFMD-Son



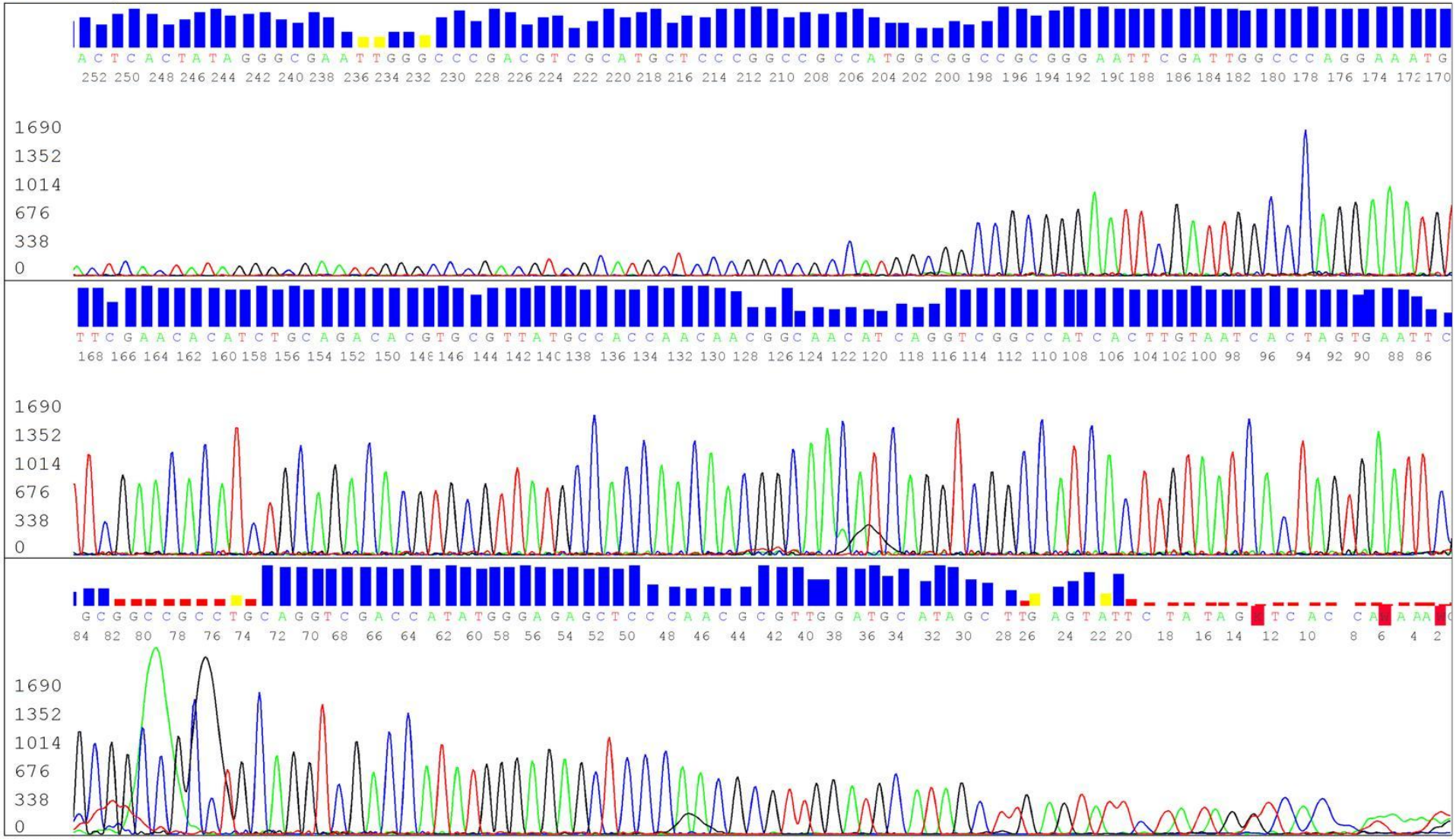
F-5’- AAT CTG TGC TGA TCC TCA AGC TGC -3’ R-5’- AAA GTG CTG GAA GGT CAC TAC CCA-3’

Plate 47 Bovine XCL1 clone PDFMD-Banganga



F-5'- GCA CAA ATC TGA TGT TTG CAT TC-3' R-5'- AGC TGG TCC TCG TTC AAA ATC T-3'

Plate 48 Bovine COX-2 clone PDFMD-Kukadi

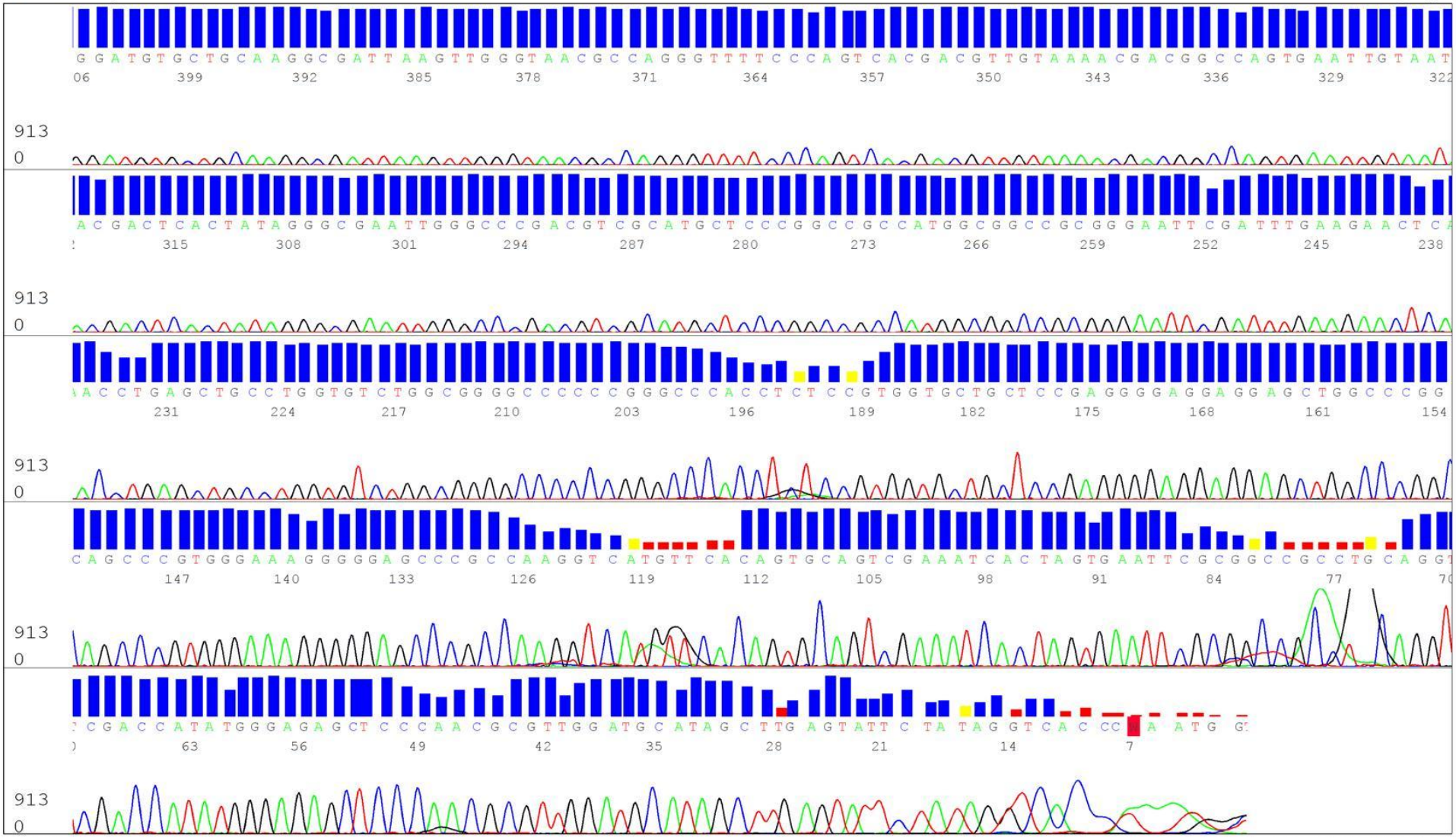


F-5’- GGC CCA GGA AAT GTT CGA A -3’ R-5’- ACA GTG ATG GCC GAC CTG AT-3’

Plate 49

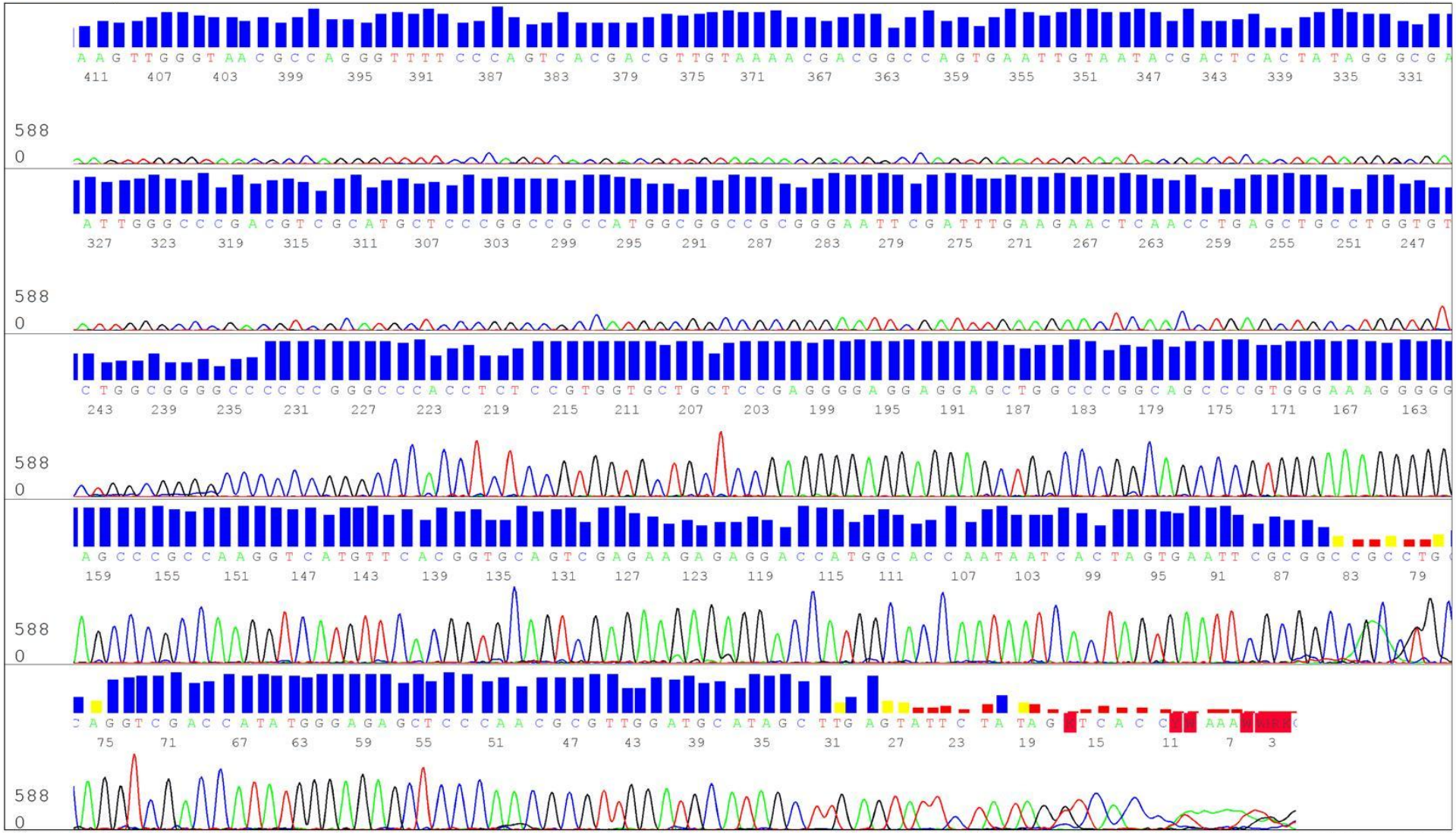
Bovine iNOS clone

PDFMD-Mayurakshi



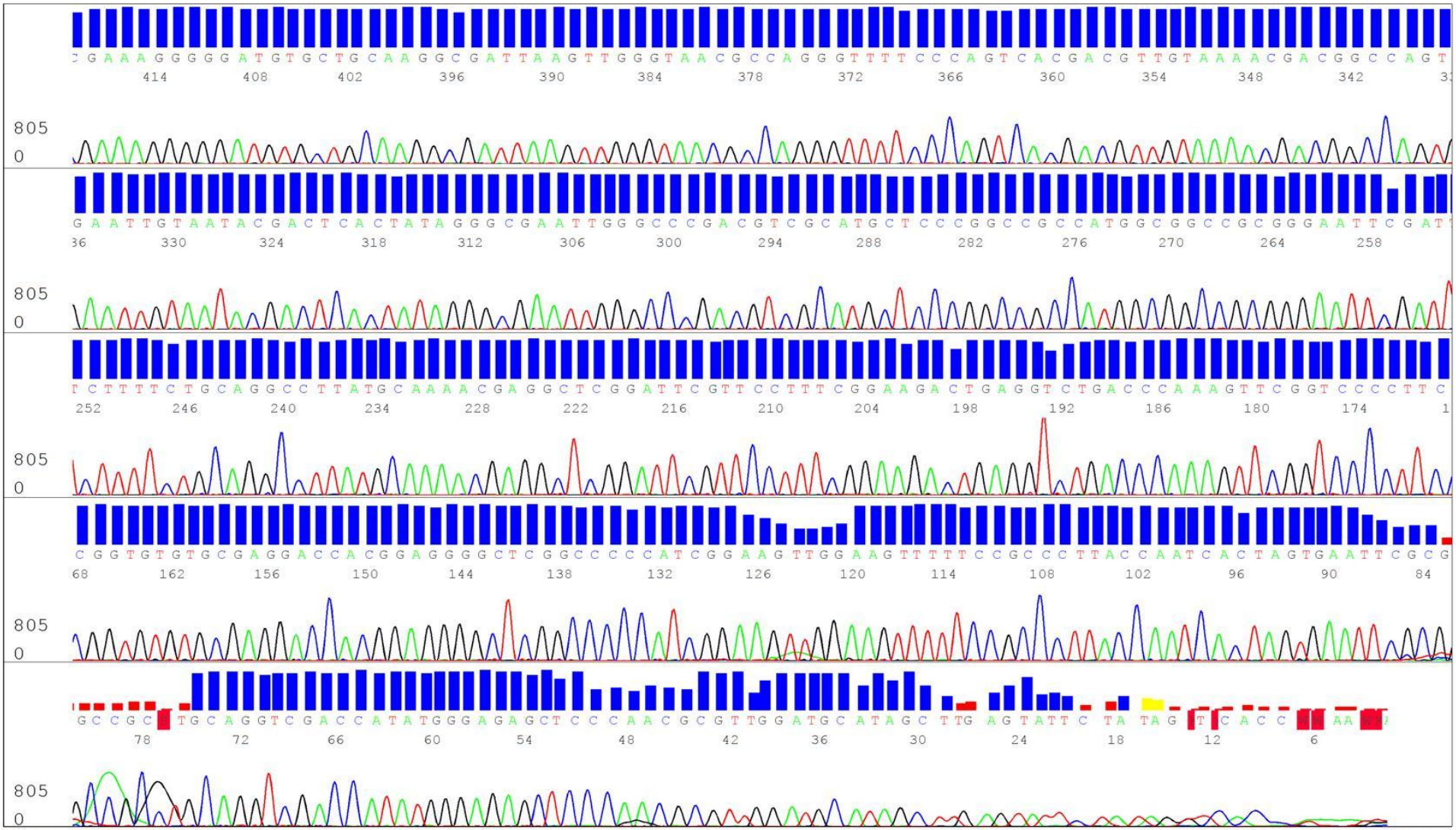
F-5’- TGA AGA ACT CAA CCT GAG CTG CCT-3’ R-5’- TCG ACT GCA CTG TGA ACA TGA CCT-3’

Plate 50 Bovine ICAM-1 clone PDFMD-Purna



F-5’- TGA AGA ACT CAA CCT GAG CTG CCT-3’ R-5’- ATT GGT GCC ATG GTC CTC TCT TCT-3’

Plate 51 Bovine ICAM-3 clone PDFMD-Tista



F-5’-CTT TTC TGC AGG CCT TAT GC-3’ R-5’-GGT AAG GGC GGA AAA ACT TC-3’

Plate 52 Bovine BAD clone PDFMD-Machkund

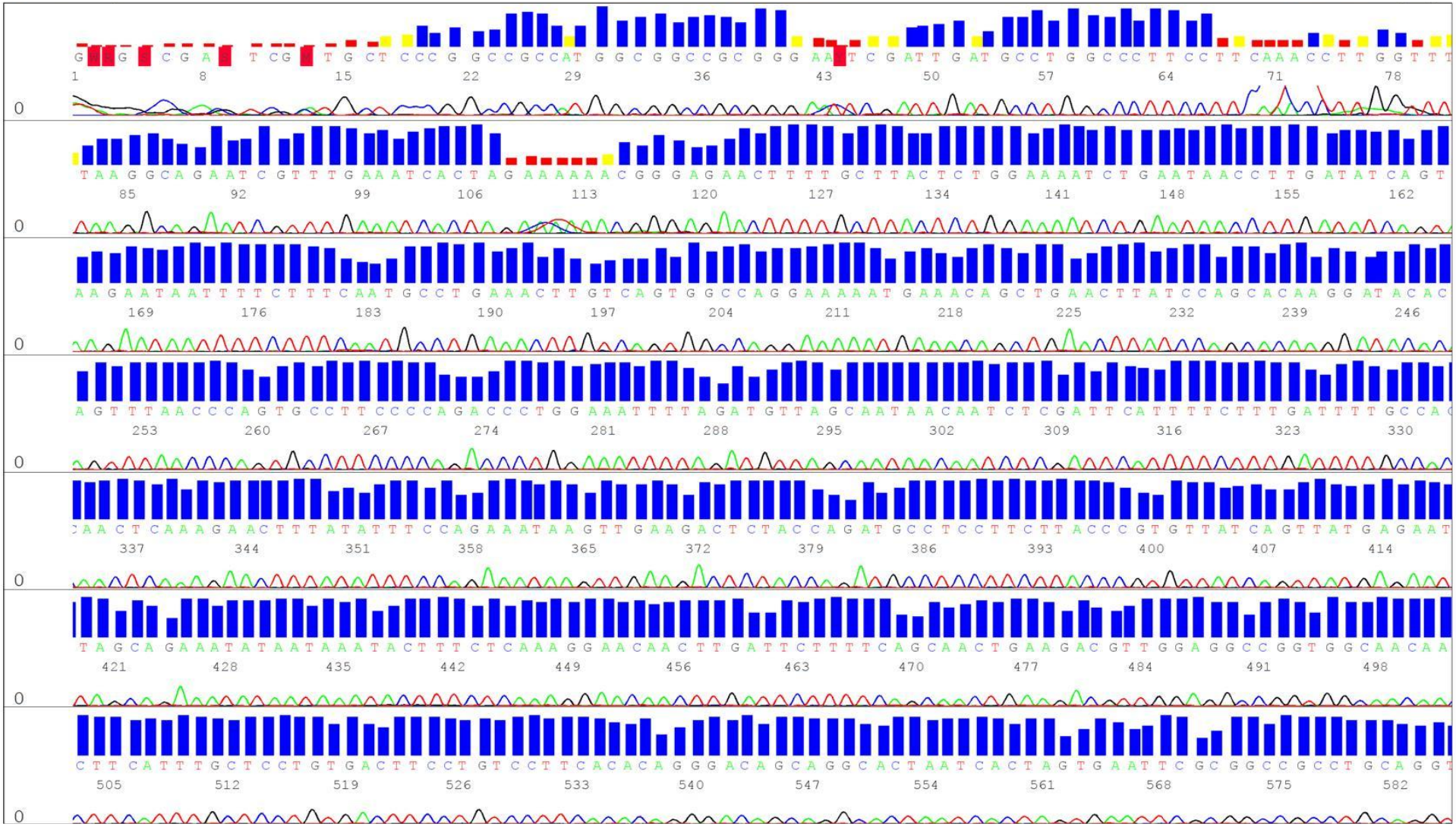


Plate 53 Bovine TLR2 clone PDFMD-Brahmputra

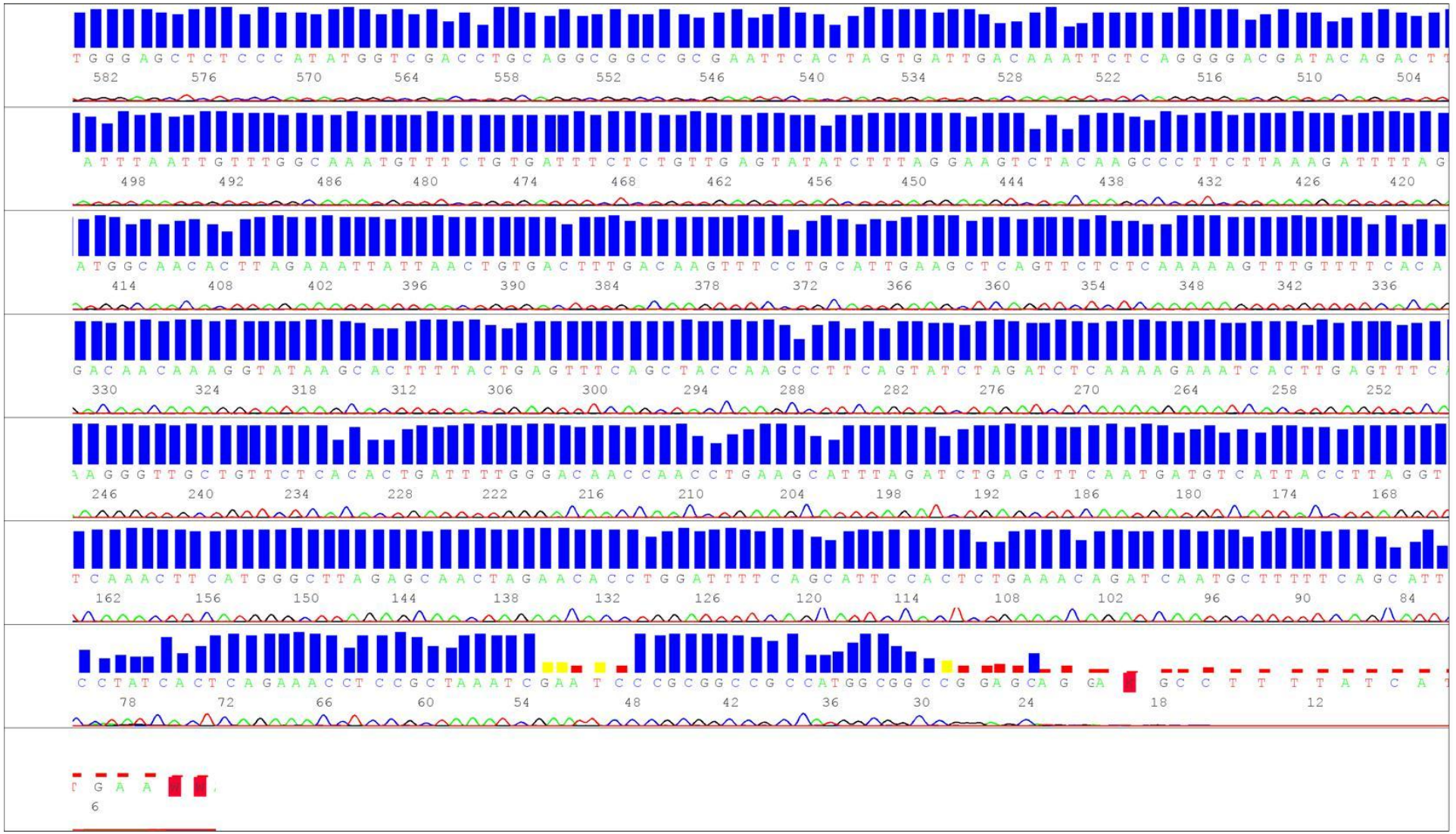
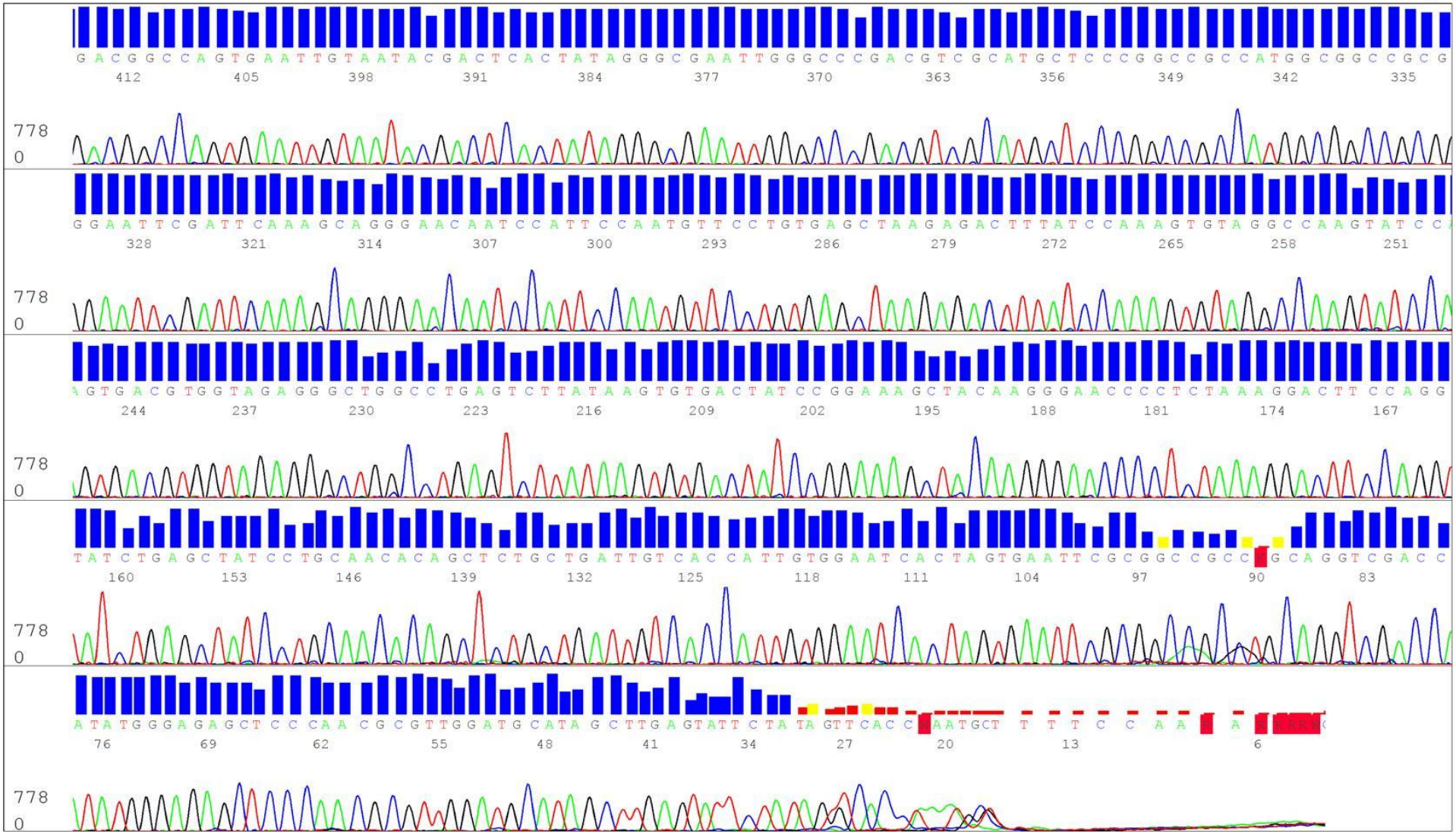


Plate 54 Bovine TLR4 clone PDFMD-Gomati



F-5’- CAA AGC AGG GAA CAA TCC AT -3’ R-5’- CCA CAA TGG TGA CAA TCA GC -3’

Plate 55 Bovine TLR6 clone PDFMD-Saraswati

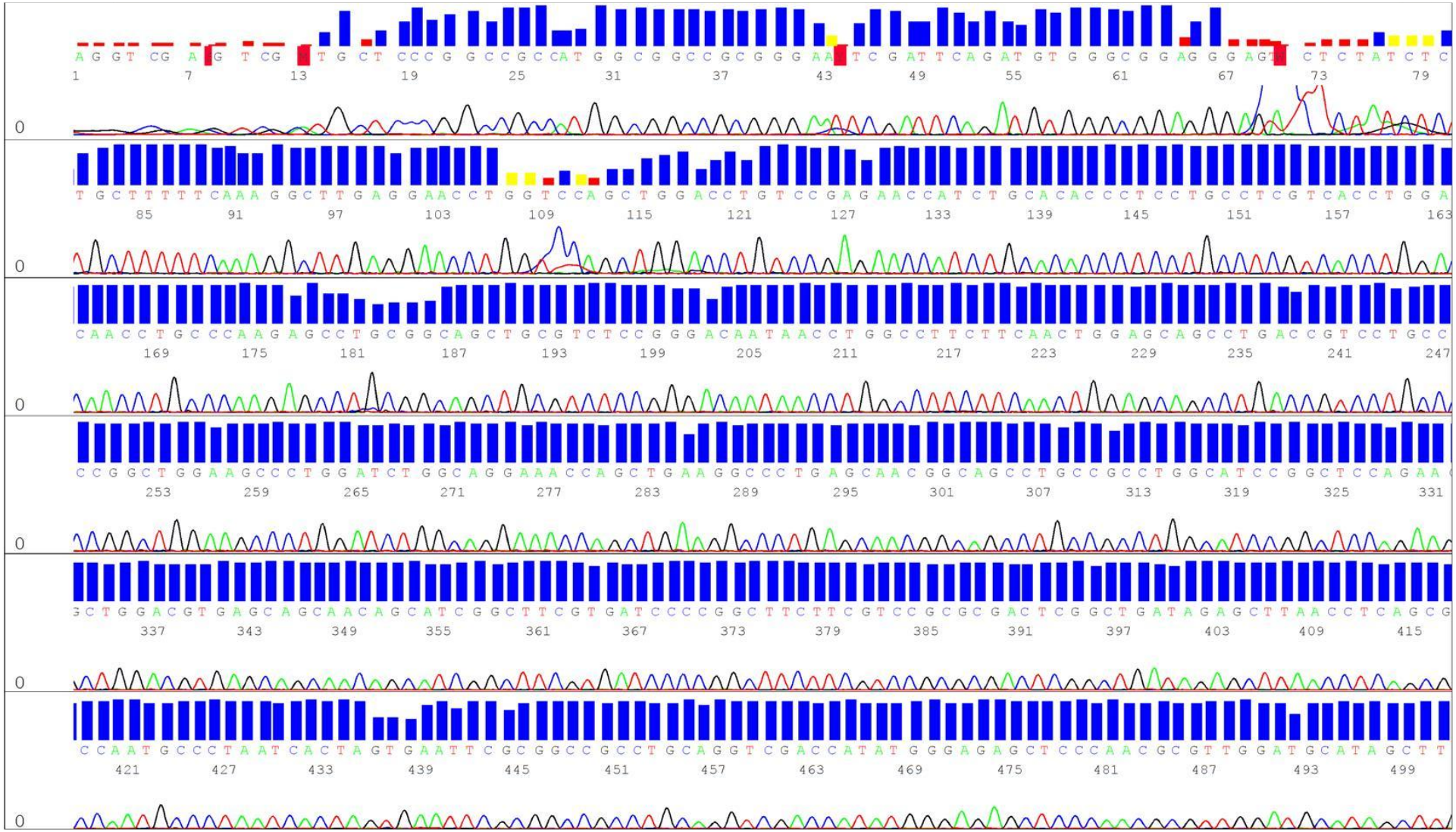
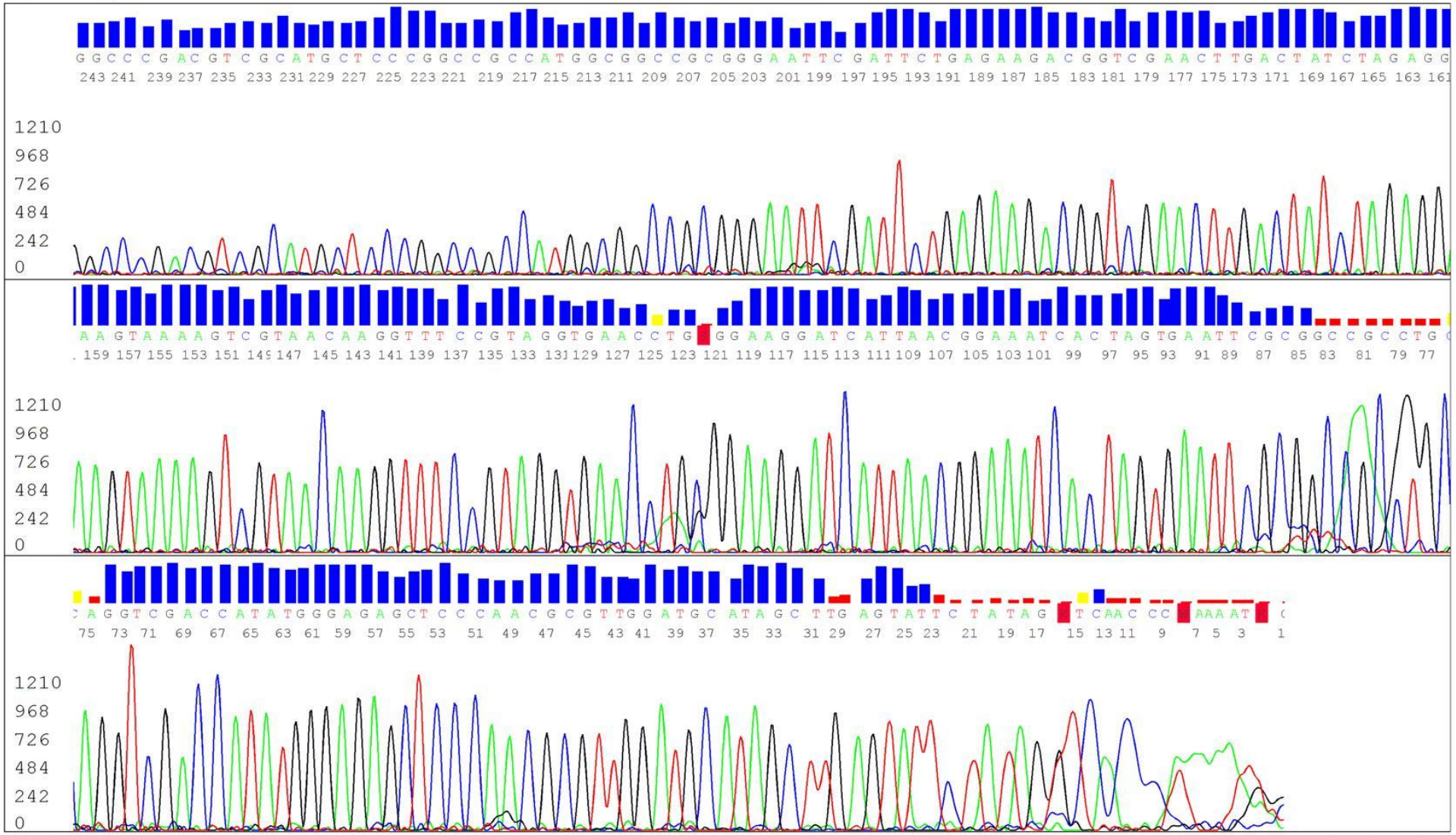
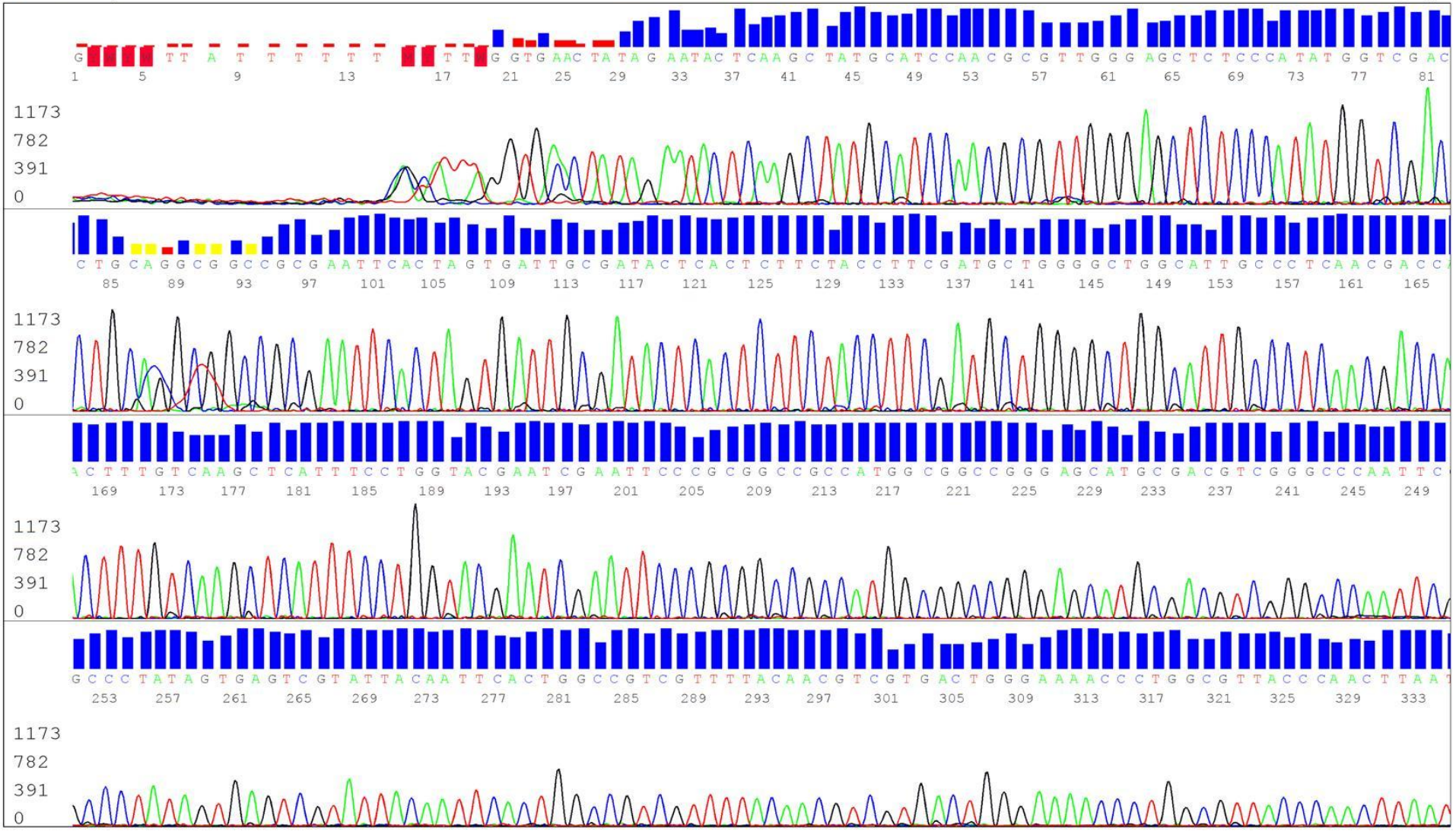


Plate 56 Bovine TLR9 clone PDFMD-Kosi



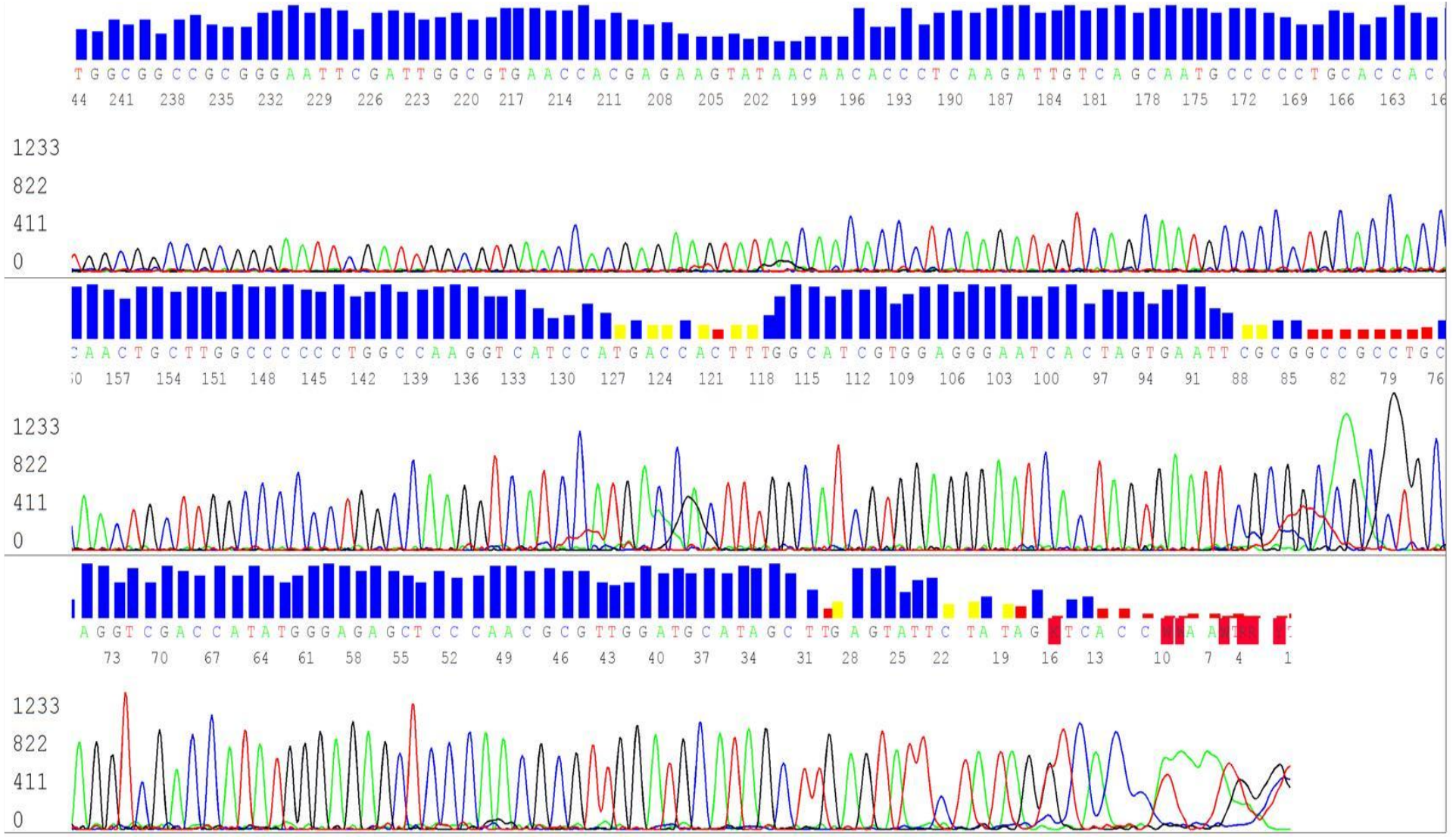
F- 5’-CTG AGA AGA CGG TCG AAC TTG ACT-3’ R-5’-TCC GTT AAT GAT CCT TCC GCA GGT-3’

Plate 57 **Bovine 18S RNA clone** PDFMD-Lohit



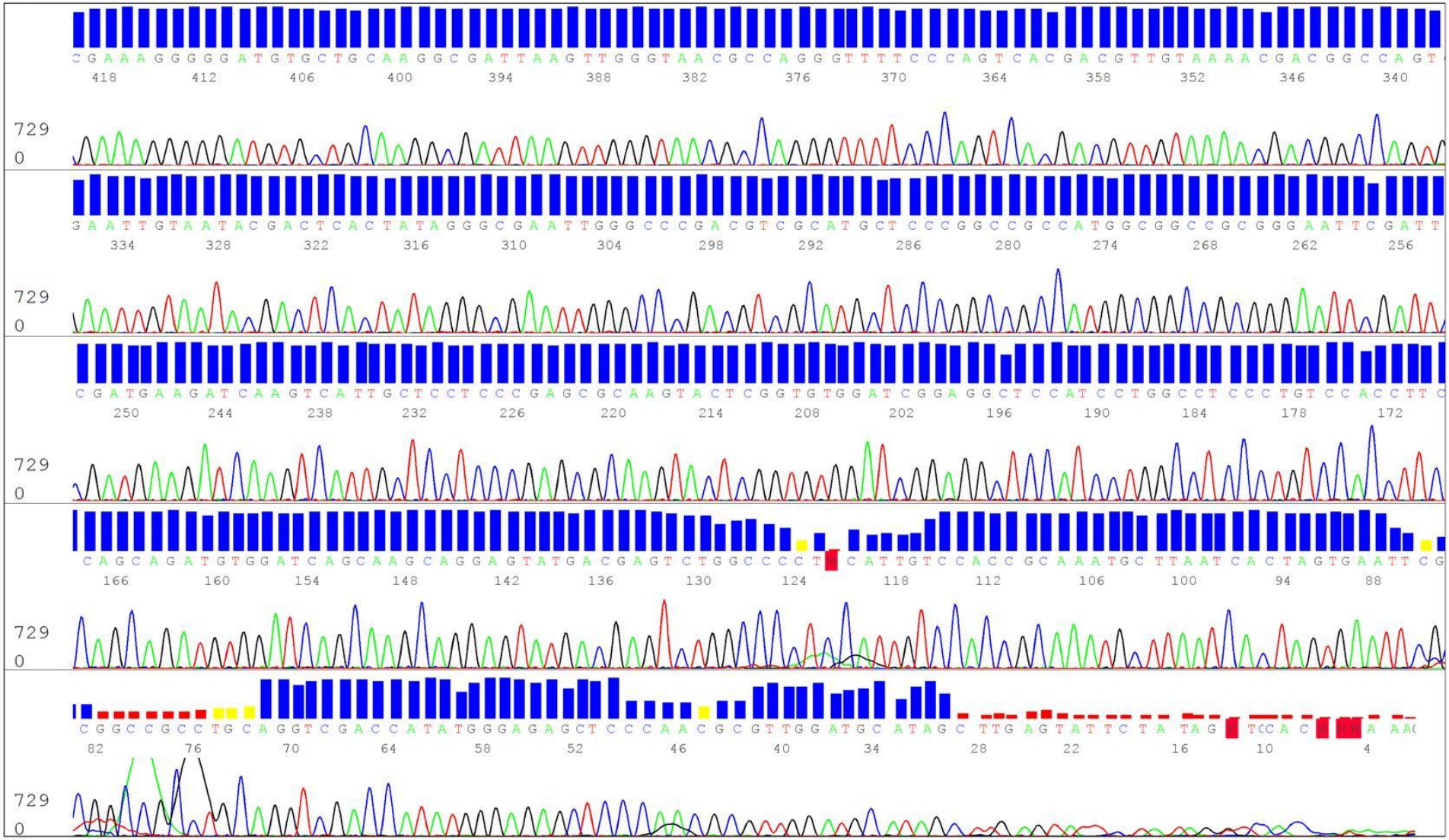
F-5’- GCG ATA CTC ACT CTT CTA CCT TCG A -3’ R-5’- TCG TAC CAG GAA ATG AGC TTG AC-3’

Plate 58 Bovine GAPDH-1 clone PDFMD-Indravati



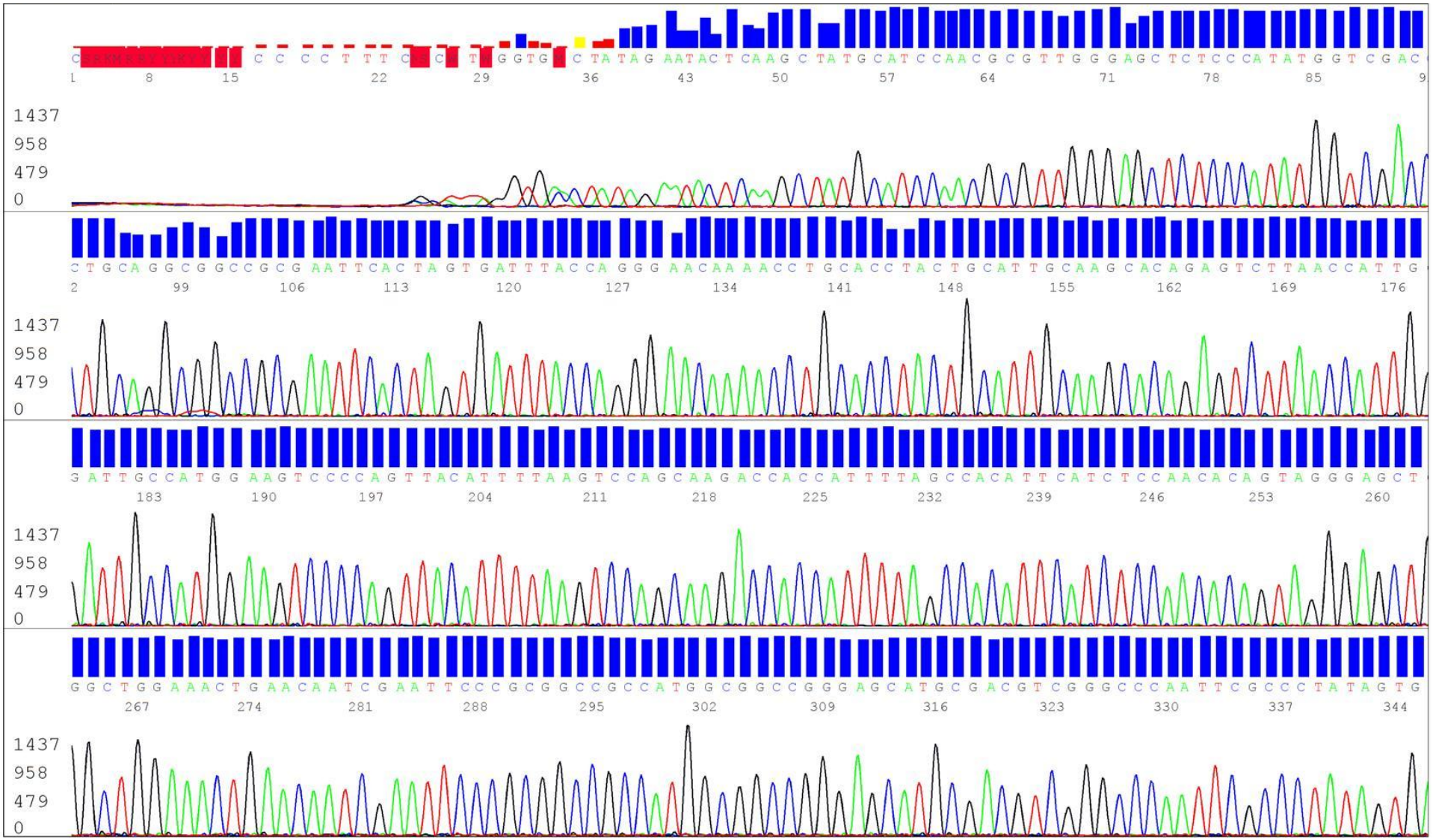
F-5’- GGC GTG AAC CAC GAG AAG TAT AA -3’ R-5’- CCC TCC ACG ATG CCA AAG T-3’

Plate 59 **Bovine GAPDH-2 clone** PDFMD-Chandrabhaga



F- 5’-CGA TGA AGA TCA ART CAT TGC-3’ R- 5’-AAG CAT TTG CGG TGG AC-3’

Plate 60 Bovine β-actin clone PDFMD-Pravra



F-5’-TAC CAG GGA ACA AAA CCT GC-3’ R-5’-GTT CAG TTT CCA GCC AGC TC-3’

Plate 61 Bovine IVD clone PDFMD-Ken

(1) PDFMD-Sindhu/Bovine IL-1α clone (124 bp)

CCACTTCGTGAGGACCAGATGAATAAGTTTATGTCCCTGGATACCTCGGAAACCTCTAAGACATC

CAAGCTTAGCTTCAAGGAGAATGTGGTGATGGTGGCAGCCAGTGGGAAGATTCTGAAGA

(2) PDFMD-Chenab/Bovine IL-1β clone (420 bp)

CACCCGTGCAGTCAATAAAGTGCAAACTCCAGGACAGAGAGCAAAAATCCCTGGTGCTGGCTA

GCCCATGTGTGCTGAAGGCTCTCCACCTCCTCTCACAGGAAATGAACCGAGAAGTGGTGTTCTG

CATGAGCTTTGTGCAAGGAGAGGAAAGAGACAACAAGATTCCTGTGGCCTTGGGTATCAAGGA

CAAGAATCTATACCTGTCTTGTGTGAAAAAAGGTGATACGCCCACCCTGCAGCTGGAGGAAGTA

GACCCCAAAGTCTACCCCAAGAGGAATATGGAAAAGCGCTTTGTCTTCTACAAGACAGAAATCA

AGAATACAGTTGAATTTGAGTCTGTCCTGTACCCTAACTGGTACATCAGCACTTCTCAAATCGAA

GAAAGGCCCGTCTTCCTGGGACATTTTCGAGGTGGCC

(3) PDFMD-Satluj/Bovine IL-2 clone (345 bp)

GGATTTACAGTTGCTTTTGGAGAAAGTTAAAAATCCTGAGAACCTCAAGCTCTCCAGGATGCAT

ACATTTGACTTTTACGTGCCCAAGGTTAACGCTACAGAATTGAAACATCTTAAGTGTTTACTAGA

AGAACTCAAACTTCTAGAGGAAGTGCTAAATTTAGCTCCAAGCAAAAACCTGAACCCCAGAGAG

ATCAAGGATTCAATGGACAATATCAAGAGAATCGTTTTGGAACTACAGGGATCTGAAACAAGAT

TCACATGTGAATATGATGATGCAACAGTAAACGCTGTAGAATTTCTGAACAAATGGATTACCTTT

TGTCAAAGCATCTACTCAACAAT

(4) PDFMD-Banas/Bovine IL-2Rα clone (130 bp)

TAGCCCAGTCCTCCAAGCTAGCCCCCCCTACTGAACCTTACAGTTTGAATTTCTAAAGAACAGAT

TCCCTGGGGGAGCAGTCTTTGGGTGACCGCAGAAGACAGCAAGCAAACCCATGGAACTGTTGA

GC

(5) PDFMD-Beas/Bovine IL-4 clone (289 bp)

CAAAACGCTGAACATCCTCACAACGAGAAAGAATTCATGCATGGAGCTGCCTGTAGCAGACGTC

TTTGCTGCCCCAAAGAACACAACTGAGAAGGAAACCTTCTGCAGGGTTGGAATTGAGCTTAGGC

GTATCTACAGGAGCCACACGTGCTTGAACAAATTCCTGGGCGGACTTGACAGGAATCTCAACAG

CTTGGCAAGCAAGACCTGTTCTGTGAATGAAGCCAAGACGAGCACAAGTACGCTGAAAGACCTC

TTGGAAAGGCTAAAGACGATTATGAAGGAGAAA

(6) PDFMD-Bhagirathi/Bovine IL-6 clone (491 bp)

CGCATGGTCGACAAAATCTCTGCAATGAGAAAGGAGATATGTGAGAAGAATGATGAGTGTGAA

AGCAGCAAGGAGACACTGGCAGAAAATAAGCTGAATCTTCCAAAAATGGAGGAAAAGGACGG

ATGCCTCCAATCTGGGTTCAATCAGGCGATTTGCTTGATCAGAACCACTGCTGGTCTTCTGGAGT

ATCAGATATACCTGGACTACCTCCAGAACGAGTATGAGGAAAATCAGGAAAATGTCAGGGATTT
GAGGAAAAATATCAGAACACTGATCCAGATCCTGAAGCAAAAGATCGCAGATCTAATAACCACT
CCAGCCACAAACACTGACCTGCTGGAGAAGATGCAGTCTTCAAACGAGTGGGTAAAGAACGCA
AAGATTATCCTCATCCTGAGAAACCTTGGGAATTTCCTGCAGTTCAGCCWGAGAGCTATTCGGA
TGAAGTAGCTGGGGCTCCCATGATTGTGGTAGTTCCTGGGCATTCC

(7) PDFMD-Ganga/Bovine IL-8 clone (209 bp)

GATGCCAATGCATAAAAACACATTCCACACCTTTCCACCCCAAATTTATCAAAGAATTGAGAGTT
ATTGAGAGTGGGCCACACTGTGAAAATTCAGAAATCATTGTTAGGCTTACCAATGGAAACGAGG
TCTGCTTAAACCCCAAGGAAAAGTGGGTGCAGAAGGTTGTGCAGGTATTTGTGAAGAGAGCTG
AGAAGCAAGATCCATGA

(8) PDFMD-Yamuna/Bovine IL-9 clone (149 bp)

GCACGAGATTCCACCTGATTGTCAACCAGCTGAAGAAAATAGTCGTGACCCTCAAGAGCAACAA
GTGCGGATCTTTTGCCTGTGAACAGCCATGCAACCGAACCACAACAGGCAATACGCTGACATTT
CTGAAGGCTCTGCTGGAAAGT

(9) PDFMD-Luni/Bovine IL-10 clone (329 bp)

TGCCAACCAGCCTGCCCCACATGCTGCGGGAGCTCCGAGCTGCTTCGGCAAGGTGAAGACTTTC
TTTCAAATGAAGGACCAACTGCACAGCTTACTGTTGACCCAGTCTCTGCTGGATGACTTTAAGGG
TTACCTGGGTTGCCAAGCCTTGTCGGAAATGATCCAGTTTTACCTGGAAGAGGTGATGCCACAG
GCTGAGAACCACGGGCCTGACATCAAGGAGCACGTGAACTCACTGGGGGAGAAGCTGTAGACC
CTCCGGCTGCGGCTGCGGCGCTGTCATCGCTTTCTGCCCTGCGAAAACAAGAGCAAGGCGGTG
GAGAAGGTGA

(10) PDFMD-Mahi/Bovine IL-12p40 clone (186 bp)

AACCTGCAACTGAGACCATTAAAGAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGAC
ACGTGGAGCACCCCGCATTCCTACTTCTCCCTGACGTTTTGTGTTCAGGTCCAGGGAAAGAACAA
GAGAGAAAAGAAACTCTTCATGGACCAAACCTCAGCCAAAGTCACATGCCACAAGGAT

(11) PDFMD-Chambal/Bovine IL-13 clone (111 bp)

CCTGTGCTGTTCTAGGCTCCATGGCGCTCTTATTGACCGCGGTCATTGTTCTTATCTGCTTTGGTG
GCCTCACCTCCCCAAGCCCTGTGCCTTCTGCTACAGCCCTCAAGG

(12) PDFMD-Sabarmati/Bovine IL-15 clone (74 bp)

GGCTGGCATTCATGTCTTCATTTTGGGCTGTATCAGTGCAAGTCTTCCCAAAACAGAAGCAAACT
GGCAGTATG

(13) PDFMD-Godavari/Bovine IL-16 clone (73 bp)

GAGGGCGGTCCCAGAAGTTAGGGCACAGTCACAGTGGGGTGGCTGATACAAACGRCAGACTG
CATCTAGAGAG

(14) PDFMD-Tapi/Bovine IL-17A clone (138 bp)

TGAGTCTGGTGGCTCTTGTGAAGGCAGGAGTCATCATCCCACAGAGTCCAGGCTGCCCACCTAC
TGAGGACAAGAACTTCCCACAGCATGTGAGGGTCAACCTAAACATCGTTAACCGGAGCACAAAC
TCCAGAAGGC

(15) PDFMD-Penganga/Bovine IL-18 clone (347 bp)

AGATAATCGACCCCAGACCATATTTATCATATATATGTATAAGGACAGCCTCACTAGAGGTCTGG
CCGTAACCATCTCTGTGCAGTGTAAGAAAATGTCTACTCTCTCCTGTGAGAACAAAATTGTTTCCT
TTAAGGAAATGAATCCTCCTGATAACATTGATAATGAAGAAAGTGACATCATATTCTTTCAAAGA
AGTGTTCCAGGACATGATGATAAGATACAATTTGAGTCTTCATTGTACAAAGGGTACTTTCTAGC
TTGTAAAAAAGAGAATGACCTTTTCAAACTCATTTTGAAAAAACAGGATGATAATAGAGATAAA
TCTGTAATGTTCACTGTTCAAA

(16) PDFMD-Narmada/Bovine IL-27p28 clone (111 bp)

CAGCCTTTTGCTGCTCTTCTTGCTTCTGGCTCGAGCTGGTGTCTGGGGATTCCCGAGGCCCCCAG
GGAGGCCCCCCCTGAGCCTGCAGGAGTTGCAGAGGGAGTTCAAGGT

(17) PDFMD-Krishna/Bovine IL-32 clone (176 bp)

GCAGGATCTCAGGTCCCTTCGGGAGGACCCTAAGCCACCATGTGCTTCGCTAAGGGAGTCCCAT
ATGACCAGGCTTCTCTGAGGTCCATAATGCACAAACGGGTGGATGATTTCTGTGATAAGATGGG
AAATGAACCAGAAGAAGCACAGATGGAGGCAGCCCTAGTGAGACGGAG

(18) PDFMD-Idukki/Bovine IL-34 clone (165 bp)

AGGGACACAGAGCCTGAGAAAGGCGCAGCTCCCCCTCTGTGGTCTCGCCTGGAGCGGCGGGG
GCTGGGGCACCTGAAGGTCGTGAAGGCAGAGCCTCTGGCTGCTCCTGGCACTGTGTGGGCTGA
CCCCTTCCCTGAAGGCTGTGCCGACCTTCTCCTGAGTCAG

(19) PDFMD-Periyar/Bovine IFN-αR1 clone (157 bp)

TCCACATGGTATGAGGTTGAGCCATTTGTACCATTTCTAGAAGCTCAGATTGGTCCCCCAGATGT
GCATTTAGAAGCTGAAGATAAGGCAATAATACTGAGTATCTCTCCCCCTGGAACAAAAGATAGT
ATCATGTGGGCTATGGATCGTTCAAGCT

(20) PDFMD-Kaladi/Bovine IFN-γ clone (509 bp)

CTACCGATTTCAACTACTCCGTGCCTAACTCTCTCCTAAACAATGAAATATACAAGCTATTTCTTA
GCTTTACTGCTCTGTGGGCTTTTGGGTTTTTCTGGTTCTTATGGCCAGGGCCAATTTTTTAGAGAA

ATAGAAAACTTAAAGGAGTATTTTAATGCAAGTAGCCCAGATGTAGCTAAGGGTGGGCCTCTCT
TCTCAGAAATTTTGAAGAATTGGAAAGATGAAAGTGACAAAAAAATTATTCAGAGCCAAATTGT
CTCCTTCTACTTCAAACTCTTTGAAAACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATA
TCATCAAGCAAGACATGTTTCAGAAGTTCTTGAATGGCAGCTCTGAGAAACTGGAGGACTTCAA
AAAGCTGATTCAAATTCCGGTGGATGATCTGCAGATCCAGCGCAAAGCCATAAATGAACTCATC
AAAGTGATGAATGACCTGTCGCCAAAATCTAACCTCAGAAAGCGGAAGAGAAGTCA

(21) PDFMD-Mahanadi/Bovine TNF-α clone (360 bp)

GTGGCCAACGGTGTGAAGCTGGAAGACAACCAGCTGGTGGTGCCTGCTGACGGGCTTTACCTC
ATCTACTCACAGGTCCTCTTCAGGGGCCAAGGCTGCCCTTCCACCCCCTTGTTCCTCACCCACACC
ATCAGCCGCATTGCAGTCTCCTACCAGACCAAGGTCAACATCCTGTCTGCCATCAAGAGCCCTTG
CCACAGGGAGACCCCAGAGTGGGCTGAGGCCAAGCCCTGGTACGAACCCATCTACCAGGGAGG
AGTCTTCCAGCTGGAGAAGGGAGATCGCCTCAGTGCTGAGATCAACCTGCCGGACTACCTGGAC
TATGCCGAGTCTGGGCAGGTCTACTTTGGGATCATCGCC

(22) PDFMD-Tungbhadra/Bovine TNF-αSF1 clone (200 bp)

GGAGCTTGGGTGGATGACTAAAGGCAGGGAGGGGGGCTTATTTATGAAGGGAAAACAATTAA
ATTATTTATTTATGGAGCATGGAGAGAAGGGAATAATAGCGAGACATCAGAAGAAAGAGCTAA
TACACCCAAGAGATGACGAGTGAGAGGGCTTAGGCACAAGGATGACCCAGTGGGAGAGAGAA
AGCGAGGCTCTGA

(23) PDFMD-Kaveri/Bovine TGFβ-1 clone (264 bp)

TACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCATGAACCCAAGGGGTACCACGCC
AATTTCTGCCTGGGGCCCTGCCCTTACATCTGGAGCCTGGATACACAGTACAGCAAGGTCCTGG
CCCTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCCTCAGGCGCTGG
AGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCAAGGTGGAGCAGTTGTCCAACATGAT
CGTGCGCTCC

(24) PDFMD-Damodar/Bovine TGFβ-2 clone (135 bp)

GAACTTGAACTCAAACGGGCCAGCAAAAGAGGTCATATTAACAAGATGAAAAATGCGAGTGAG
ATCTATTATATGACCGAGTGAGTCTGCATAAAGAGAGAGACCCTGCAAATACATGCTTTGTACC
AACTGCCC

(25) PDFMD-Ghaghra/Bovine TGFβ-3 clone (116 bp)

CTCTCTGGTCTGCTCCTTGGGTTCCTCCTCTCGCCTTCTTCCTTGATCGTTATTTCTCCTTGGACATT
TGGCTAGACACCTTCCAGGTCAGGGCGCACACGACTGGAGTTTGGGTC

(26) PDFMD-Wardha/Bovine GM-CSF clone (87 bp)

AATGACACAGAAGTCGTCTCTGAAAAGTTTGACTCCCAGGAACCAACGTGCCTGCAGACTCGCC
TGAAGCTGTACAAGAACGGCCTG

(27) PDFMD-Penneru/Bovine CSF clone (158 bp)

ATTTTGACTCCTCGTGTGGGGAGCCCAGGCATCAGTGACGCCTCTTGTTTTTCTGGATGCACTAG
AGACGCCATGAGGGTCATGACCTTGCTCCACCCTACCGGGGTTAGGGTCAGGATTCTGACTCTC
TTTTGGGCTGGGCAGATGGTCGTATGTCT

(28) PDFMD-Hooghly/Bovine MCP-1 clone (209 bp)

CCAAGTCGCCTGCTGCTATACATTCAACAGTAAGAAGATCTCCATGCAGAGGCTGATGAACTAC
AGAAGAGTCACCAGCAGCAAGTGTCCTGAAGAGGCTGTGATTTTCAAGACCATCCTGGGCAAG
GAGTTATGTGCAGACCCCAAGCAGAAATGGGTCCAGGACTCCATAAACTATCTTAACAAGAAAA
ACCAAACTCCAAAGCCTT

(29) PDFMD-Jhelum/Bovine CCL3 clone (81 bp)

TCAAGCCTGGTGTCATCTTCCAGACCAAAAAGGCCGGCAGGTCTGTGCCAACCCCACTGAGGAC
TGGGTCCAGGAATACAT

(30) PDFMD-Sharda/Bovine CCL5 clone (82 bp)

AACGCTTTGGAGTTGAGCTAGGGTGGAGGACGCCTTGAACCTGAACTTGCGCCAACTCTGCTTC
TCGCTCTTGTCCTAAGCA

(31) PDFMD-Manjari/Bovine CCR5 clone (119 bp)

ACATGCTGGTTGTCCTCATCCTGATAAACTGCAAAAAGCTGAAGAGCATGACTGACATCTATCTG
CTCAACTTGGCCATCTCTGACCTACTTTTCATCATCACCATCCCATTCTGGGCT

(32) PDFMD-Sabari/Bovine CXCL1 clone (93 bp)

ACATCCAGAGCGTGAAGGTGACAACCCCCGGCCCCCACTGCGACCAAACTGAAGTCATAGCCAC
TCTCAAGACTGGTCAGGAAGTGTGTCTCA

(33) PDFMD-Ghaggar/Bovine CXCL2 clone (91 bp)

AGAAGCTCTTGGATGGCTGTTCCAGAAGTAGACCCTGCCCCTACAGAAATCAAAGAGGGAAAA
GAGGAATCACCACCTCCTAAGGCCATCT

(34) PDFMD-Gandak/Bovine CXCL6 clone (139 bp)

TTGTGAGAGAGCTGCGTTGTGTGTGTTTAACCACCMCMCCGGGAATTCATCCCAAAACGGTCA
GTGATCTGCAGGTGATCGCCGCGGGACCGCAGTGCTCCAAGGTGGAAGTGATAGCCMCCTTGA
AGAATGGAAGGGA

(35) PDFMD-Wainganga/Bovine CXCR4 clone (113 bp)

ACCTGTGGCTAGTGGTGTTTCAGTTTCAGCACATCGTGGTCGGCCTTCTCCTGCCTGGTATCGTC
ATCCTGTCCTGCTACTGCGTTATCATCTCCAAGCTGTCCCACTCCAAA

(36) PDFMD-Chitravati/Bovine CD11a clone (102 bp)

TGAACTCCATGAGGGTGAGGATCATAGCTGTCTTGTTCACCTTGGGACCCCTGGTGATATCACA
GAGCCAGATACATAGTAGGTGCTTGGCACAGTTTCACT

(37) PDFMD-Vaigai/Bovine CD11b clone (139 bp)

AGGAAATCTCGACAGGAGCTCGATACTATTGCATCTAAGCCCCCTGCTGACCACGTGTTCCAGG
TGAATAACTTCGAAGCTCTGAAGACCATTCAGAACCAGCTTCAGGAAAAGATCTTTGCGATTGA
GGGTACGCAGA

(38) PDFMD-Kollidam/Bovine CD11c clone (158 bp)

TGTCAACCTGAGCTTCTTAGGGCTGGAGACCCTGGTGGTGGGGAGCTCCCTGGAGCTCAATGTT
GCAGTGATGGTGTCCAATGAGGGCGAGGATTCCTATGGAACGGTGATCAGCTTCTACTATCCAG
CAGGGCTGTCCTATCGACGCACGTTAGCAA

(39) PDFMD-Manas/Bovine CD14 clone (81 bp)

TCTAGCGCCGTTCAGTGTATGGTTGCCGTCGAGGTGGAGATCAGTGCCGGCGGCCGCAGCCTG
GAACAGTTTCTCAAGGGA

(40) PDFMD-Varna/Bovine CD18 clone (133 bp)

AACTGGCAGAAAGCAACMTCCAGCCCATCTTTGCAGTAACCAAGAAGATGGTGAAAACGTACG
AGAAGCTGACAGAGATCATCCCCAAGTCTGCAGTCGGGGAGCTGTCTGAAGATTCCAGGAACG
TGGTGGA

(41) PDFMD-Girna/Bovine CD68 clone (164 bp)

TGATGAGAGGCAGCAAGATGGACTTATCATTGGGACAAGAGAAACTTGGTCCAAAGGCCCCTG
AGGGGGACAGCTGAGCAGCTTGTAGCTTCAGGGAGAGCAGATCAAGGTGTAAAGCTGGTGAA
ACAATGATGCTTGCATTTCTGCAACTGAAGCTATGGCCC

(42) PDFMD-Palar/Bovine Integrin β3 clone (137 bp)

TCCCACTTGCTGGTGTTTACCACTGATGCCAAGACCCATATAGCGCTGGATGGAAGGCTGGCAG
GCATCGTCCAGCCCAATGATGGGCAGTGTCATGTGGGCAGTGACAACCATTATTCTGCCTCCAC
CACCATGGA

(43) PDFMD-Ramganga/Bovine Galectin-3 clone (97 bp)

TCGCATTGGGCTTTACTGTACCCAGGATTGTTATCAGCATGCGAGGCCTGATTCCTCCAGGAAAA
GGCAGGTCATAAGGCACATTCAGTGGTCCGGA

(44) PDFMD-Vaitarni/Bovine Granzyme A clone (121 bp)

ATGACTCAGTCTTTGCTGATCGGGGAAGAGTGACAGCAGGAGGTCATTTCTTTCATTGCTGAATT
TATTTTAGACAGGCGGCATTTTGTATGTGAAGTTCAGAGGGCTGCAAGATGCTTCT

(45) PDFMD-Manipur/Bovine Granulysin clone (128 bp)

ACGCTGTGGTTCTTGTCGGAGAATAATACAACATCTGATGGACAAGTTGGGAGATCAGCCCGAT
GAGAATACCGTTATCGAGGAGGCCTCCAAGGTGTGCAGCAAGATGAGGCTGCTGAAAGGTCTG
T

(46) PDFMD-Son/Bovine Perforin clone (178 bp)

TCCTTTCCAGTTTGGCACAGAGGTCCTCTCAGGACCCACTTCTCTCACCACACTGGCCCACTCCGG
TTTCCTGGAGGATTCCCCAGAAGCCCTTGGGGGGCATACTGCAGGCACCTCTCCCCCGTCAGGT
GAGGCAAGCATTTGACCTGGTAGGAGAATTTCAGGAGACCGTGGTCCA

(47) PDFMD-Banganga/Bovine XCL1 clone (147 bp)

AATCTGTGCTGATCCTCAAGCTGCCTGGGTGAAAAAAGCCGTCCAAAAGATAGACAGGAAAAAT
ATAAGCCAGGCCAAGCCTACAGGAGCCTAGCAATCCACCAATACAACTGTGACCCTGACTGGGT
AGTGACCTTCCAGCACTTT

(48) PDFMD-Kukadi/Bovine COX-2 clone (80 bp)

GCACAAATCTGATGTTTGCATTCTTTGCCCAGCACTTCACCCATCAATTTTTCAAGACAGATTTTG
AACGAGGACCAGCT

(49) PDFMD-Mayurakshi/Bovine iNOS clone (82 bp)

GGCCCAGGAAATGTTCGAACACATCTGCAGACACGTGCGTTATGCCACCAACAACGGCAACATC
AGGTCGGCCATCACTTGT

(50) PDFMD-Purna/Bovine ICAM-1 clone (146 bp)

TGAAGAACTCAACCTGAGCTGCCTGGTGTCTGGCGGGGCCCCCCGGGCCCACCTCTCCGTGGTG
CTGCTCCGAGGGGAGGAGGAGCTGGCCCGGCAGCCCGTGGGAAAGGGGGAGCCCGCCAAGG
TCATGTTCACAGTGCAGTCGA

(51) PDFMD-Tista/Bovine ICAM-3 clone (169 bp)

TGAAGAACTCAACCTGAGCTGCCTGGTGTCTGGCGGGGCCCCCCGGGCCCACCTCTCCGTGGTG
CTGCTCCGAGGGGAGGAGGAGCTGGCCCGGCAGCCCGTGGGAAAGGGGGAGCCCGCCAAGG
TCATGTTCACGGTGCAGTCGAGAAGAGAGGACCATGGCACCAAT

(52) PDFMD-Machkund/Bovine BAD clone (151 bp)

CTTTTCTGCAGGCCTTATGCAAAACGAGGCTCGGATTCGTTCCTTTCGGAAGACTGAGGTCTGAC
CCAAAGTTCGGTCCCCTTCCGGTGTGTGCGAGGACCACGGAGGGGCTCGGCCCCCATCGGAAG

TTGGAAGTTTTTCCGCCCTTACC

(53) PDFMD-Brahmputra/Bovine TLR2 clone (503 bp)

GATGCCTGGCCCTTCCTTCAAACCTTGGTTTTAAGGCAGAATCGTTTGAAATCACTAGAAAAAAC
GGGAGAACTTTTGCTTACTCTGGAAAATCTGAATAACCTTGATATCAGTAAGAATAATTTTCTTTC
AATGCCTGAAACTTGTCAGTGGCCAGGAAAAATGAAACAGCTGAACTTATCCAGCACAAGGATA
CACAGTTTAACCCAGTGCCTTCCCCAGACCCTGGAAATTTTAGATGTTAGCAATAACAATCTCGA
TTCATTTTCTTTGATTTTGCCACAACTCAAAGAACTTTATATTTCCAGAAATAAGTTGAAGACTCT
ACCAGATGCCTCCTTCTTACCCGTGTTATCAGTTATGAGAATTAGCAGAAATATAATAAATACTTT
CTCAAAGGAACAACTTGATTCTTTTCAGCAACTGAAGACGTTGGAGGCCGGTGGCAACAACTTC
ATTTGCTCCTGTGACTTCCTGTCCTTCACACAGGGACAGCAGGCACT

(54) PDFMD-Gomati/Bovine TLR4 clone (473 bp)

GACAAATTCTCAGGGGACGATACAGACTTATTTAATTGTTTGGCAAATGTTTCTGTGATTTCTCT
GTTGAGTATATCTTTAGGAAGTCTACAAGCCCTTCTTAAAGATTTTAGATGGCAACACTTAGAAA
TTATTAACTGTGACTTTGACAAGTTTCCTGCATTGAAGCTCAGTTCTCTCAAAAAGTTTGTTTTCA
CAGACAACAAAGGTATAAGCACTTTTACTGAGTTTCAGCTACCAAGCCTTCAGTATCTAGATCTC
AAAAGAAATCACTTGAGTTTCAAGGGTTGCTGTTCTCACACTGATTTTGGGACAACCAACCTGAA
GCATTTAGATCTGAGCTTCAATGATGTCATTACCTTAGGTTCAAACTTCATGGGCTTAGAGCAAC
TAGAACACCTGGATTTTCAGCATTCCACTCTGAAACAGATCAATGCTTTTTCAGCATTCCTATCAC
TCAGAAACCTCCGCTA

(55) PDFMD-Saraswati/Bovine TLR6 clone (206 bp)

CAAAGCAGGGAACAATCCATTCCAATGTTCCTGTGAGCTAAGAGACTTTATCCAAAGTGTAGGC
CAAGTATCCAGTGACGTGGTAGAGGGCTGGCCTGAGTCTTATAAGTGTGACTATCCGGAAAGCT
ACAAGGGAACCCCTCTAAAGGACTTCCAGGTATCTGAGCTATCCTGCAACACAGCTCTGCTGATT
GTCACCATTGTGG

(56) PDFMD-Kosi/Bovine TLR9 clone (377 bp)

CAGATGTGGGCGGAGGGAGACCTCTATCTCTGCTTTTTCAAAGGCTTGAGGAACCTGGTCCAGC
TGGACCTGTCCGAGAACCATCTGCACACCCTCCTGCCTCGTCACCTGGACAACCTGCCCAAGAGC
CTGCGGCAGCTGCGTCTCCGGGACAATAACCTGGCCTTCTTCAACTGGAGCAGCCTGACCGTCC
TGCCCCGGCTGGAAGCCCTGGATCTGGCAGGAAACCAGCTGAAGGCCCTGAGCAACGGCAGCC
TGCCGCCTGGCATCCGGCTCCAGAAGCTGGACGTGAGCAGCAACAGCATCGGCTTCGTGATCCC
CGGCTTCTTCGTCCGCGCGACTCGGCTGATAGAGCTTAACCTCAGCGCCAATGCCCT

(57) PDFMD-Lohit/Bovine 18S RNA clone (90 bp)

CTGAGAAGACGGTCGAACTTGACTATCTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGG
TGAACCTGSGGAAGGATCATTAACGGA

(58) PDFMD-Indravati/Bovine GAPDH-1 clone (82 bp)

GCGATACTCACTCTTCTACCTTCGATGCTGGGGCTGGCATTGCCCTCAACGACCACTTTGTCAAG
CTCATTTCCTGGTACGA

(59) PDFMD-Chandrabhaga/Bovine GAPDH-2 clone (100 bp)

GGCGTGAACCACGAGAAGTATAACAACACCCTCAAGATTGTCAGCAATGCCCCCTGCACCACCA
ACTGCTTGGCCCCCCTGGCCAAGGTCATCCATGACCACTTTGGCATCGTGGAGGG

(60) PDFMD-Pravra/Bovine β-actin clone (153 bp)

CGATGAAGATCAAGTCATTGCTCCTCCCGAGCGCAAGTACTCGGTGTGGATCGGAGGCTCCATC
CTGGCCTCCCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCTGGCC
CCTMCATTGTCCACCGCAAATGCTT

(61) PDFMD-Ken/Bovine IVD clone (156 bp)

TACCAGGGAACAAAACCTGCACCTACTGCATTGCAAGCACAGAGTCTTAACCATTGGATTGCCAT
GGAAGTCCCCAGTTACATTTTAAGTCCAGCAAGACCACCATTTTAGCCACATTCATCTCCAACAC
AGTAGGGAGCTGGCTGGAAACTGAAC

4.4 Investigation of natural FMD outbreaks at Izatnagar and Papum Pare and comparison with FMDV vaccinated cattle at Mukteshwar

PBMCs extraction was unsuccessful but 500 µl of whole blood was collected in RNAprotect® Animal blood tubes (Qiagen) and total RNA was extracted. IL-2 cytokine mRNA expression level in the total RNA was detected in one-step real-time PCR using comparative CT (ΔΔ) type of experiment. Amplification plot (Figure 25) and Melt curve analysis (Figure 27, Figure 28) were depicted. Expression levels of IL-2 mRNA were depicted (Figure 26) in the form of Relative Quantitation (RQ) values vs Target/Sample. Increased mRNA expression levels of IL-2 were found in FMDV infected breeding bulls than the vaccinated cattle (No. – 223, 411, 418, 766). One odd sample of Mithun did not show higher expression level of IL-2 mRNA.

Table 7. List of cloned, sequenced and preserved bovine cytokines, chemokines, chemokine receptors, toll-like receptors and others

Sr. No.	Item	Product Size, bp	Sr. No.	Item	Product Size, bp	Sr. No.	Item	Product Size, bp
1	IL-1α [L]	124	23	IFN-γ	509	45	Integrin β3[L]	137
2	IL-1β	420	24	TNF-α[L]	360	46	Galectin-3[L]	97
3	IL-2	345	25	TNF-αSF1[A]	200	47	Granzyme A[L]	121
4	IL-2Rα [A]	130	26	TGFβ-1	264	48	Granulysin[L]	128
5	IL-4	289	27	TGFβ-2[A]	135	49	Perforin[L]	178
6	IL-6	491	28	TGFβ-3[A]	116	50	XCL1[L]	147
7	IL-8	209	29	GM-CSF [T]	87	51	COX-2 [T]	80
8	IL-9 [A]	149	30	CSF[A]	158	52	iNOS [T]	82
9	IL-10	329	31	MCP-1	209	53	ICAM-1[L]	146
10	IL-12p40 [K]	186	32	CCL3	81	54	ICAM-3[L]	169
11	IL-13[A]	111	33	CCL5[L]	82	55	BAD	151
12	IL-15[We]	74	34	CCR5[L]	119	56	TLR2	503
13	IL-16[C]	73	35	CXCL1[L]	93	57	TLR4	473
14	IL-17A[A]	138	36	CXCL2[L]	91	58	TLR6 [M]	206
15	IL-18	347	37	CXCL6[L]	139	59	TLR9	377
16	*IL-20[A]	149	38	CXCR4[L]	113	60	18S RNA[L]	90
17	*IL-21[A]	132	39	CD11a[L]	102	61	GAPDH-1[T]	82
18	*IL-25[A]	100	40	CD11b[L]	139	62	GAPDH-2[T]	100
19	IL-27p28[A]	111	41	CD11c[L]	158	63	β-actin	153
20	IL-32[A]	176	42	CD14[L]	81	64	IVD[A]	156
21	IL-34[A]	165	43	CD18[L]	133			
22	IFN-αR1[L]	157	44	CD68[L]	164			

Primers designed with the help of NCBI Primer-Blast: [A];
Primers reported by: [C] - Coussens *et al.*, 2004; [K] - Konnai *et al.*, 2003; [L] - Lahmers *et al.*, 2006; [M] - Menzies and Ingham, 2005; [T] - Taubert and Hermosilla, 2008; [We] - Werling, 2002; Other primers or sequences were procured commercially from Pierce-Endogen or provided by laboratory
*Sequencing not done or machine unable to read

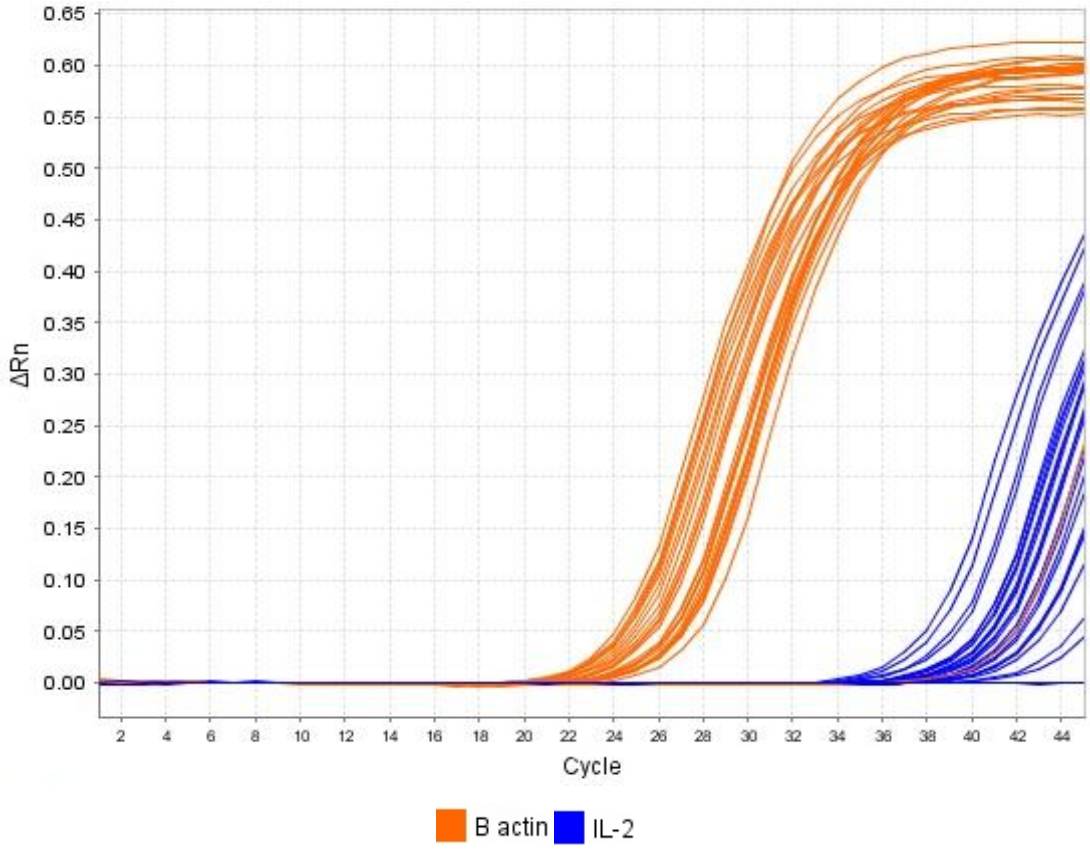


Figure 25. Amplification plot

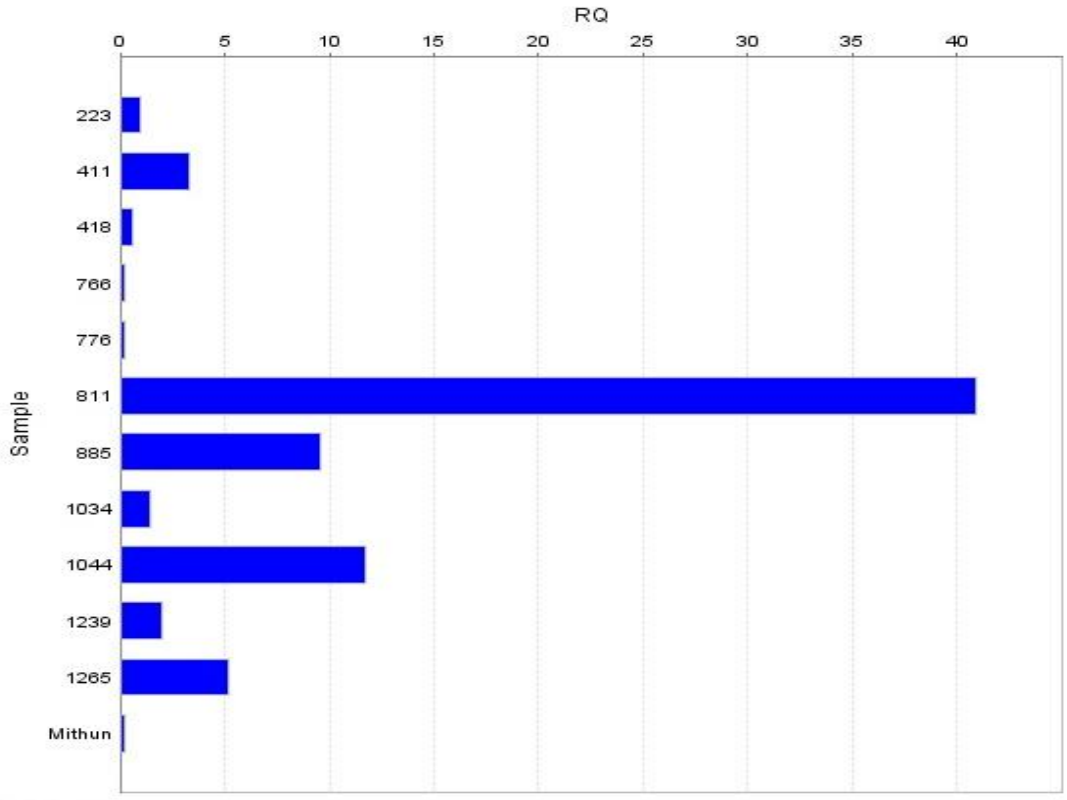


Figure 26. IL-2 mRNA expression levels in FMD infected breeding bulls and cattle vaccinated against FMD

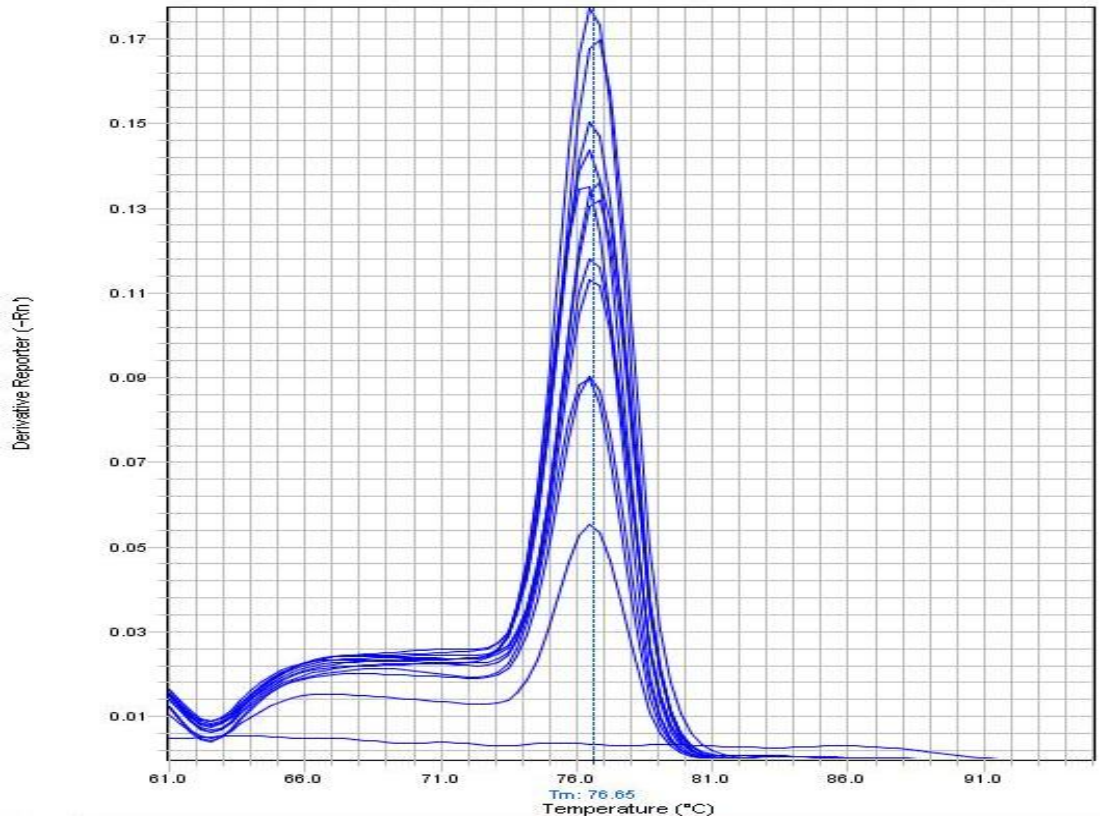


Figure 27. Melt curve of IL-2

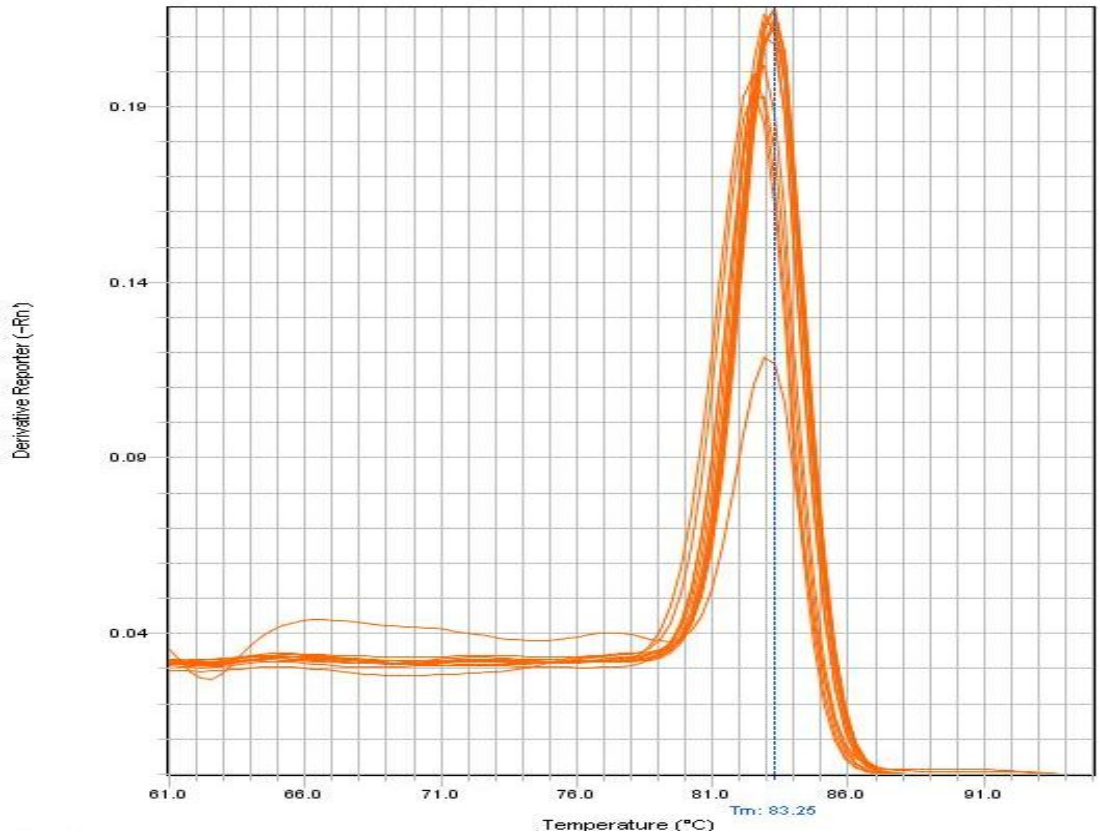


Figure 28. Melt curve of β-actin

4.5 Investigation of natural FMD outbreak in dairy cows at Kashipur

Collected serum samples (20, crossbred adult cows) were from either i) in-contact apparently healthy, ii) freshly infected showing clinical signs, iii) attacked once before 1 to 4 months before sample collection and iv) those attacked twice by FMD clinically. Laboratory findings showed that cows were infected with FMDV Type 'A' virus. These cows were vaccinated against FMDV using commercially available vaccine which was regularly used for prevention at the farm. History indicated that some of the cows (R-54, R-57) were introduced in the farm from distant areas. So, first to check serum antibody levels, representative samples were tested in liquid phase blocking enzyme linked immunoassay (LPBE). LPBE titers of Type 'O', 'A' and 'Asia 1' were indicated in the brackets respectively for individual cow serum samples tested, A-12 (2.28, 2.28, 1.98), A-29 (1.98,>2.4, 1.98), B-1 (1.98, 2.28, 2.28), B-4 (>2.4, >2.4, >2.4), M-51 (>2.4, >2.4, >2.4), M-53 (2.28, 2.28, >2.4), M-54 (1.98, >2.4, 2.28), R-57 (1.68, 1.98, 1.98). Results indicated good protective level of antibody titers in most of the cows to all three types including one which was introduced in the farm.

When tested in differentiation between infected and vaccinated animals (DIVA) most of the animals irrespective of their group indicated presence of NSP (titers more than 0.3) but in few infected animals H-65, R-57 DIVA titers were below 0.3. Hence, the division of groups was made on the basis of clinical outcome. History was utilized while comparing the results obtained in real-time PCR studies.

1. In-contact apparently healthy cows – A-12, A-29, B-1, M-53, M-54
2. Clinical FMD noticed early in cows – T-61, T-69, B-4, H-65, R-54, R-57
3. Clinical FMD noticed only once in cows – B-6, B-10, B-17, B-40, M-57, T-70
4. Clinical FMD noticed twice in cows (re-infected) – B-19, M-51, M-56

20 ng of extracted total RNA was used in one-step real-time PCR using comparative CT (ΔΔ) type of experiment to test cytokine mRNA expression (IFN-α, IFN-β, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-16, IL-21, IL-27p28, TLR-7). Amplification plots (Figure 29, Figure 30, Figure 31; Figure 32) and Melt curve analysis were shown (Figure 33, Figure 34, Figure 35, Figure 36, Figure 37, Figure 38, Figure 39, Figure 40, Figure 41, Figure 42, Figure 43, Figure 44, Figure 45). Representative of the some of the products amplified in real-time PCR were electrophoresed. Specific amplified products were visualized and recorded with help of

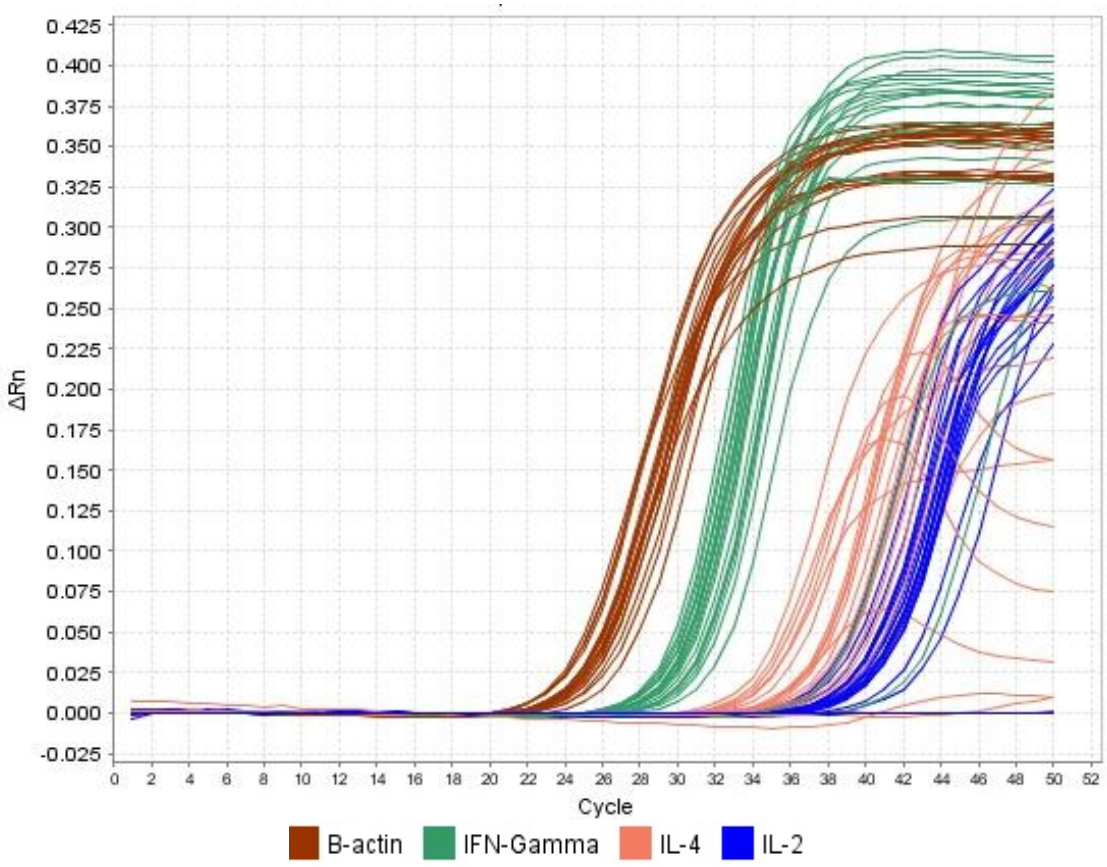


Figure 29. Amplification plot

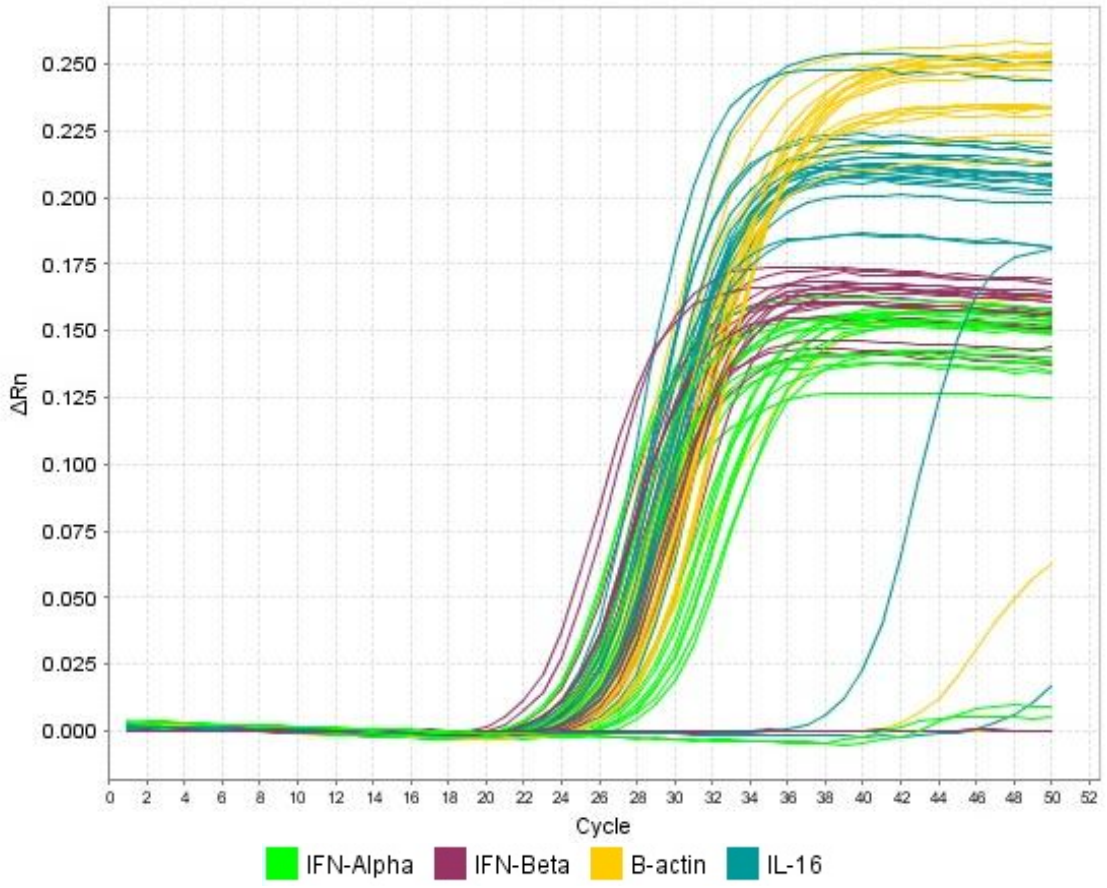


Figure 30. Amplification plot

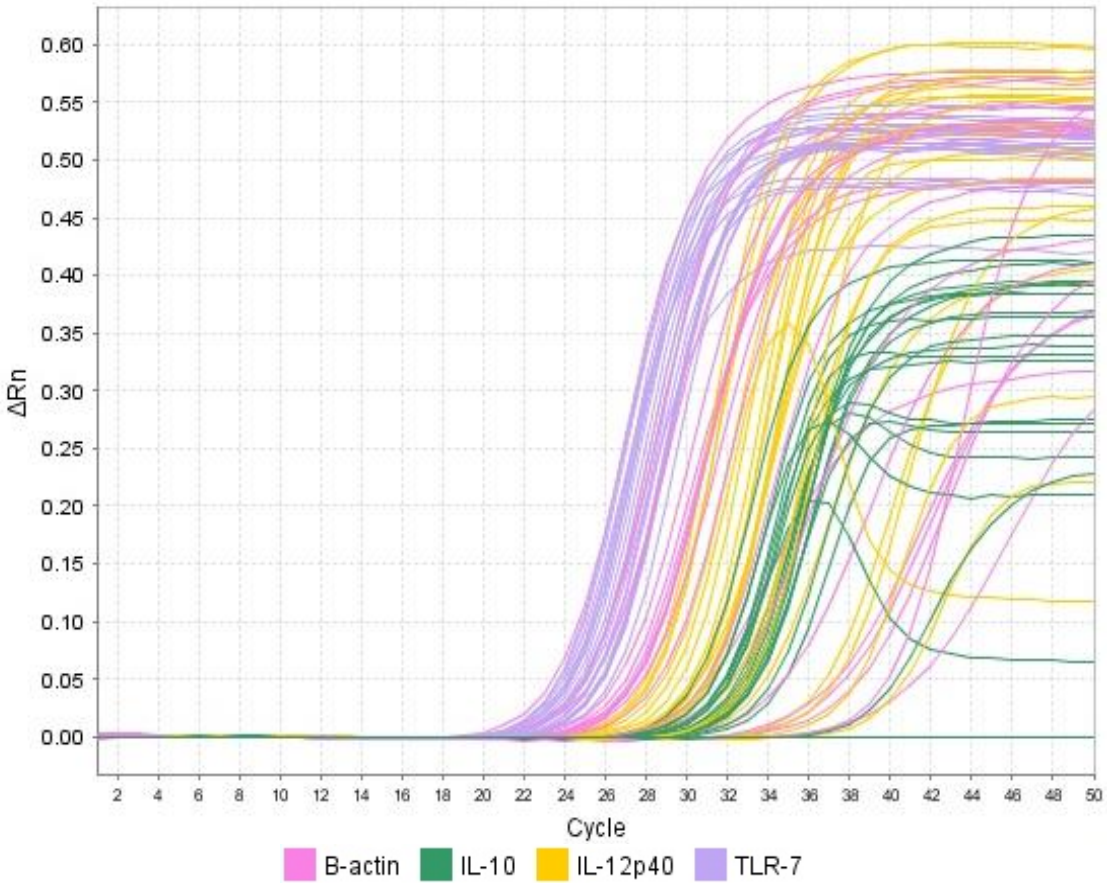


Figure 31. Amplification plot

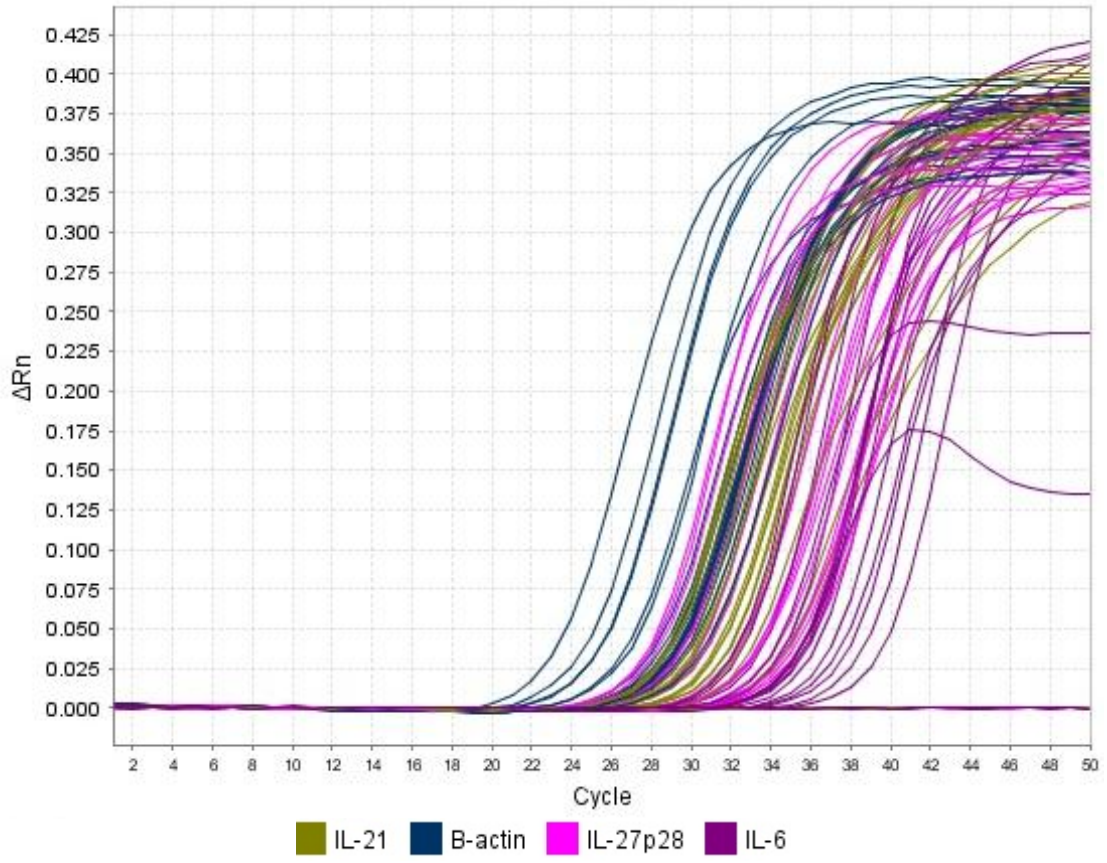


Figure 32. Amplification plot

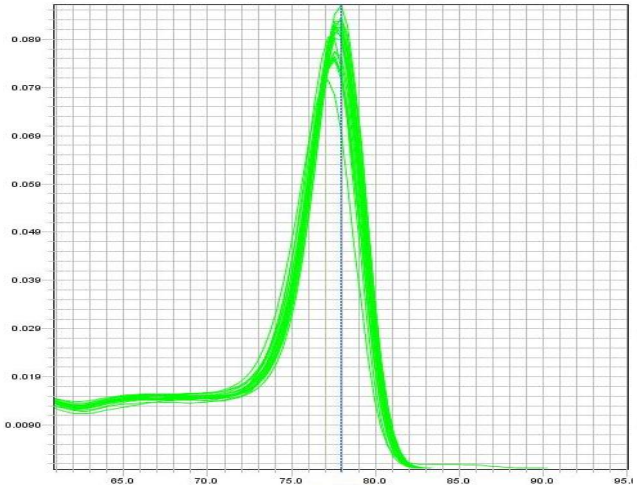


Figure 33. Melt curve of IFN-α

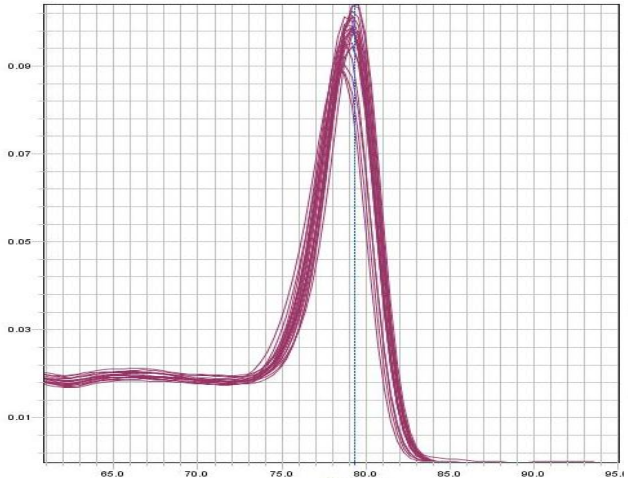


Figure 34. Melt curve of IFN-β

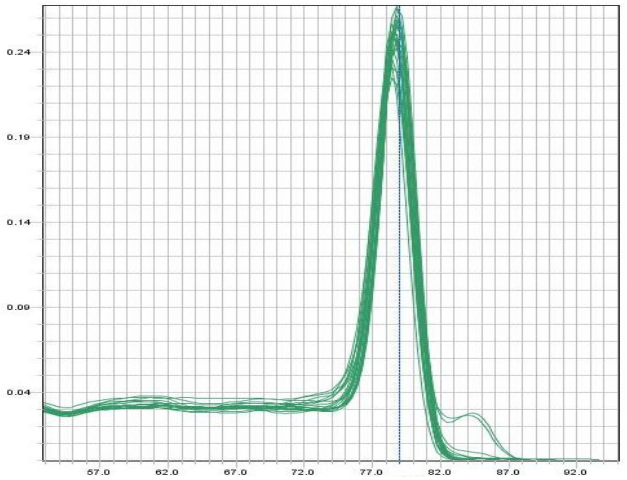


Figure 35. Melt curve of IFN-γ

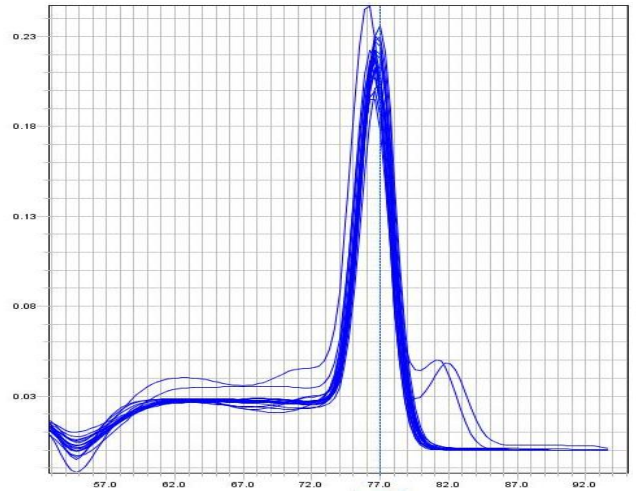


Figure 36. Melt curve of IL-2

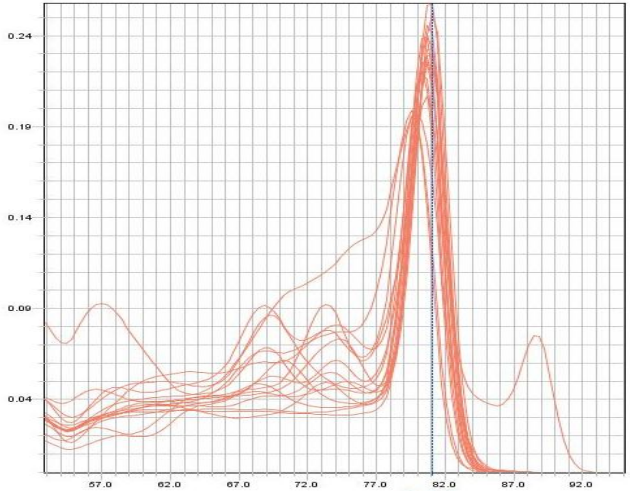


Figure 37. Melt curve of IL-4

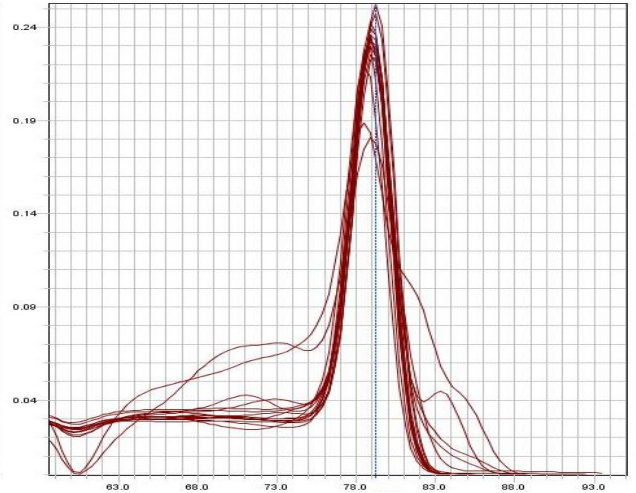


Figure 38. Melt curve of IL-6

For all figures: Y axis – Derivative Reporter (-Rn), X axis – Temperature

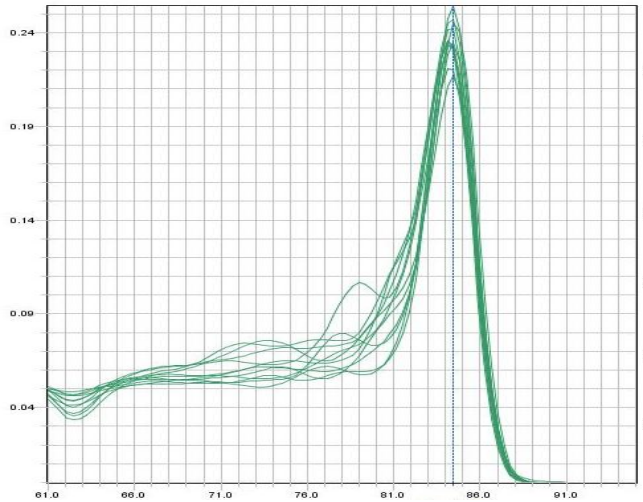


Figure 39. Melt curve of IL-10

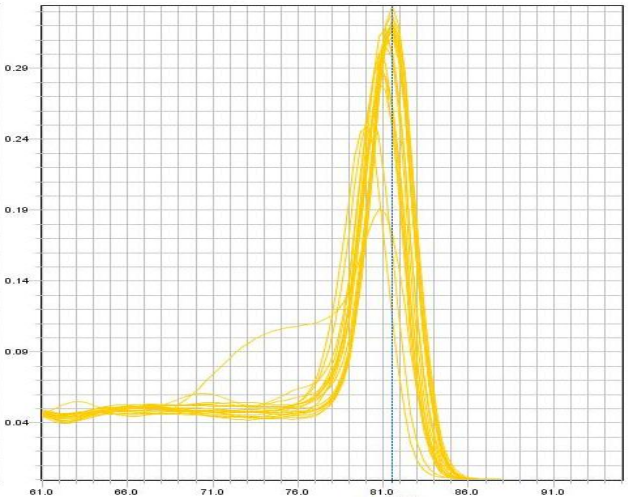


Figure 40. Melt curve of IL-12p40

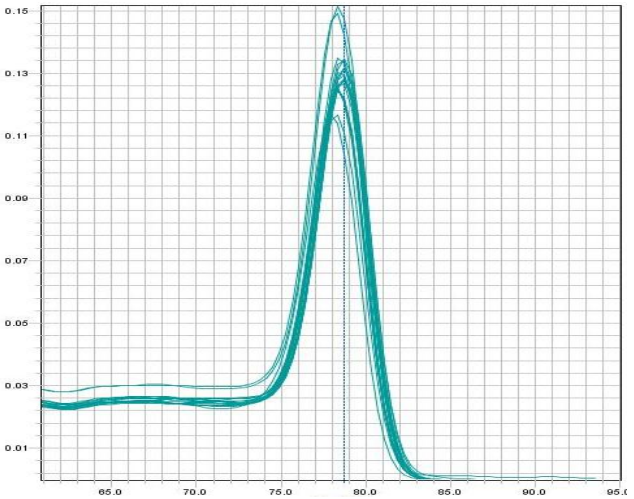


Figure 41. Melt curve of IL-16

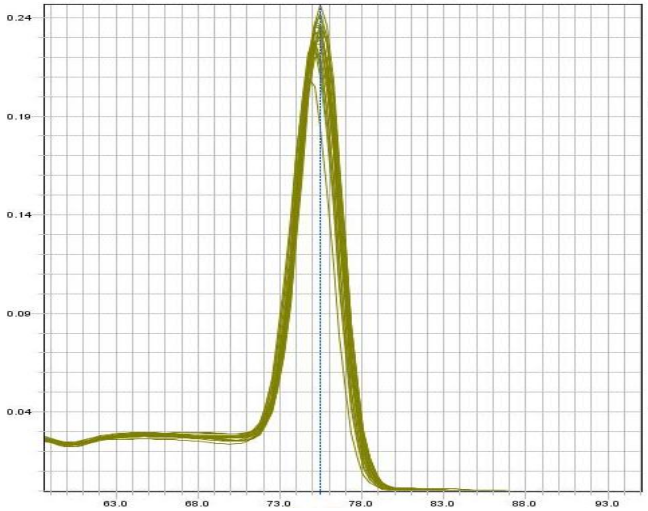


Figure 42. Melt curve of IL-21

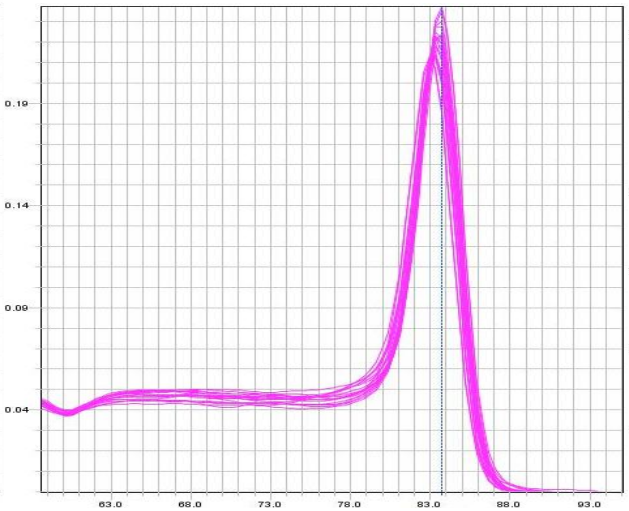


Figure 43. Melt curve of IL-27p28

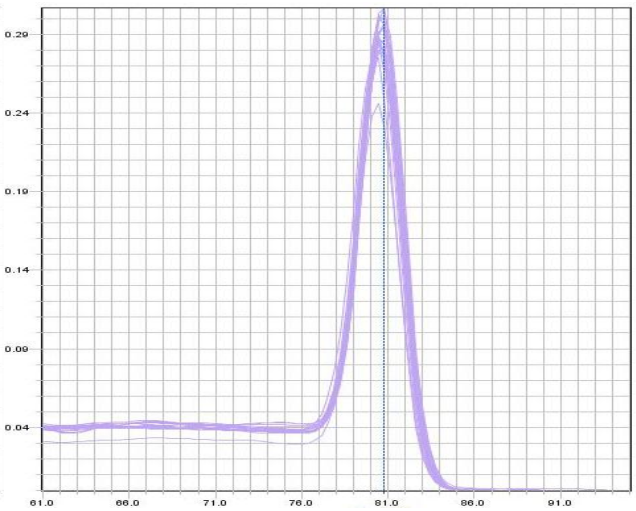


Figure 44. Melt curve of TLR7

For all figures: Y axis – Derivative Reporter (-Rn), X axis – Temperature

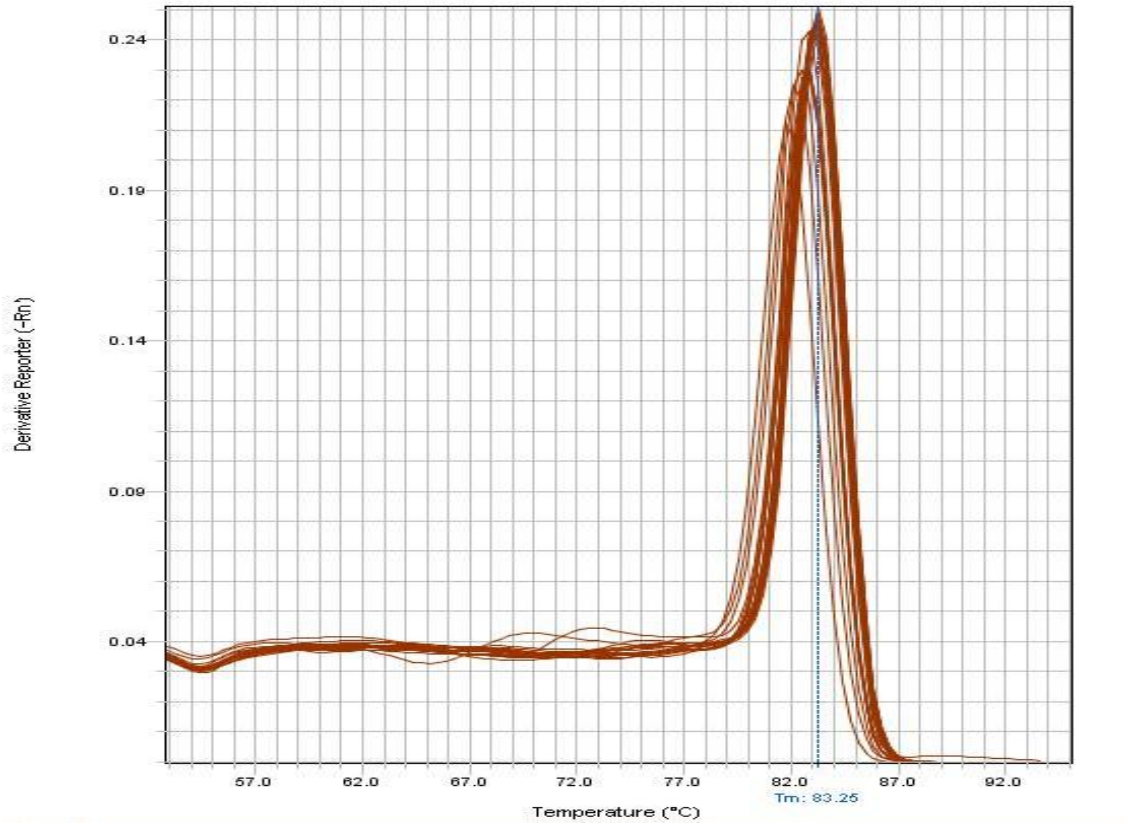


Figure 45. Melt curve of β-actin

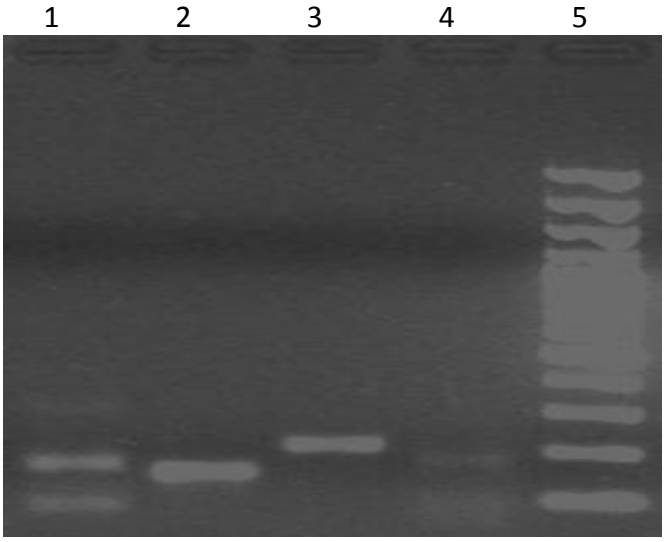


Figure 46. Amplified products in real time polymerase chain reaction

Lane 5: Marker

Lanes: 1 - IFN-γ (171 bp); 2 - IFN-β (97 bp);

3 - IL-2 (217 bp); 4 - IL-4 (181 bp)

gel documentation system (Figure 46). mRNA expression levels of 12 different cytokines studied were shown (Figure 47, Figure 48, Figure 49, Figure 50).

4.6 Investigation of vaccination and/or challenge study at Hyderabad

4.6.1 Serology

LPBE and DIVA results for plasma sample collected during the study are shown (Table 8). 3 AB3 based DIVA results indicated FMDV free status of vaccinated and control animals before challenge. Plasma samples showed gradual increase in the antibody titer against FMDV in vaccinated animals.

4.6.2 Level of mRNA expression after three days post FMDV infection

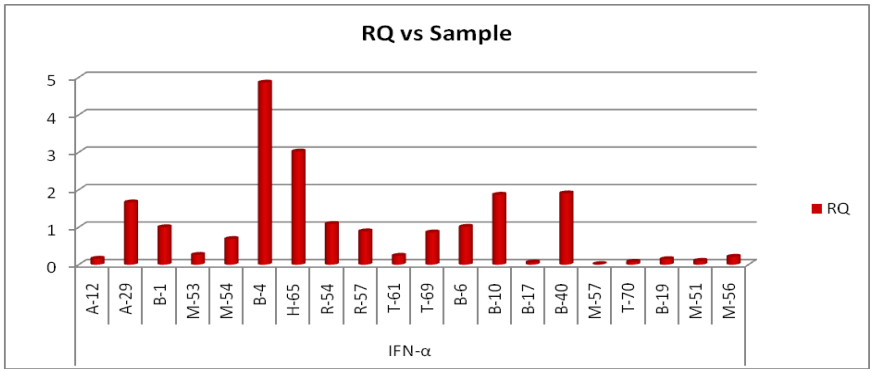
Total RNA was extracted from PBMCs of Animal No. 1022 (at 0 day before infection and 3 day after infection – FMDV Type 'O') by the use of RNeasy kit. Total RNA was quantified and diluted to 10 ng/µl. 30 ng of RNA was used in 10 µl of total volume of reaction and one-step real-time PCR was performed for 92 items and GAPDH-2, β-actin, multiple endogenous controls were used to analyze data by comparative CT (ΔΔ) type of experiment. Gene expression patterns were generated by the machine itself (RQ vs Target/Sample) in figure format. Amplification plots were shown (Figure 51). By manual notice of increase or decrease in the pattern was noticed in 3 dpi sample against 0 day sample as reference standard (Table 9). Most of the genes are appeared to be downregulated. Some of the cytokine mRNA was found increased when GAPDH-2 is taken as endogenous control (IL-1β, IL-2, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-15, IFN-αR1, MSR1, TLR10, CCL8) as compared to β-actin as endogenous control.

4.6.3 Investigation of cattle calves used in vaccination and challenge study at Hyderabad

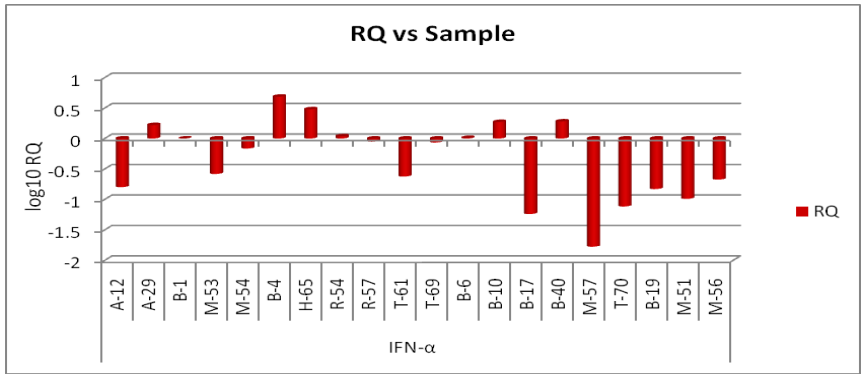
PBMCs from young cattle calves in the Vaccine Potency testing at Indian Immunological Limited, Hyderabad were used to extract RNA. Plasma/PBMCs samples were collected after 10 days post vaccination (DPV), 15 DPV, 21 DPV, 3 days post challenge (DPC), 5 DPC, 12 DPC, 0 days post infection (DPI) , 3 DPI, 5 DPI and 12 DPI from calves (vaccinated against FMD with monovalent inactivated Type 'O' FMDV vaccine and

Figure 47. mRNA expression levels of IFN-α (A, B), IFN-β (C, D), IFN-γ (E, F) in peripheral blood mononuclear cells of dairy cows experiencing natural Foot-and-Mouth disease virus infection

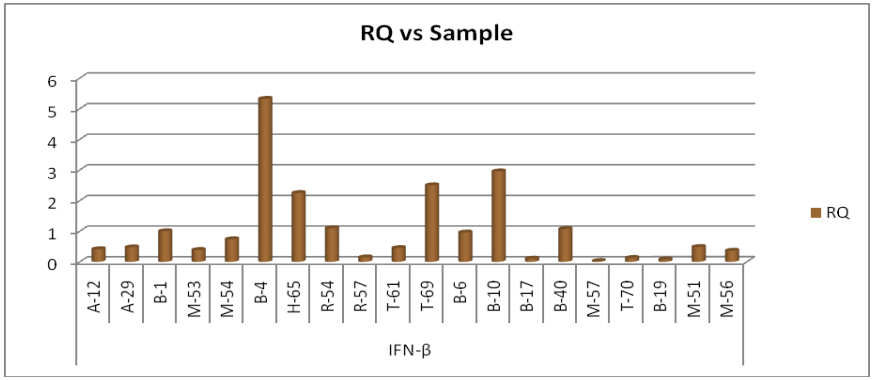
A-12	A-29	B-1	M-53	M-54	B-4	H-65	R-54	R-57	T-61	T-69	B-6	B-10	B-17	B-40	M-57	T-70	B-19	M-51	M-56
In-contact apparently healthy					Clinical FMD noticed early (10 days of sample collection)						Clinical FMD noticed only once						Clinical FMD noticed twice/Re-infected		


RQ vs Sample
5
4
3
2
1
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IFN-α
RQ

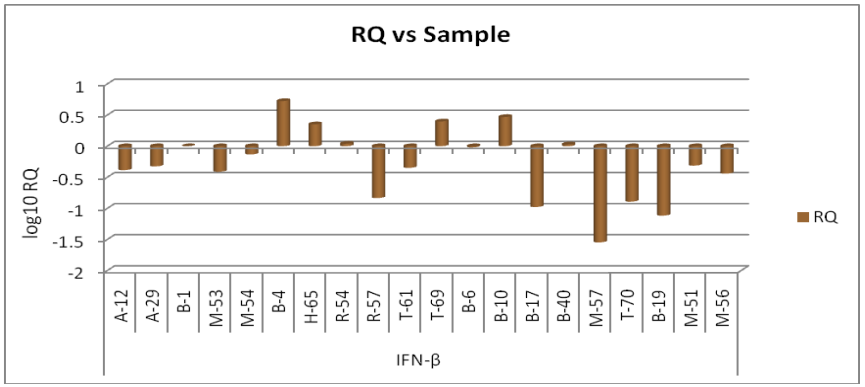
(A)


RQ vs Sample
1
0.5
0
-0.5
-1
-1.5
-2
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IFN-α
RQ

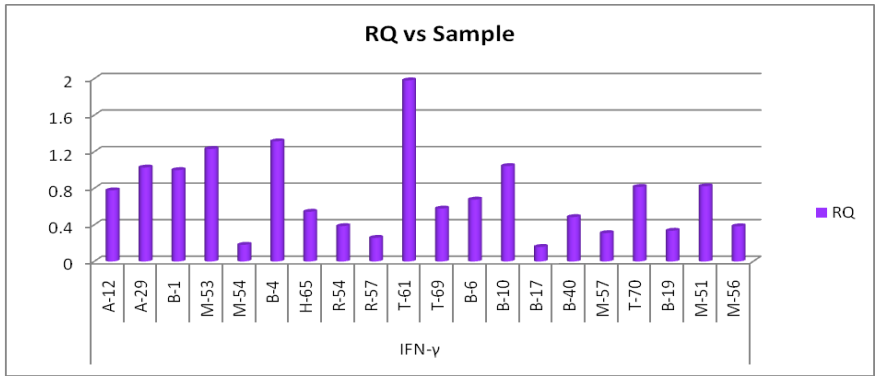
(B)


RQ vs Sample
6
5
4
3
2
1
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IFN-β
RQ

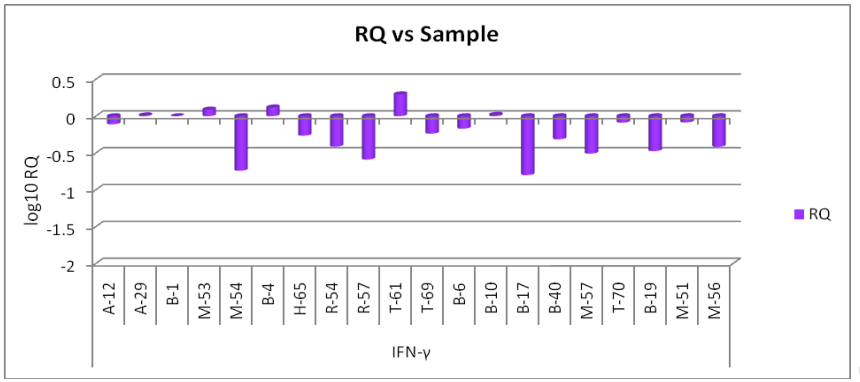
(C)


RQ vs Sample
1
0.5
0
-0.5
-1
-1.5
-2
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IFN-β
RQ

(D)


RQ vs Sample
2
1.6
1.2
0.8
0.4
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IFN-γ
RQ

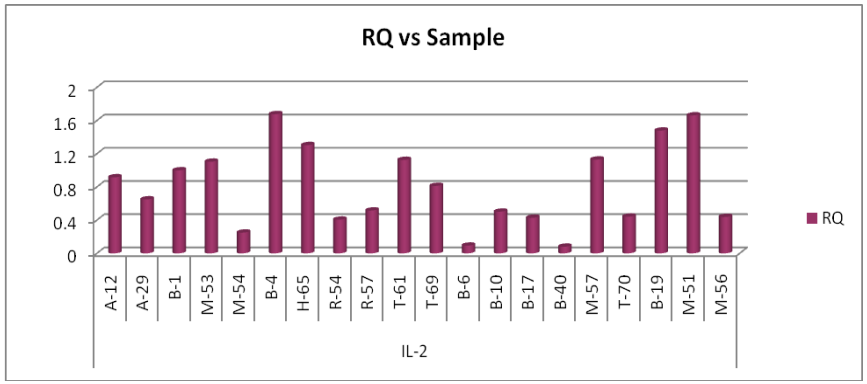
(E)


RQ vs Sample
0.5
0
-0.5
-1
-1.5
-2
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IFN-γ
RQ

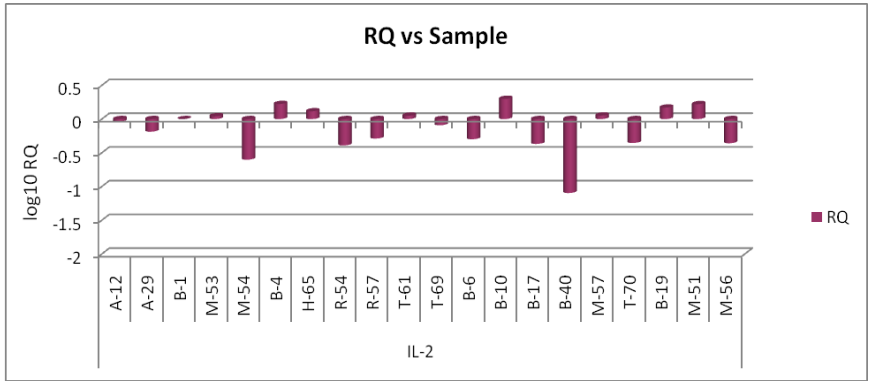
(F)

Figure 48. mRNA expression levels of IL-2 (A, B), IL-4 (C, D), IL-6 (E, F) in peripheral blood mononuclear cells of dairy cows experiencing natural Foot-and-Mouth disease virus infection

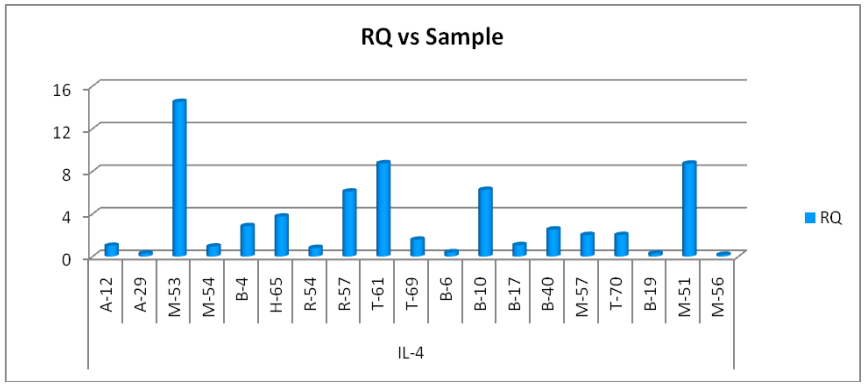
A-12	A-29	B-1	M-53	M-54	B-4	H-65	R-54	R-57	T-61	T-69	B-6	B-10	B-17	B-40	M-57	T-70	B-19	M-51	M-56
In-contact apparently healthy					Clinical FMD noticed early (10 days of sample collection)						Clinical FMD noticed only once						Clinical FMD noticed twice/Re-infected		


RQ vs Sample
2
1.6
1.2
0.8
0.4
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-2
RQ

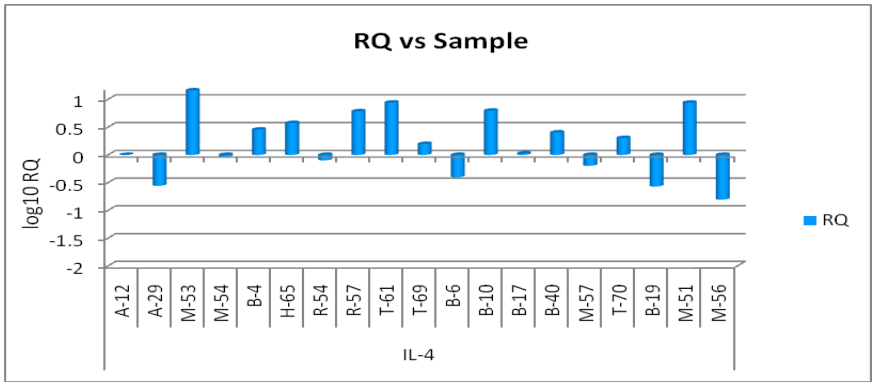
(A)


RQ vs Sample
0.5
0
-0.5
-1
-1.5
-2
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-2
RQ

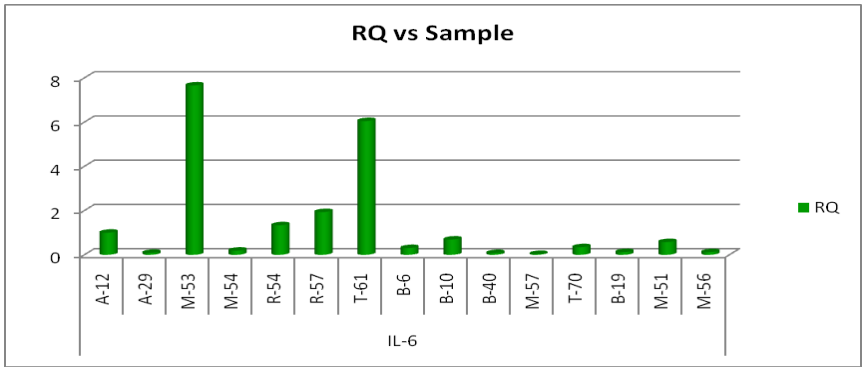
(B)


RQ vs Sample
16
12
8
4
0
A-12
A-29
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-4
RQ

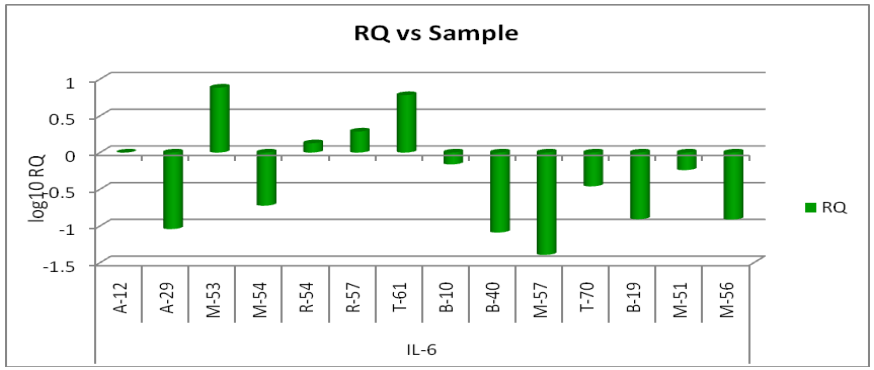
(C)


RQ vs Sample
1
0.5
0
-0.5
-1
-1.5
-2
log10 RQ
A-12
A-29
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-4
RQ

(D)


RQ vs Sample
8
6
4
2
0
A-12
A-29
M-53
M-54
R-54
R-57
T-61
B-6
B-10
B-40
M-57
T-70
B-19
M-51
M-56
IL-6
RQ

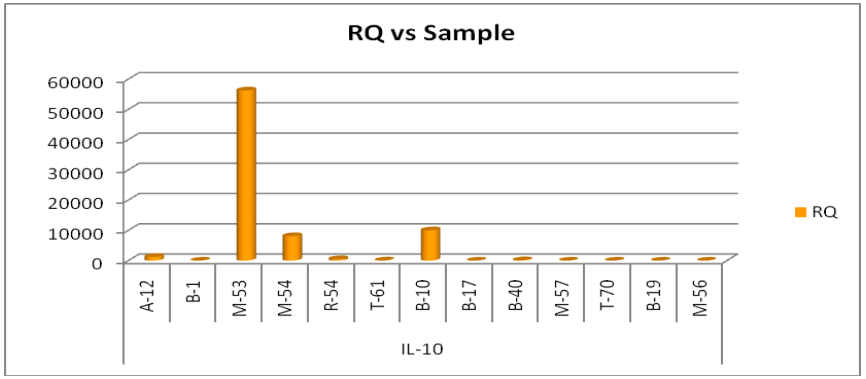
(E)


RQ vs Sample
1
0.5
0
-0.5
-1
-1.5
log10 RQ
A-12
A-29
M-53
M-54
R-54
R-57
T-61
B-10
B-40
M-57
T-70
B-19
M-51
M-56
IL-6
RQ

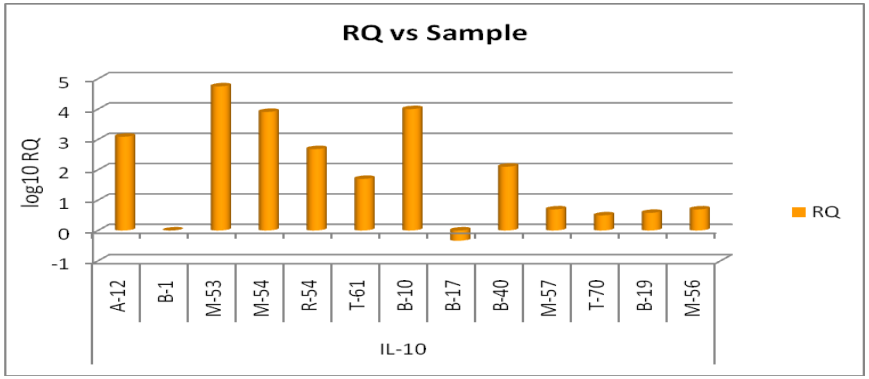
(F)

Figure 49. mRNA expression levels of IL-10 (A, B), IL-12p40 (C, D), IL-16 (E, F) in peripheral blood mononuclear cells of dairy cows experiencing natural Foot-and-Mouth disease virus infection

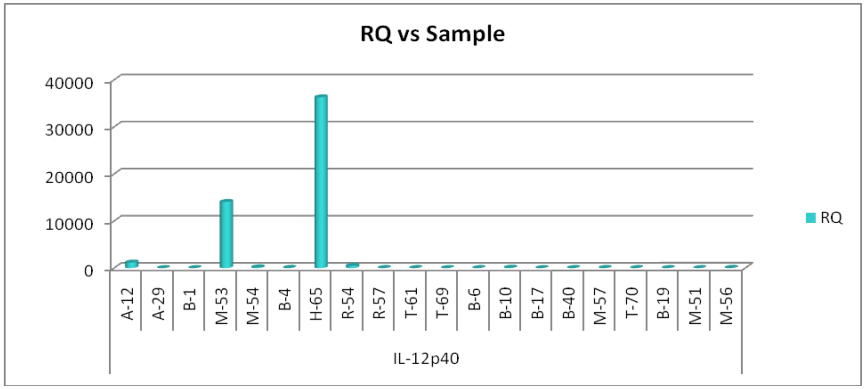
A-12	A-29	B-1	M-53	M-54	B-4	H-65	R-54	R-57	T-61	T-69	B-6	B-10	B-17	B-40	M-57	T-70	B-19	M-51	M-56
In-contact apparently healthy					Clinical FMD noticed early (10 days of sample collection)						Clinical FMD noticed only once						Clinical FMD noticed twice/Re-infected		


RQ vs Sample
60000
50000
40000
30000
20000
10000
0
A-12
B-1
M-53
M-54
R-54
T-61
B-10
B-17
B-40
M-57
T-70
B-19
M-56
IL-10
RQ

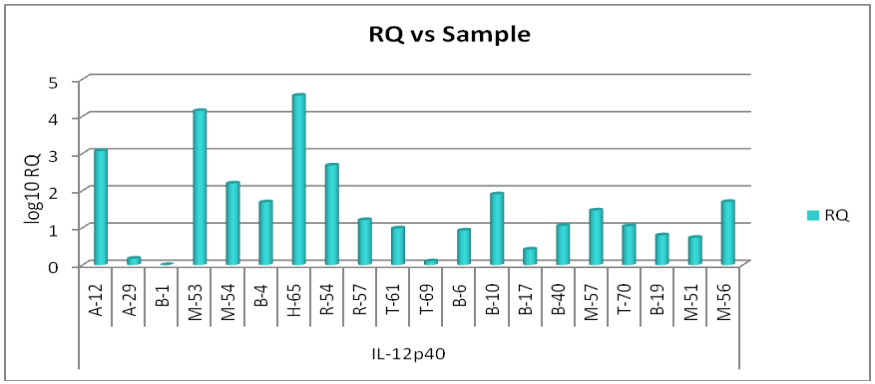
(A)


RQ vs Sample
log10 RQ
5
4
3
2
1
0
-1
A-12
B-1
M-53
M-54
R-54
T-61
B-10
B-17
B-40
M-57
T-70
B-19
M-56
IL-10
RQ

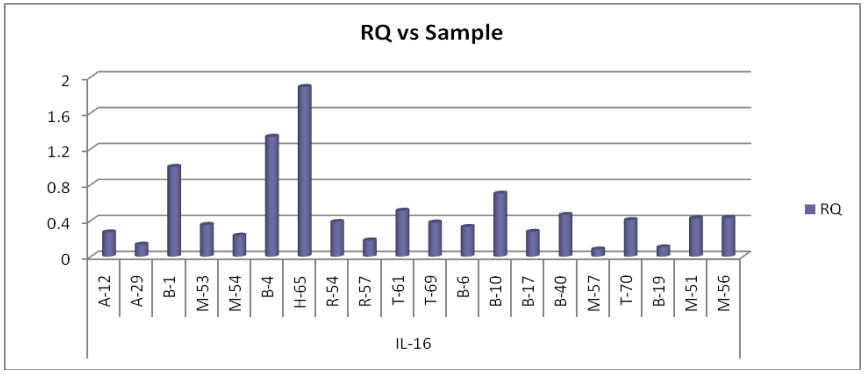
(B)


RQ vs Sample
40000
30000
20000
10000
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-12p40
RQ

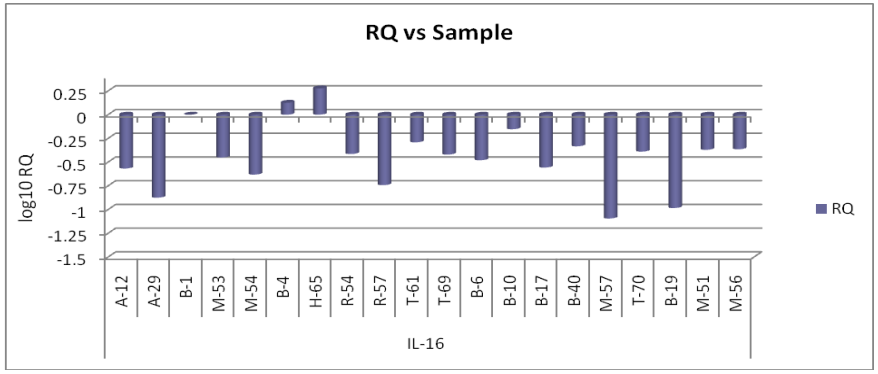
(C)


RQ vs Sample
log10 RQ
5
4
3
2
1
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-12p40
RQ

(D)


RQ vs Sample
2
1.6
1.2
0.8
0.4
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-16
RQ

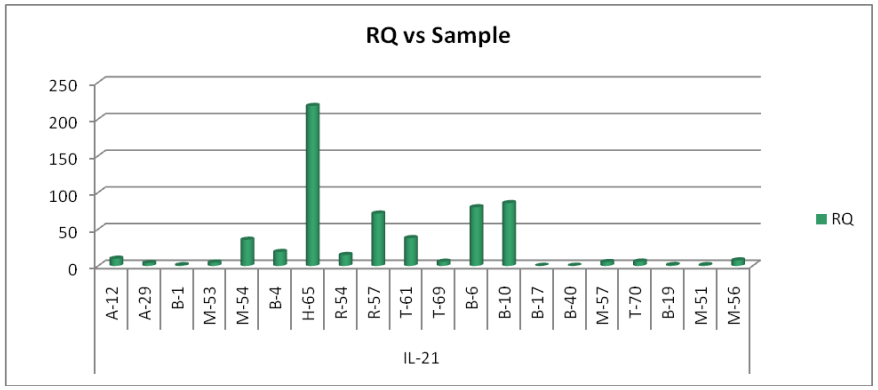
(E)


RQ vs Sample
log10 RQ
0.25
0
-0.25
-0.5
-0.75
-1
-1.25
-1.5
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-16
RQ

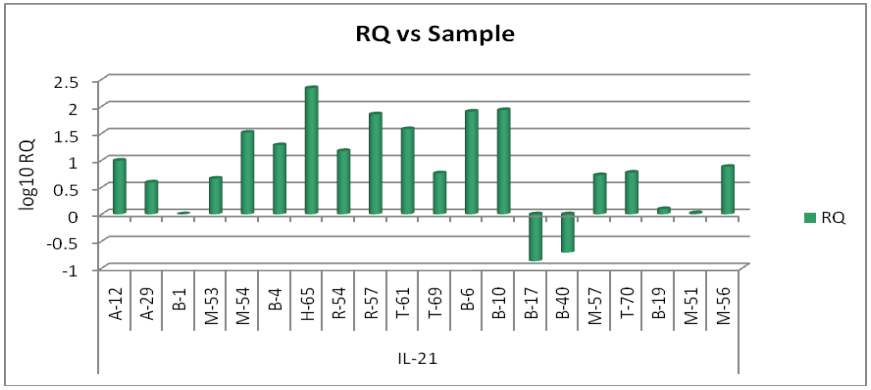
(F)

Figure 50. mRNA expression levels of IL-21 (A, B), IL-27p28 (C, D), TLR7 (E, F) in peripheral blood mononuclear cells of dairy cows experiencing natural Foot-and-Mouth disease virus infection

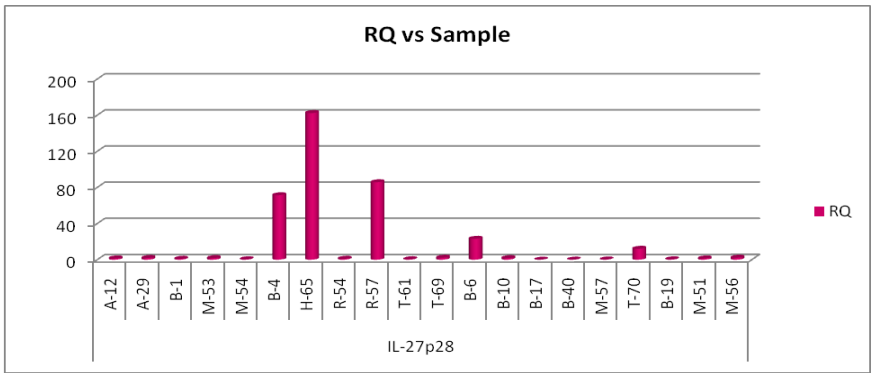
A-12	A-29	B-1	M-53	M-54	B-4	H-65	R-54	R-57	T-61	T-69	B-6	B-10	B-17	B-40	M-57	T-70	B-19	M-51	M-56
In-contact apparently healthy					Clinical FMD noticed early (10 days of sample collection)						Clinical FMD noticed only once						Clinical FMD noticed twice/Re-infected		


RQ vs Sample
250
200
150
100
50
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-21
RQ

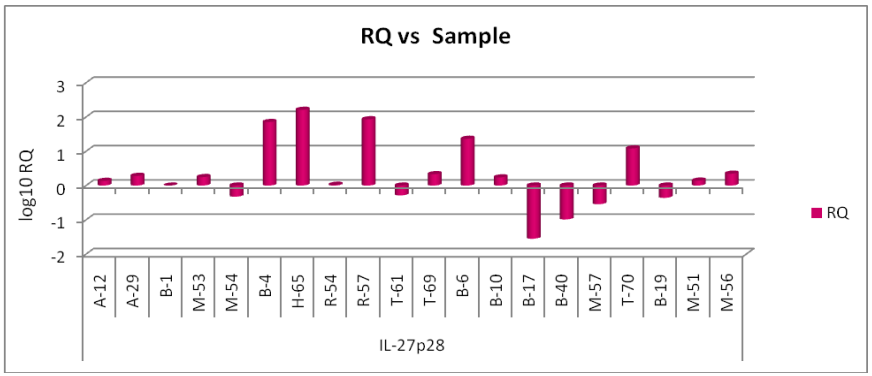
(A)


RQ vs Sample
2.5
2
1.5
1
0.5
0
-0.5
-1
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-21
RQ

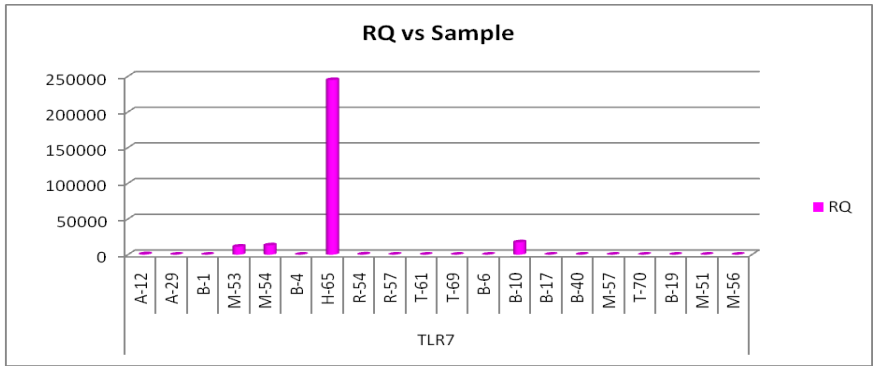
(B)


RQ vs Sample
200
160
120
80
40
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-27p28
RQ

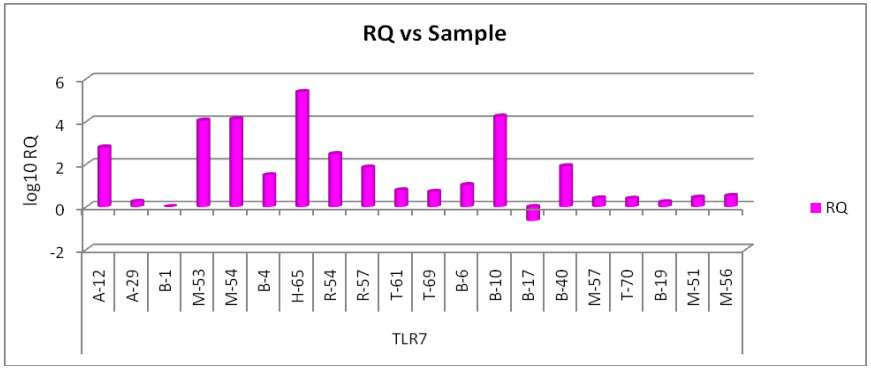
(C)


RQ vs Sample
3
2
1
0
-1
-2
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
IL-27p28
RQ

(D)


RQ vs Sample
250000
200000
150000
100000
50000
0
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
TLR7
RQ

(E)


RQ vs Sample
6
4
2
0
-2
log10 RQ
A-12
A-29
B-1
M-53
M-54
B-4
H-65
R-54
R-57
T-61
T-69
B-6
B-10
B-17
B-40
M-57
T-70
B-19
M-51
M-56
TLR7
RQ

(F)

challenged with Type 'O' FMDV). cDNA was prepared from extracted RNA and used to evaluate IFN-γ mRNA expression levels by employing two-step real-time PCR using comparative CT (ΔΔ) type of experiment. Five animals were included in vaccination and challenge study (No. – 209, 211, 212, 257, 1004) while two were kept as infection control (No. – 202, 1022). Amplification plot (Figure 52) and Melt curve analysis (Figure 53, Figure 54) were recorded. Gene expression data generated by the machine was recorded (Figure 55, Figure 56).

4.7 Vaccination study at Mukteshwar

4.7.1 Serology

LPBE and DIVA results for plasma and serum samples collected from young crossbred cattle calves (at Latoli farm of Dairy Section, Mukteshwar) vaccinated against FMD with trivalent inactivated FMDV vaccine (Types – 'O', 'A', 'Asia 1') are shown (Table 10).

4.7.2 IFN-γ expression levels in peripheral blood mononuclear cells and white blood cells after vaccination with trivalent inactivated FMDV vaccine

cDNA prepared from 500 ng of extracted RNA from PBMCs and WBCs was used in two-step real-time PCR using comparative CT (ΔΔ) type of experiment to analyze expression levels of IFN-γ mRNA. Amplification plots (Figure 57), Melting curve analysis (Figure 58, Figure 59) and IFN-γ mRNA expression data (Figure 60) when cDNA from PBMCs used were shown. Amplification plots (Figure 61), Melting curve analysis (Figure 62, Figure 63) and IFN-γ mRNA expression data (Figure 64) when cDNA from WBCs used were recorded.

Table 8. Results of plasma samples collected from young calves used in vaccine potency testing at Hyderabad in liquid phase blocking enzyme linked immunoassay and differentiation between infection and vaccination tests

	10 DPV				15 DPV				21 DPV				3 DPI				5DPI				12 DPI			
	Type 'O'	**Type 'A'**	**Type 'Asia 1'**	**DIVA OD**	**Type 'O'**	**Type 'A'**	**Type 'Asia 1'**	**DIVA**	**Type 'O'**	**Type 'A'**	**Type 'Asia 1'**	**DIVA OD**	**Type 'O'**	**Type 'A'**	**Type 'Asia 1'**	**DIVA OD**	**Type 'O'**	**Type 'A'**	**Type 'Asia 1'**	**DIVA OD**	**Type 'O'**	**Type 'A'**	**Type 'Asia 1'**	**DIVA OD**
209	1.68	<1.5	<1.5	0.089	1.98	<1.5	<1.5	0.146	1.98	<1.5	<1.5	0.125	1.98	<1.5	<1.5	0.115	2.28	<1.5	<1.5	0.105	>2.4	1.98	2.28	0.492
211	1.68	<1.5	<1.5	0.168	1.68	<1.5	<1.5	0.244	1.68	<1.5	<1.5	0.271	1.68	<1.5	<1.5	0.216	2.28	<1.5	<1.5	0.14	>2.4	1.98	1.68	1.678
212	<1.5	<1.5	<1.5	0.186	1.68	<1.5	<1.5	0.451	1.68	<1.5	<1.5	0.273	1.68	<1.5	<1.5	0.288	2.28	<1.5	<1.5	0.181	>2.4	1.68	<1.5	0.78
257	1.98	1.68	<1.5	0.104	>2.4	<1.5	1.68	0.107	>2.4	1.68	<1.5	0.098	>2.4	1.68	1.68	0.106	>2.4	<1.5	<1.5	0.101	>2.4	1.98	2.28	1.403
1004	<1.5	<1.5	<1.5	0.04	1.98	<1.5	<1.5	0.056	1.98	<1.5	<1.5	0.061	1.98	<1.5	<1.5	0.051	2.28	<1.5	<1.5	0.052	>2.4	<1.5	1.68	1.802
202	<1.5	<1.5	<1.5	0.037	<1.5	<1.5	<1.5	0.12	<1.5	<1.5	<1.5	0.035	<1.5	<1.5	<1.5	0.039	2.28	<1.5	<1.5	0.04	Animal Died			
1022	<1.5	<1.5	<1.5	0.062	<1.5	<1.5	<1.5	0.078	<1.5	<1.5	<1.5	0.094	<1.5	<1.5	<1.5	0.131	1.68	<1.5	<1.5	0.167	>2.4	<1.5	1.68	1.654

209, 211, 212, 257, 1004 – Vaccinated with inactivated monovalent FMDV vaccine Type 'O'- R2/75-then-challenged with FMDV Type 'O'- R2/75

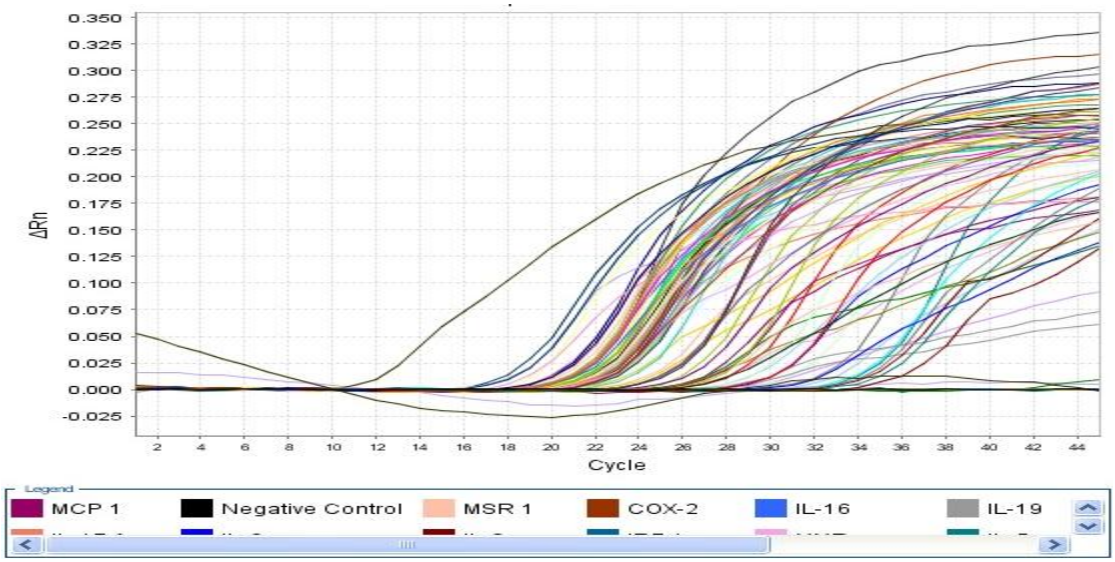
202, 1022 – Control calves experimentally infected with FMDV Type 'O'- R2/75

In LPBE: More than 1.68 Log_{10} titers considered as protective

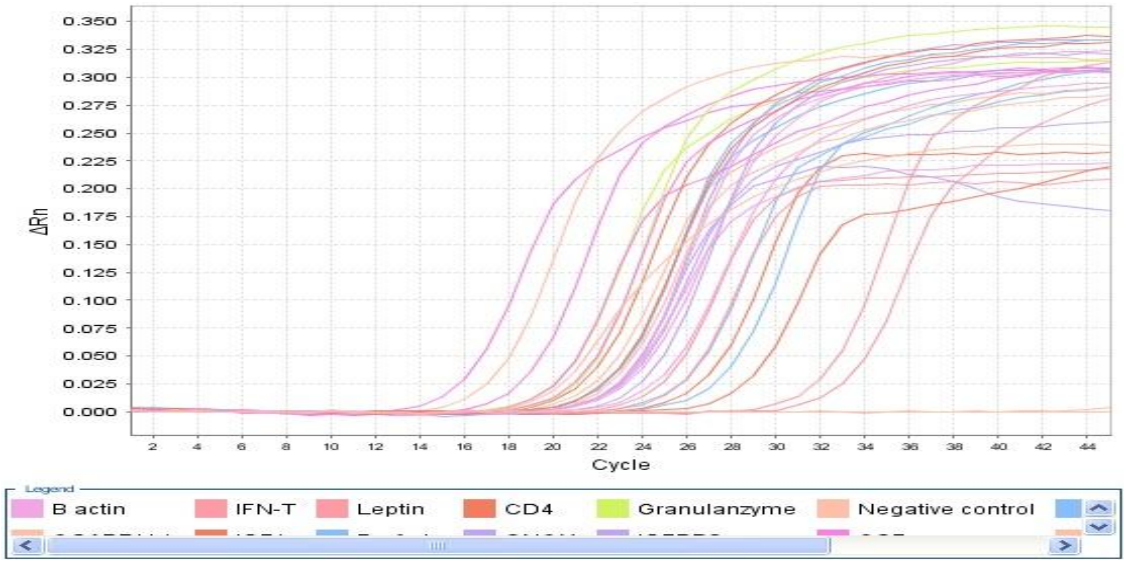
In DIVA: Positive control - 2.537 (Average value)

Negative control - 0.0825 (Average value)

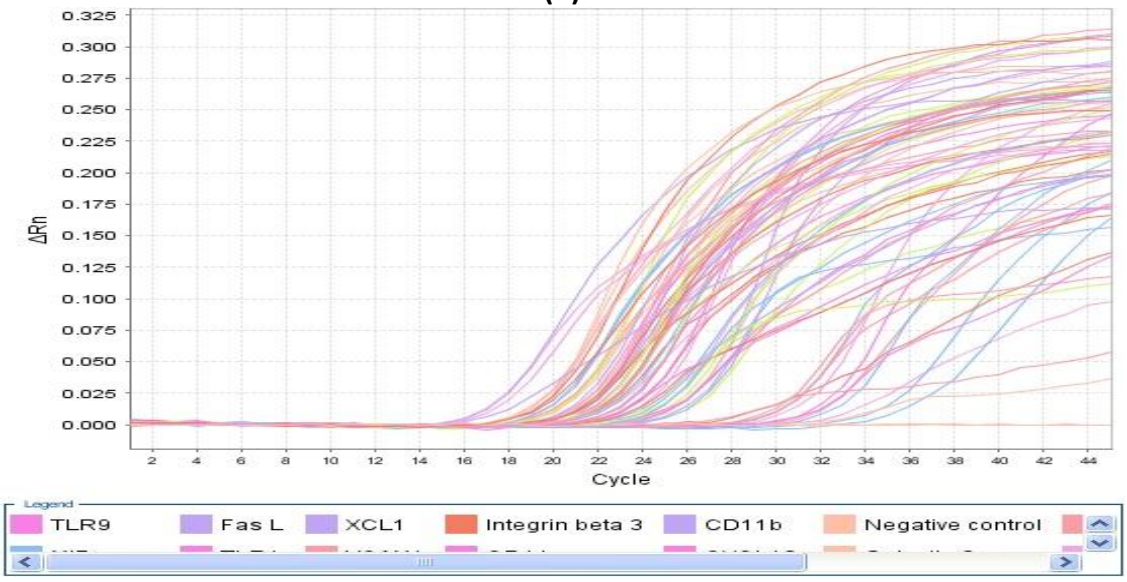
Values above 0.3 considered positive



(A)



(B)



(C)

Figure 51 (A, B, C). Amplification plots for different cytokines and others

Table 9. Change in mRNA expression level of cytokines, chemokines, toll-like receptors and others after three days post Foot-and-Mouth disease virus infection

Items	GAPDH^	β-actin^	Multiple^	Items	GAPDH	β-actin	Multiple
IL-1α				CCL3			
IL-1β				CCL5			
IL-2				CCL8			
IL-2Rα				CCR5			
IL-3*				CXCL1			
IL-4*				CXCL2			
IL-5				CXCL6			
IL-6				CXCL10			
IL-8				CXCR4			
IL-9				CD11a			
IL-10				CD11b			
IL-12p35				CD11c			
IL-12p40				CD14			
IL-13				CD18			
IL-15				CD68			
IL-16				Integrin β3			
IL-17A				FasL			
IL-18**				Galectin-3			
IL-19				Granzyme A			
IL-20				Granulysin			
IL-21				Perforin			
IL-22				Sel'protein P			
IL-25				XCL1			
IL-26**				MMR			
IL-27p28				MSR1			
IL-32				COX-2			
IL-34				iNOS			
IFN-α				LIF			
IFN-αR1				OSM			
IFN-β				CT-1			
IFN-γ				L-Selectin			
IFN-T				VCAM-1			
IRF-1#				ICAM-1			
TNF-α				ICAM-3*			
TNF-αSF1				BAX			
TGFβ-1				BAD			
TGFβ-2				TLR1			
TGFβ-3				TLR2			
GM-CSF				TLR3			
CSF				TLR4			
IGF-1				TLR5			
IGFBP-2				TLR6*			
IGFBP-3				TLR7			
VEGF				TLR8			
CD4?/IVD?				TLR9			
Leptin				TLR10			

Note: ^-Endogenous controls

Green colour indicates increased level of expression Red colour indicates decreased level of expression

Blue colour indicates – *Not amplified/unreadable (0 days); ** Not amplified (3 days); # No amplification

Table 10. Results of plasma and serum samples collected from young calves at Mukteshwar after vaccination in liquid phase blocking enzyme linked immunoassay and differentiation between infection and vaccination tests

No.	0 DPV				7 DPV				14 DPV				21 DPV			
	Type 'O'	Type 'A'	Type 'Asia 1'	DIVA OD	Type 'O'	Type 'A'	Type 'Asia 1'	DIVA OD	Type 'O'	Type 'A'	Type 'Asia 1'	DIVA OD	Type 'O'	Type 'A'	Type 'Asia 1'	DIVA OD
415p	<1.5	<1.5	<1.5	0.185	>2.4	<1.5	1.68	0.196	>2.4	1.98	1.98	0.352	>2.4	1.98	1.68	0.333
423p	<1.5	<1.5	<1.5	0.114	>2.4	<1.5	<1.5	0.147	>2.4	<1.5	<1.5	1.169	>2.4	<1.5	<1.5	1.023
424p	<1.5	<1.5	<1.5	0.123	>2.4	1.68	1.68	0.187	>2.4	1.68	1.98	0.618	>2.4	1.68	1.98	0.461
425p	>2.4	<1.5	<1.5	0.095	>2.4	<1.5	<1.5	0.109	>2.4	<1.5	1.98	1.2	>2.4	<1.5	1.98	1.036
444p	<1.5	<1.5	<1.5	0.16	>2.4	1.98	1.98	0.987	>2.4	1.98	1.98	1.903	>2.4	1.68	1.68	1.516
415s	<1.5	<1.5	<1.5	0.185	>2.4	1.68	1.68	0.207	>2.4	1.98	1.68	0.385	>2.4	1.98	1.68	0.303
423s	<1.5	<1.5	<1.5	0.114	>2.4	<1.5	<1.5	0.141	>2.4	1.68	1.68	1.008	>2.4	<1.5	<1.5	1.005
424s	<1.5	<1.5	<1.5	0.123	>2.4	1.68	1.98	0.218	>2.4	1.98	1.98	0.75	>2.4	1.68	1.98	0.472
425s	>2.4	<1.5	<1.5	0.095	>2.4	<1.5	<1.5	0.122	>2.4	<1.5	1.98	0.827	>2.4	<1.5	1.98	1.059
444s	<1.5	<1.5	<1.5	0.16	>2.4	1.98	1.98	0.752	>2.4	1.68	1.68	1.328	>2.4	1.98	1.98	1.512

415, 423, 424, 425, 444 – Young calves vaccinated on 05/03/2010 with inactivated trivalent (Type 'O', Type 'A' and 'Asia 1') Foot-and-Mouth disease virus vaccine (0 DPV LPBE titer and DIVA OD values of plasma samples were used to compare rest of the serum and plasma samples);

(p)-plasma sample; (s)-serum sample; DPV-days post vaccination

In LPBE: More than 1.68 Log_{10} titers considered as protective

In DIVA: Positive control-2.537(Average value); Negative control-0.0825 (Average value); Values above 0.3 considered positive

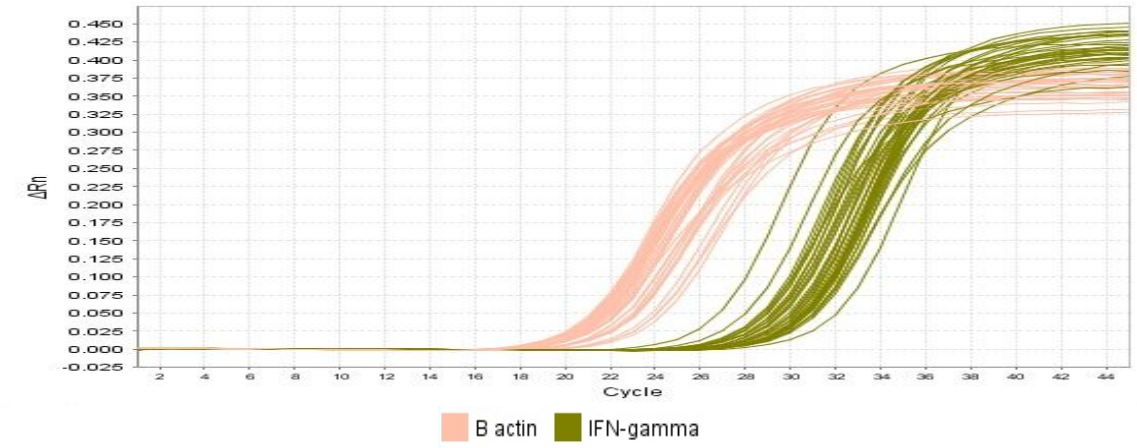


Figure 52. Amplification plot

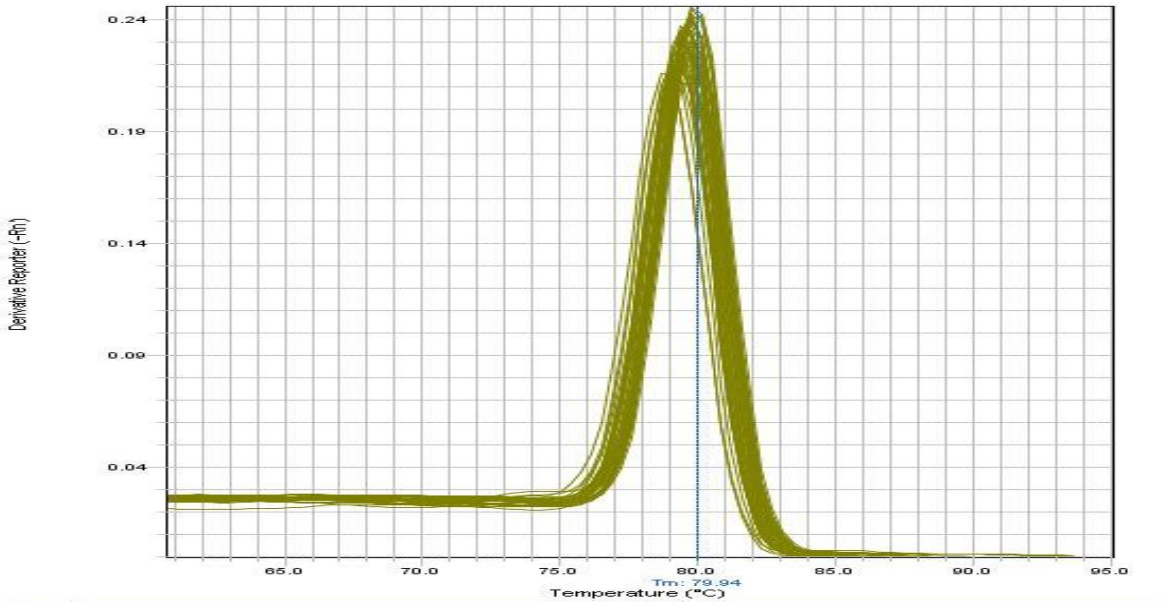


Figure 53. Melt curve of IFN-γ

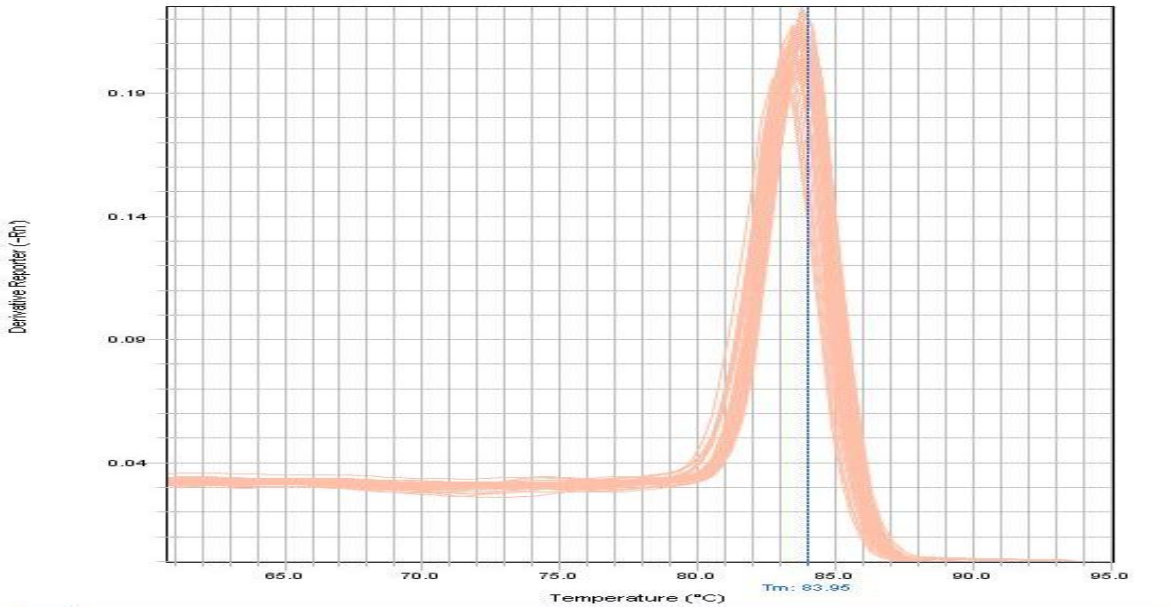


Figure 54. Melt curve of β-actin

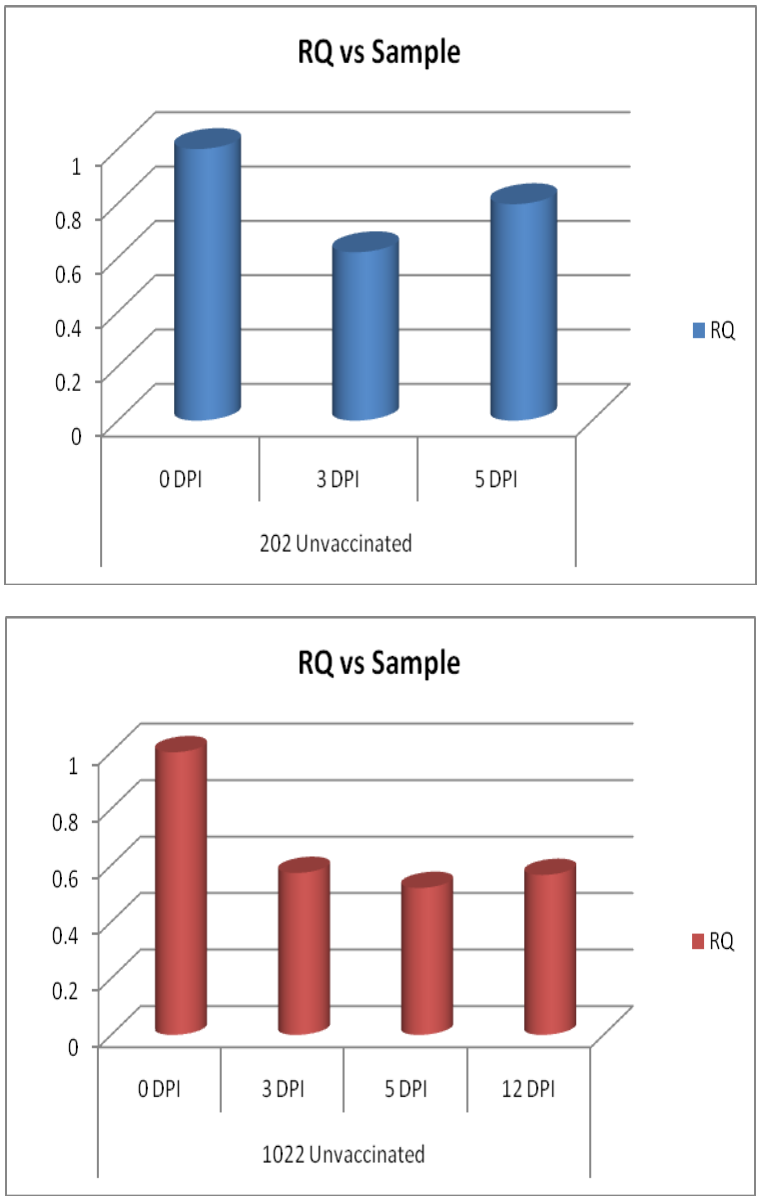


Figure 55. mRNA expression level of IFN-γ in peripheral blood mononuclear cells from control calves after Foot-and-Mouth disease virus Type 'O' infection

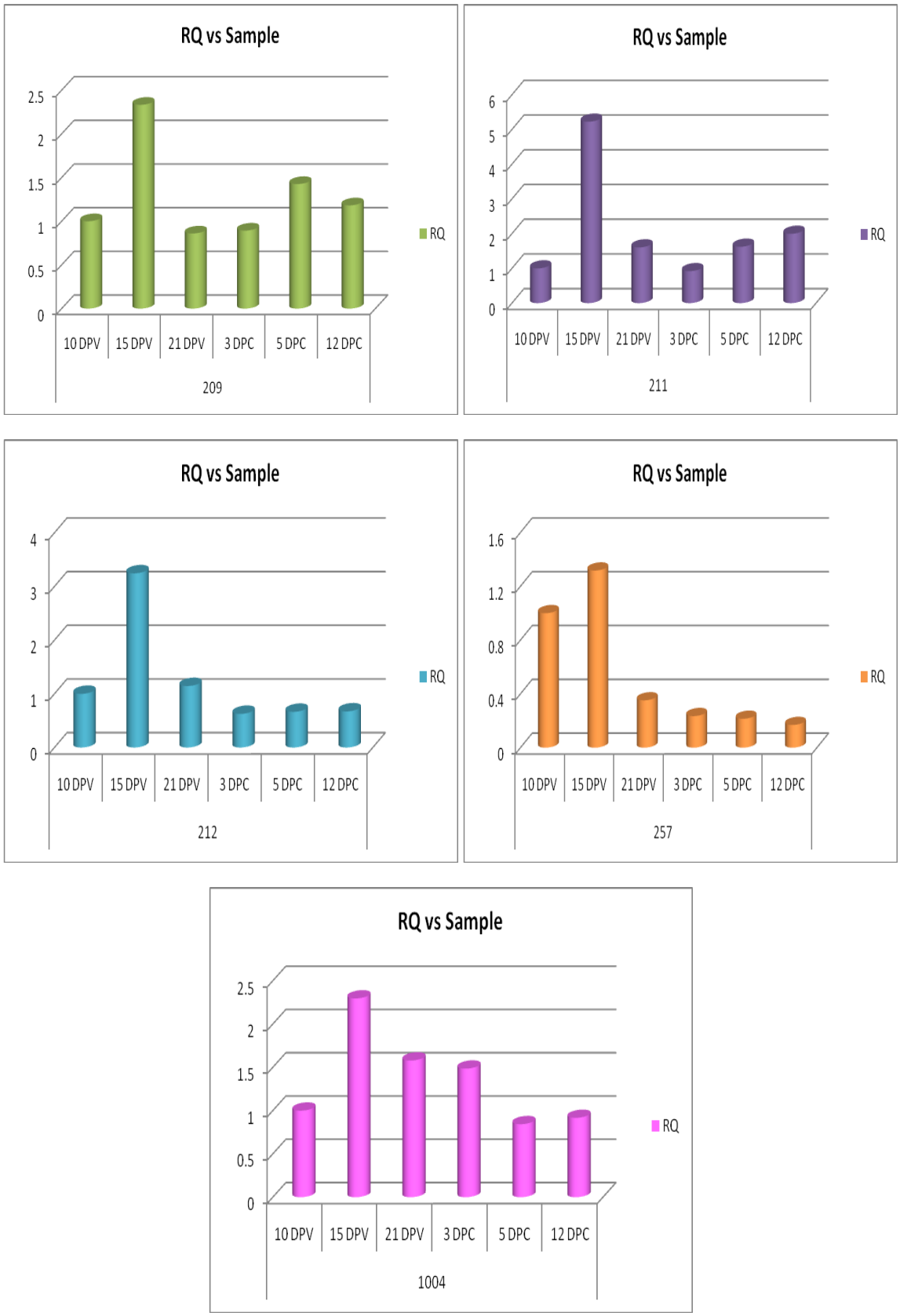


Figure 56. mRNA expression level of IFN-γ in peripheral blood mononuclear cells from calves used in Foot-and-Mouth disease virus vaccination-then-challenge study

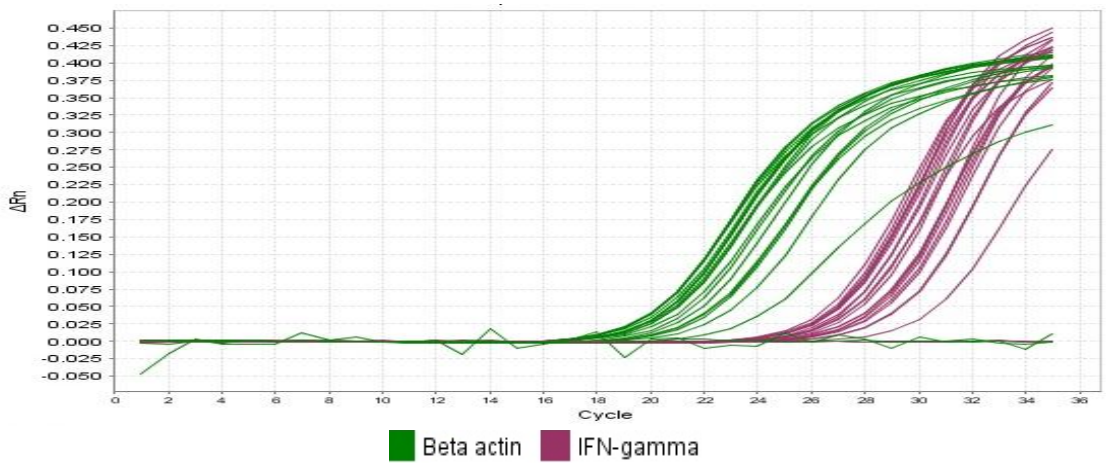


Figure 57. Amplification plot

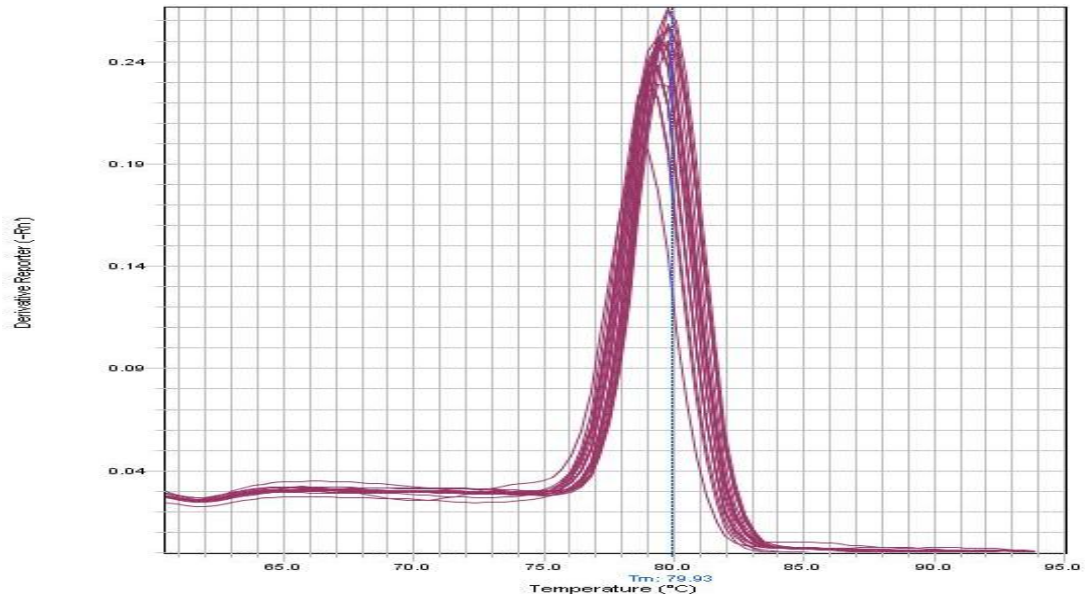


Figure 58. Melt curve of IFN-γ

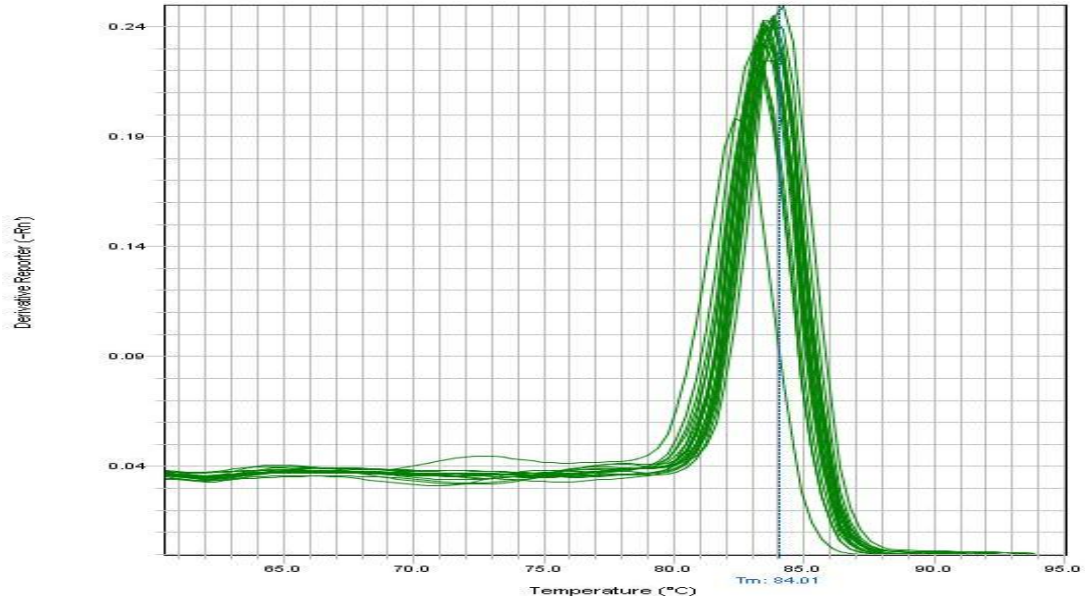


Figure 59. Melt curve of β-actin

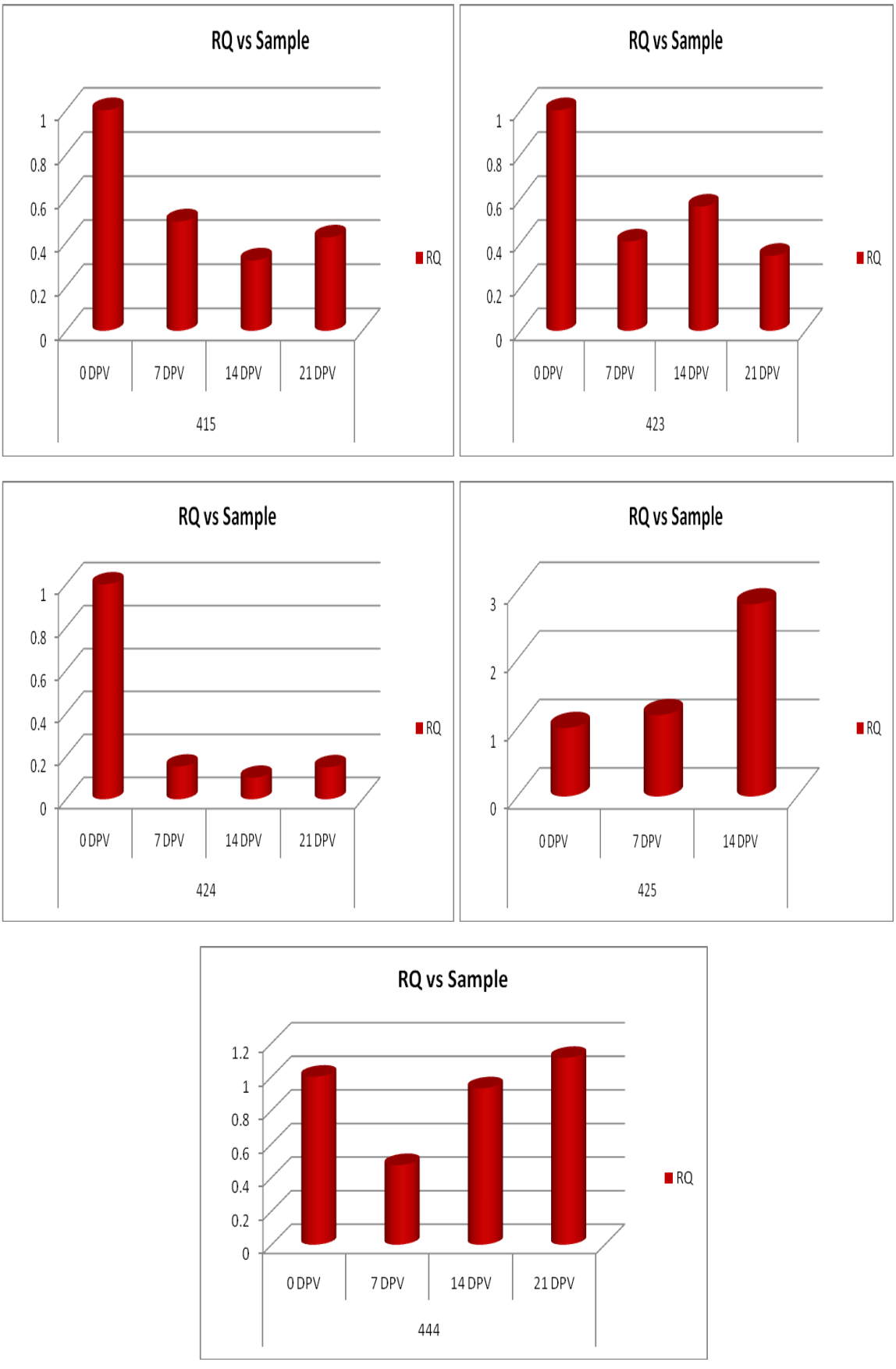


Figure 60. Expression level of IFN-γ mRNA in peripheral blood mononuclear cells after vaccination against Foot-and-Mouth disease with trivalent inactivated vaccine

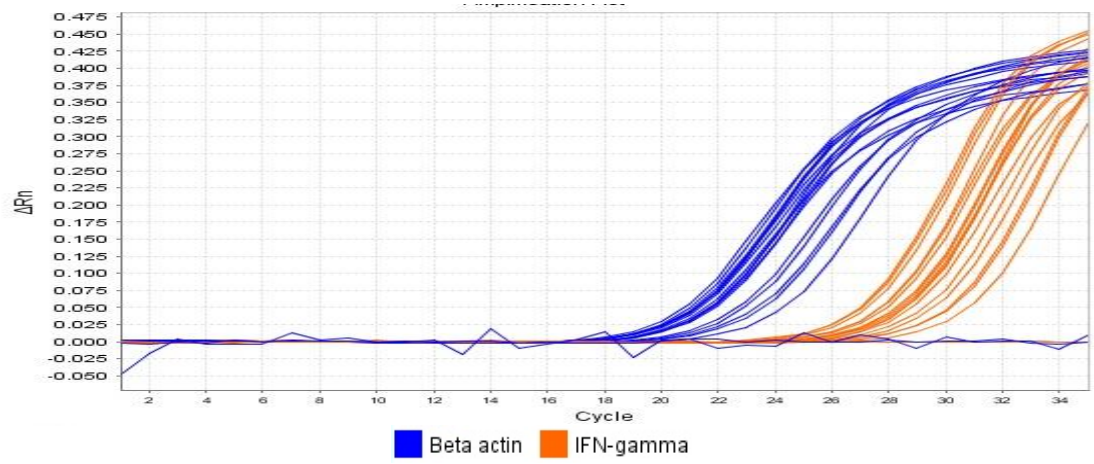


Figure 61. Amplification plot

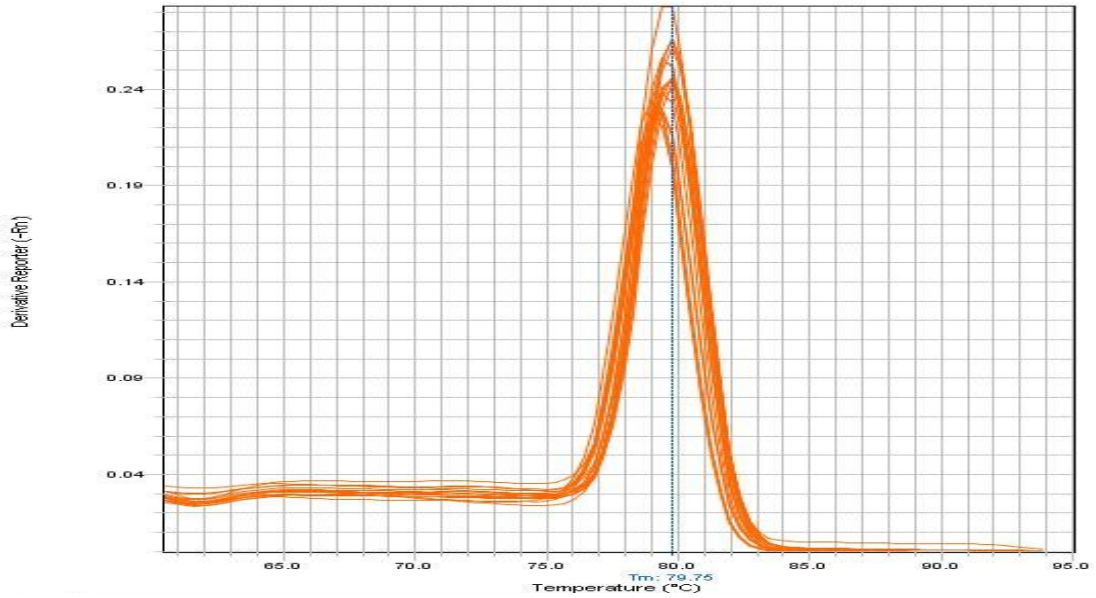


Figure 62. Melt curve of IFN-γ

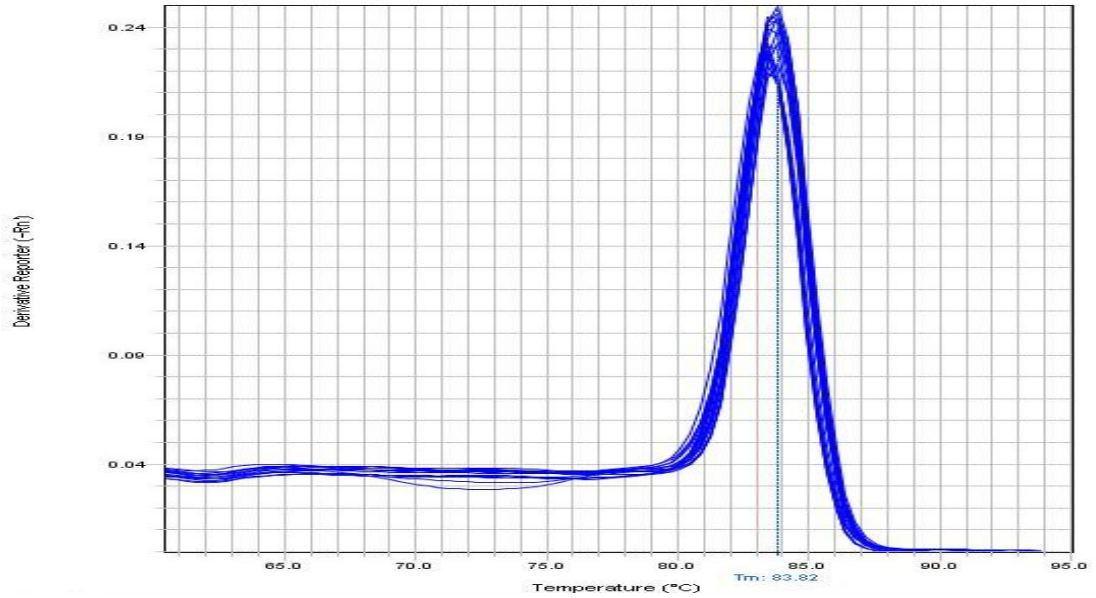


Figure 63. Melt curve of β-actin

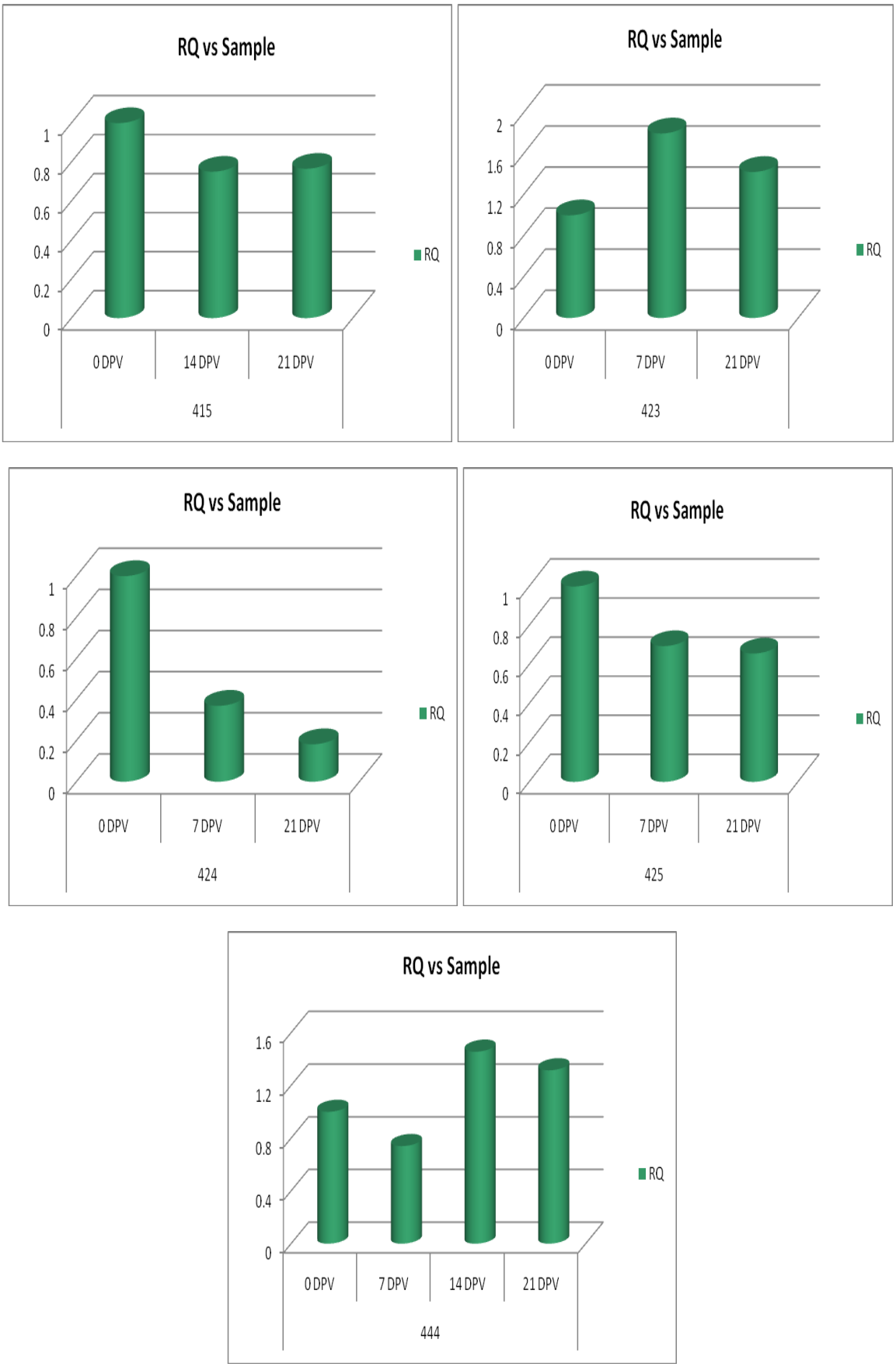


Figure 64. Expression level of IFN-γ mRNA in white blood cells after vaccination against Foot-and-Mouth disease with trivalent inactivated vaccine

DISCUSSION

“The years teach much which the days never know”

…Ralph Waldo Emerson

Chapter-5

DISCUSSION

Recently, scarcity of fodder impacted on milk production and its cascading effect on the milk price making the milk beyond the reach of the common household consumers in India (Kaur, 2010). Deliberate attempts have to be made to minimize the production losses by protecting the animals against infectious agents including the most dreadful one Foot-and-Mouth disease (FMD) by immunization. Vaccination limits Foot-and-Mouth disease virus (FMDV) excretion and transmission (Eble *et al.*, 2007; Orsel *et al.*, 2007). Summerfield and others (2008) opined that investigations in the immune responses elicited by FMDV were needed to improvise the existing vaccines and to sketch novel control strategies against FMD. Zhang and others (2009) also stated that the factors affecting the pathogenesis and persistence of FMDV were not completely understood.

Cytokines, chemokines and toll-like receptors (TLRs) and their agonists in therapeutics and autoimmune diseases and their applications were documented (Blacha, 1991; Wood and Seow, 1996; Rollins, 1997; Alluwaimi, 2004; Krieg, 2007; Brennan and McInnes, 2008; Kovarik *et al.*, 2008; Feldmann, 2008; Tayal and Kalra, 2008). Role of interferons, cytokines and chemokine receptors in response to inflammatory diseases and viral infections is well described (Lan *et al.*, 1995; Brown *et al.*, 2000; Goodbourn *et al.*, 2000; Murdoch and Finn, 2000; Ali, 2003; Colmenares *et al.*, 2003; Hedges *et al.*, 2003; Schroder *et al.*, 2004; Lee *et al.*, 2006; Haller *et al.*, 2007; Kim *et al.*, 2007; Cai, 2008; Kruif *et al.*, 2008). Cytokines play an impotant role in FMDV pathogenesis and prevalence of cytokines in the blood is one of the first indications of infection (Flint et al., 2004; Zhang *et al.*, 2009). So in the present investigation efforts have been made to understand the cytokine response elicited after FMDV infection and vaccination.

Commercially available primers and primers reported by various researchers were procured for amplification of some cytokines. For other cytokines for which primers were unavailable (not reported), nucleotide sequence data was aligned and with the help of NCBI-Primer blast software the primers were designed. Initially, unstimulated/stimulated (either by Conconavalin A or FMDV) peripheral blood

mononuclear cells (PBMCs) from male crossbred calf (FMDV uninfected, apparently healthy and unvaccinated with no history of FMD) were used to identify the cytokine genes amplified after reverse-transcriptase polymerase chain reaction (RT-PCR). Amplified cytokine products were gel-purified and cloned by T-A cloning in pGEM® - T Easy Vector System I. Out of 64 cloned cytokines and others (of 96 different interleukins, interferons, growth factors, toll-like receptors, chemokines, chemokine receptors and others), 61 were sequenced (IL-1α, IL-β, IL-2, IL-2Rα, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-13, IL-15, IL-16, IL-17A, IL-18, IL-20, IL-21, IL-27p28, IL-32, IL-34, IFN-αR1, IFN-γ, TNF-α, TNF-αSF1, TGFβ-1, TGFβ-2, TGFβ-3, GM-CSF, CSF, MCP-1, CCL3, CCL5, CCR5, CXCL1, CXCL2, CXCL6, CXCR4, CD11a, CD11b, CD11c, CD14, CD18, CD68, Integrin β3, Galectin-3, Granzyme A, Granulysin, Perforin, XCL1, COX-2, iNOS, ICAM-1, ICAM-3, BAD, TLR2, TLR4, TLR6, TLR9, 18S RNA, GAPDH-1, GAPDH-2, β-actin and CD4), successfully. Sequencing was performed to reconfirm the results of RT-PCR. Barring few (namely CD4 (sequenced to be IVD) and MCP-1 (extra stretch of nucleotides at 5')) cytokines, all of the other cytokines revealed specific amplification. IL-20, IL-21 and IL-25 clones were positive in PCR.

Applications of real-time PCR in virology have been thoroughly elaborated (Mackay *et al.*, 2002). To identify presence or absence of exotic viral disease in India this test was employed (Vanamayya *et al.*, 2009). Applications of real-time PCR based identification and measurement of cytokine mRNA response in whole blood, PBMCs, white blood cells (WBCs), dendritic cells and sputum have increased significantly (Grone *et al.*, 1998; Blaschke *et al.*, 2000; Yin *et al.*, 2001; Murphy *et al.*, 2002; Konnai *et al.*, 2003; Mamessier *et al.*, 2003; Coussens *et al.*, 2004; Lagrelius *et al.*, 2006; Taubert and Hermosilla, 2008; VanGuilder *et al.*, 2008).

Unavailability of protein based detection systems for many of the bovine cytokines lead to increased dependency on real-time PCR based detection of cytokines (Koyama *et al.*, 2009). Few reports are available on the study of *in-vivo* cytokine response after FMDV infection and vaccination in experimental models in cattle and swine (Barnett *et al.*, 2002; Cox *et al.*, 2003; Barnard *et al.*, 2005; Bautista *et al.*, 2005; Zhang *et al.*, 2006; Mingala *et al.*, 2009; Zhang *et al.*, 2009). But no reports are available on investigation of cattle during natural FMD outbreak. As per the author's knowledge this will be the first study describing the cytokine response in cattle during natural FMD

outbreaks. In one of the experiments to identify cytokine expression in natural FMD outbreak, breeding bulls (vaccinated against FMD before 2 months of FMD outbreak) infected with FMDV before 4 months of blood collection was investigated for mRNA expression level of IL-2 using total RNA extracted from the whole blood preserved in RNAprotect® Animal blood tubes. SYBR Green based one step real-time PCR (comparative CT (ΔΔ) type of experiment) was employed to study mRNA expression level of IL-2 using β-actin as endogenous control. Increased level of IL-2 mRNA expression was found in infected bulls than the vaccinated cattle (FMDV uninfected) indicative of build up of Th1 type of immune response. One odd sample of Mithun infected with FMDV did not show increased level of IL-2 mRNA expression.

In the other part of the study, all three interferons and representative for Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-6, IL-10) response, cytokines having impact on IFN-γ production and other cytokines (IL-12p40, IL-16, IL-21, IL-27p28 and TLR7) were targeted. mRNA expression level of these 12 selected cytokines in dairy cows experiencing natural FMD (Type 'A') outbreak was investigated. A total of 20 RNA samples extracted from PBMCs from the dairy cows experiencing natural FMD outbreak having different history of attack of FMDV infection (in-contact apparently healthy, clinical FMD noticed early (upto 10 days post FMDV infection), clinical FMD noticed only once, clinical FMD noticed twice/reinfected) were investigated. One of the RNA samples from in-contact apparently healthy cow was used as reference standard. SYBR Green based one step real-time PCR (comparative CT (ΔΔ) type of experiment) was employed to study mRNA expression levels of cytokines and others. β-actin was used as endogenous control. mRNA expession levels of IFN-α, IL-2, IL-6, IL-10, IL-21, IL-27p28 and TLR7 were found at lower side in dairy cows which had experienced clinical FMD twice compared to in-contact apparently healthy dairy cows (Table 11). IFN-α level of mRNA was higher in FMDV infected than in-contact apparently healthy or re-infected dairy cows. Increased IFN-β and IL-16 mRNA expression level was observed in a few early infected dairy cows (10 days post FMDV infection). Zhang and others (2009) found significant increase in IL-1α, TNF-α, IFN-α, IFN-β and IFN-γ in the coronary band and tongue at 1 to 2 days post FMDV infection and increased cytokine mRNA induction found in the coronary band and tongue coincided with the increased viral RNA load. Leader proteinase (L^{pro}) of FMDV inhibits induction of IFN-α and IFN-β mRNA and blocks

host innate immune response (Santos *et al.*, 2006). L^{pro} of Theiler's virus also reported to inhibit IFN-α and IFN-β production (Pesch *et al.*, 2001). Nfon and others (2008) reported inhibition of IFN-α production by dendritic cells during acute infection with FMDV in swine. IL-6, IL-10, 12p40, IL-21, IL-27p28 and TLR7 mRNA expression levels were increased in FMDV infected than in-contact apparently healthy or re-infected dairy cows. The level of IFN-γ mRNA expression was higher in in-contact apparently healthy dairy cows than the infected animals. IL-4 results were inconclusive.

In another study, total RNA was used to detect specific increase or decrease in level of cytokine mRNA expression after FMDV infection. SYBR Green based one step real-time PCR (comparative CT (ΔΔ) type of experiment) was employed to study mRNA expression levels of different cytokines and others. RNA samples extracted from PBMCs of one of the control calf used in the Vaccine Potency testing were investigated. Compared to 0 days post FMDV infection (DPI), in 3 DPI there were discripancies in expression of cytokine mRNA levels when GAPDH or β-actin or both were used as endogenous controls. mRNA expression levels of 13 cytokines and others were found increased when GAPDH was used as endogenous control (IL-1β, IL-2, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-15, IFN-αR1, CCL8, MSR1 and TLR10) after FMDV infection. mRNA expression levels of 17 cytokines and others; IGF-1, IGFBP-3, VEGF, CCL3, CCL8, CXCL1, CXCL10, CD14, CD68, FasL, TLR1, TLR2, TLR5, TLR7, TLR8, TLR9, TLR10 were found increased when β-actin was used as endogenous control. Although there was no positive correlation between the results obtained when both endogenous controls were used for above mentioned cytokines, but there was an agreement for decrease in mRNA expression level of 56 cytokines and others (IL-1α, IL-2Rα, IL-12p35, IL-13, IL-16, IL-17A, IL-19, IL-20, IL-21, IL-22, IL-25, IL-27p28, IL-32, IL-34, IFN-α, IFN-β, IFN-γ, IFN-T, TNF-α, TNF-αSF1, TGF-β1, TGF-β2, TGF-β3, GM-CSF, CSF, IGFBP-2, Leptin, CCL5, CCR5, CXCL2, CXCL6, CXCR4, CD11a, CD11b, CD11c, CD18, Integrin β3, Galectin-3, Granzyme A, Granulysin, Perforin, Selenoprotein P, XCL1, MMR, COX-2, iNOS, LIF, OSM, CT-1, L-Selectin, VCAM-1, ICAM-1, BAX, BAD, TLR3, TLR4) and increase in mRNA expression level of 2 cytokines and others (CCL8, TLR10) when GAPDH and β-actin were used separately or taken together as endogenous controls. Use of two endogenous controls is advisable. Hence these cytokines can be targeted to study cytokine response in FMDV infection

and vaccination. Significantly, there was an agreement for decrease in mRNA expression level of IFN-γ after FMDV infection which was found in vaccination and challenge study.

PBMC samples were isolated from blood samples collected at different intervals from individual crossbred cattle calves (FMDV uninfected, apparently healthy and unvaccinated with no history of FMD) from Foot-and-Mouth disease virus Vaccine Potency testing (vaccination and challenge study). No published reports are available in the literature on mRNA expression level of IFN-γ in cattle after FMDV vaccination and challenge study. SYBR Green based two-step real-time PCR (comparative CT (ΔΔ) type of experiment) was employed using β-actin as endogenous control. The study indicated increase in IFN-γ mRNA expression level after vaccination with monovalent inactivated FMDV vaccine (Type 'O'). Increase in mRNA expression level of IFN-γ after 15 days post FMDV vaccination was found unequivocally in all five calves (more than two-fold increase in expression level in 4 out of 5 vaccinated calves) used in vaccination and challenge study (Table 12). Humoural antibody response was also higher on 15 days post FMDV vaccination. In both, FMDV infected cattle and cattle vaccinated with inactivated FMDV, there was predominance of IgG1 antibodies over IgG2 (Mulcahy *et al.*, 1990). Although increase in mRNA expression in 15 days post vaccinated calves was observed, upon challenge of these vaccinated calves there were no fixed trends of increase or decrease in IFN-γ mRNA expression level. After infection, in control unvaccinated cattle calves there were decrease in IFN-γ mRNA expression. Mingala and others (2009) have observed increased IFN-γ mRNA expression in buffaloes on 21 days of vaccination with monovalent inactivated FMDV vaccine (Type 'O').

In the next part of the study, complementary deoxyribonucleic acid (cDNA) transcribed from PBMCs and WBCs from calves vaccinated against FMD with trivalent inactivated vaccine (Type 'O', Type 'A' and Type 'Asia 1') was used to determine whether the trends in IFN-γ mRNA expression reported in vaccination and challenge study exists (Table 13). SYBR Green based two-step real-time PCR (comparative CT (ΔΔ) type of experiment) was employed using β-actin as endogenous control. cDNA of 0 days post vaccination (DPV) was used as reference standard. A total of 3-4 out of 5 calves indicated lower level of IFN-γ mRNA expression on 7 DPV compared to 0 DPV, though increase in humoural antibody response was observed on 7 DPV in most of the calves. Results did not correlate with earlier results obtained in vaccination and challenge study where

increase in humoural antibody and increase in IFN-γ mRNA expression level was positively correlated. One of the reasons for this may be due to the fact that the calves used in this study were vaccinated against FMD before 2 months of blood collection though the antibody titer at that time was much below than the protective level.

It is possible that in cattle IFN-γ response is earlier than buffalo (15 DPV in comparison to 21 DPV in buffalo) after monovalent inactivated FMDV vaccination. But trivalent inactivated vaccination against FMD did not exhibit significant increase in IFN-γ response after 7 DPV in most of the calves. It is possible that more quantum of inactivated whole virus particle available in monovalent vaccine compared to trivalent vaccine elicits better stimulation of IFN-γ. Immune response to FMDV is affected by host (species, breed, age, health, physiological states and FMD immune status and others) and viral variables (dose, route, volume, viral strain and others) (Doel, 1996). Similarly, mRNA expression levels of IFN-γ and other cytokines may also be influenced by the above mentioned factors. In the present, study some of the cytokines (mainly IFN-α, IFN-β, IFN-γ, IL-2, IL-6, IL-10, IL-12p40, IL-16, IL-21, IL-27p28, TLR7 and others) showed difference in mRNA expression levels after FMDV infection or vaccination. Combination of two or more cytokines can be used as a marker in differentiating vaccination and infection. Currently, studies have been done on the cytokines as adjuvants in the vaccine (Botton *et al.*, 2006; Shi *et al*., 2007; Su *et al*., 2008; Wang *et al.*, 2008) against FMD.

Most sensitive route of FMDV infection in cattle and sheep is respiratory tract. Inhalatory dose of 10-25 infectious units is sufficient to produce FMDV infection compared to oral dose of 1 million infectious units (Donaldson, 1987). Hence, cytokine response in the respiratory tract and blood in response to FMDV infection in cattle need to be studied in order to devise a test helpful in early diagnosis of the infection before onset of clinical sickness. To conclude,

- Following monovalent FMDV vaccination IFN-γ mRNA level was peak at 15 days and decreased thereafter.
- Following experimental challenge with live FMDV in unvaccinated cattle calves there was about two-fold decrease in IFN-γ mRNA level.
- IFN-γ is a potential marker to understand FMDV pathogenesis and immune response. With IFN-γ; IFN-α, IFN-β, IL-2, IL-6, IL-10, IL-12p40, IL-16, IL-21, IL-27p28 and TLR7 can be useful.

Table 11. Salient points observed in dairy cows during FMD outbreak (Type 'A')

Item	Level of cytokine mRNA expression in peripheral blood mononuclear cells
IFN-α	Compared to most of the clinically free cows, some of the early infected and once affected cows (B-4, H-65, B-10, B-40) showed increased levels. Cows in which clinical FMD noticed twice/re-infected showed decreased levels.
IFN-β	Many early infected (B-4, H-65, T-69) and once affected (B-6, B-10, B-40) cows showed increased level. Low levels were noticed in all of the twice affected cows.
IFN-γ	Two cows with early infection (B-4 and T-61) experienced higher levels compared to clinically free cows. Contrastingly, few animals in the same group (H-65, R-54, R-57) indicated lower expression levels. Twice affected cows (B-19, M-51, M-56) showed lower levels than most of the apparently healthy cows.
IL-2	Compared to most of the clinically free cows, early infected (B-4, H-65, T-61) and twice affected (B-19 and M-51) cows showed increased levels. Most of the cows affected for once indicated lower levels.
IL-4	No specific pattern was observed. One or two cows from each group showed increased level.
IL-6	In one of the apparently healthy clinically free (M-53) and most of the early affected cows (R-54, R-57, T-61) increased levels were observed to that of once/twice affected cows.
IL-10	Higher levels were observed in apparently healthy cows (A-12, M-53, M-54), early infected (R-54 and T-61) and few of the once infected cows (B-10, B-40) than those clinically affected twice.
IL-12p40	Higher levels were observed in few cows from each of the apparently healthy (M-53, M-54) and early infected (H-65, R-54) cows. Levels in many of the cows from once/twice affected were at lower side.
IL-16	Three cows (FMD noticed early - B-4, H-65 and apparently healthy - B-1) showed increased levels.
IL-21	Except one of the apparently healthy cows (M-54), increased levels were observed in early infected (B-4, H-65, R-54, R-57, T-61) and two of the once affected cows (B-6, B-10) to that of others in apparently healthy and once/twice affected.
IL-27p28	Increased level of expression was observed in most of the early infected (B-4, H-65, R-57) and few of the once affected cows than most of the clinically free and twice affected.
TLR7	Most of the once affected (B-6, B-17, M-57, T-70) and all of the twice affected cows (M-51, M-56) experienced lower levels than most of the apparently healthy or early infected (A-12, M-53, M-54, B-4, H-65, R-54, R-57, B-10, B-40).

Table 12. Salient points noticed in cattle calves used in vaccination and challenge study

No.	IFN-γ expression level in peripheral blood mononuclear cells
Controls unvaccinated (Infected clinically with FMDV, Type 'O' R2/75)	
202	Compared to 0 DPV decreased after infection.
1022	Compared to 0 DPV decreased after infection.
Vaccination and challenge (protection upon challenge)	
209	Compared to 10 DPV increased on 15 DPV then decreased. Compared to 21 DPV level increased upon challenge.
211	Compared to 10 DPV increased on 15 DPV then decreased. Compared to 21 DPV fallen down on 3 DPC but subsequently increased.
212	Compared to 10 DPV increased on 15 DPV then decreased. Decreased on 3 DPC than 21 DPV but subsequently increased on 12 DPC.
257	Compared to 10 DPV increased on 15 DPV then decreased. Decreased on 12 DPC to that of 21 DPV and 3DPC.
1004	Compared to 10 DPV increased on 15 DPV then decreased. Maintained on 3 DPC compared to 21 DPV but subsequently decreased.

DPV – Days post vaccination

DPC – Days post challenge

DPI – Days post infection

Table 13. Salient points noticed in cattle calves administered with trivalent inactivated Foot-and-Mouth disease virus vaccine

No.	IFN-γ expression level
	In peripheral blood mononuclear cells
415	Compared to 0 DPV decreased on 7 DPV, 14 DPV and 21 DPV.
423	Compared to 0 DPV decreased on 7 DPV, 14 DPV and 21 DPV. Compared to 7 DPV increased on 14 DPV then decreased.
424	Compared to 0 DPV decreased on 7 DPV, 14 DPV and 21 DPV.
425	Compared to 0 DPV gradually increased on 7 DPV and 14 DPV.
444	Compared to 0 DPV decreased on 7 DPV thereafter gradually increased.
	In white blood cells
415	Decreased on 14 DPV compared to 0 DPV.
423	Incresed on 7 DPV than 0 DPV, thereafter decreased.
424	Decreased on 7 DPV and 21 DPV than 0 DPV.
425	Decreased on 7 DPV and 21 DPV than 0 DPV.
444	Increased on 21 DPV than 0 DPV, 7DPV and 21 DPV.

DPV- Days post vaccination

SUMMARY

“A journey of a thousand miles
begins with a single step”
…Confucius

Chapter-6

SUMMARY

Foot-and-Mouth disease (FMD) is most important viral diseases of bovines in India. India stands first in milk production in the world. Infection with Foot-and-Mouth disease virus (FMDV) adversely affects animal health and production. Immune responses elicited after FMDV infection and vaccination against FMD are not completely understood hence present investigation was aimed at - To understand and analyze expression of various cytokines involved in the FMDV infection vis-à-vis vaccination so that will be of help in differentiating pathogenesis of the disease from immunization.

Commercially available primers, primers reported by various researchers and designed primers were used in the study. Specific amplification was observed after reverse-transcriptase polymerase chain reaction (RT-PCR) from complementary deoxyribonucleic acid (cDNA) transcribed from ribonucleic acid (RNA) of unstimulated/stimulated (either by mitogen Conconavalin A or FMDV) peripheral blood mononuclear cells (PBMCs). Cytokine products were gel-purified and cloned by T-A cloning in pGEM® - T Easy Vector System I. Out of 64 cloned cytokines, chemokines, chemokine receptors, toll-like receptors and others (of 96 different interleukins, interferons, growth factors, toll-like receptors, chemokines, chemokine receptors and others), 61 (IL-1α, IL-β, IL-2, IL-2Rα, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-13, IL-15, IL-16, IL-17A, IL-18, IL-20, IL-21, IL-27p28, IL-32, IL-34, IFN-αR1, IFN-γ, TNF-α, TNF-αSF1, TGFβ-1, TGFβ-2, TGFβ-3, GM-CSF, CSF, MCP-1, CCL3, CCL5, CCR5, CXCL1, CXCL2, CXCL6, CXCR4, CD11a, CD11b, CD11c, CD14, CD18, CD68, Integrin β3, Galectin-3, Granzyme A, Granulysin, Perforin, XCL1, COX-2, iNOS, ICAM-1, ICAM-3, BAD, TLR2, TLR4, TLR6, TLR9, 18S RNA, GAPDH-1, GAPDH-2, β-actin, IVD) were sequenced, successfully. Three unsequenced clones were positive in PCR (IL-20, IL-21, and IL-25). Wherever appropriate, peripheral blood mononuclear cells (PBMCs) or white blood cells (WBCs) isolated from blood using Histopaque®-1077 or Ammonium chloride treatment of blood samples collected from animals. Whole blood samples were used to extract total RNA at some occasions. SYBR Green based one-step or two-step real-time PCR (comparative CT (ΔΔ) type of experiment) was used to study mRNA levels of different cytokines and others.

Total RNA extracted from whole blood samples of breeding bulls (which have been vaccinated against FMD prior to FMDV infection) affected with FMD and cattle vaccinated against FMD were used in one-step real-time PCR. Increased mRNA level of IL-2 was found in FMDV infected breeding bulls than the vaccinated cattle.

RNA extracted from PBMCs of dairy cows at Kashipur during natural FMD outbreak was used in one-step real-time PCR. These samples were divided according to their history for analysis. When total RNA from these dairy cows were used in one-step real-time PCR following points were observed, i) Increased IFN-γ expression levels were found in in-contact apparently healthy cows than FMDV affected cows, ii) IFN-α and IFN-β levels were higher in FMDV infected than in-contact apparently healthy iii) IL-2 and IL-4 results were not indicative of any significance, iv) increased IL-16 levels were observed in early FMDV infection in few dairy cows, v) IL-6, IL-10, IL-12p40, IL-21, IL-27 and TLR7 levels were increased in infected than apparently healthy and re-infected.

PBMCs isolated from cattle calves used in vaccination and challenge study was used to extract total RNA. Total RNA (0 days post infection (DPI) and 3 DPI) were used in one-step real-time PCR to compare the expression of mRNA levels of 92 different cytokines and others when GAPDH and β-actin or both were used as endogenous controls. After FMDV infection mRNA expression levels of most of the cytokines, chemokines, chemokine receptors and toll-like receptors and others were found downregulated (56 out of 92). There was increase in mRNA expression level of others (CCL8, TLR10 increased when multiple endogenous controls were used). IFN-γ mRNA was found decreased after FMDV infection irrespective of endogenous controls. cDNA prepared from total RNA was used in two-step real-time PCR. IFN-γ mRNA levels were found increased on 15 days post vaccination with monovalent inactivated vaccine (FMDV - Type 'O'), thereafter the level decreased. Increased IFN-γ level was correlated with the humoural antibody response. On experimental infection with live FMDV (Type - 'O') in control cattle calves there was about two-fold decrease in IFN-γ level.

When cDNA prepared from PBMCs and WBCs of trivalent inactivated vaccine (Type 'O', Type 'A', Type 'Asia 1') administered calves were used in two-step real-time PCR there was decrease in IFN-γ mRNA expression in most of the calves at 7 days post vaccination, though no specific pattern of IFN-γ expression and humoural antibody response was observed.

MINI-ABSTRACT

(ENGLISH)

“Facts do not cease to exist
because they are ignored”
…Aldous Huxley

MINI-ABSTRACT

Foot-and-Mouth disease (FMD) is most important viral diseases of bovines in India. India stands first in milk production in the world. Infection with Foot-and-Mouth disease virus (FMDV) adversely affects animal health and production. Immune responses elicited after FMDV infection and vaccination against FMD are not completely understood hence present investigation was aimed at - To understand and analyze expression of various cytokines involved in the FMDV infection vis-à-vis vaccination so that will be of help in differentiating pathogenesis of the disease from immunization.

Specific primers were used to amplify cytokines, chemokines, chemokine receptors, toll-like receptors and others in reverse-transcriptase polymerase chain reaction (RT-PCR) from complementary deoxyribonucleic acid (cDNA) prepared from total RNA of stimulated/unstimulated PBMCs. Of 96 cytokines and others, 64 were cloned, of which 61 cytokine and others were successfully sequenced.

Real-time PCR was used to study messenger ribonucleic acid (mRNA) expression levels of different cytokines and others in natural and experimental FMDV infection and vaccination. In natural outbreak at Izatnagar, IL-2 levels were found increased in FMD affected bulls than vaccinated cows. In dairy cows at Kashipur during natural outbreak of FMD (Type 'A'), increased IFN-γ levels were found in apparently healthy than infected. IFN-α / β levels were higher in infected than apparently healthy. IL-2, IL-4 results were inconclusive. Increased IL-16 levels were observed in early infection. IL-6, IL-10, IL-12p40, IL-21, IL-27 and TLR7 levels were increased in infected than apparently healthy and re-infected. Peripheral blood mononuclear cells (PBMCs) were isolated (from cattle calves in experimental vaccination then challenge study at Hyderabad), preserved and brought to Mukteshwar. In total RNA on three days post FMDV infection most of the cytokines and others levels were found downregulated including IFN-γ. When cDNA is used increased IFN-γ level was found on 15 days post FMDV vaccination (monovalent inactivated vaccine - Type 'O') and thereafter decreased. No specific pattern of IFN-γ increase was found in PBMCs/white blood cells (WBCs) of calves vaccinated with trivalent inactivated FMDV vaccine though in most of the animals expression levels of IFN-γ mRNA was found decreased on 7 DPV. Similar studies are required to understand the immune responses FMDV vaccination and infection.

MINI-ABSTRACT

(HINDI)

“Where observation is concerned,
chance favours only the prepared mind”
…Louis Pasteur

लघु–सारांश

खुरपका–मुँहपका रोग एक विषाणुजन्य रोग है। यह पालतू रोमंथी पशुओं का आर्थिक दृष्टि से महत्वपूर्ण संक्रामक रोग है। भारत में रोमंथी पशुओं की संख्या विश्व में शीर्ष स्थान पर है। इन पशुओं से बहुमूल्य उत्पाद प्राप्त होते हैं, जिनमें से एक दूध है। यह रोग होने पर पशुओं की उत्पादक क्षमता कम हो जाती है और इसका प्रतिकूल असर दूध उत्पादन पर भी पड़ता है। टीकाकरण एक प्रभावी तरीका है जिससे इस विषाणु के प्रकोप को नियंत्रण में लाया जा सकता है।

खुरपका–मुँहपका रोग होने के उपरान्त तथा टीकाकरण के पश्चात् पशुओं में कोशिकीय द्रव्य उत्पन्न होते हैं, जो पशुओं की प्रतिरक्षण क्षमता पर असर डालते हैं। प्रस्तुत अध्ययन में इस रोग अथवा टीकाकरण के पश्चात् उत्पन्न होने वाले कोशिकीय द्रव्यों के एम.–आर. एन. ए. के बारे में प्रकाश डाला गया है।

श्वेत रक्त कोशिकाओं का विघटन करके इनमें से केवल एककेन्द्रकी कोशिकाओं को पृथक किया गया। इन कोशिकाओं को विषाणु अथवा मायटोजन से उद्दीपित किया गया और उसके उपरान्त आर. टी.–पी. सी. आर. करके एम.–आर. एन. ए. के, अधिक मात्रा में उत्पन्न होने वाले, प्रकार का अध्ययन किया गया। परन्तु कोई भी एक कोशिकीय द्रव्य अधिक मात्रा में नहीं पाया गया।

एक अन्य अध्ययन में काशीपुर (उत्तराखण्ड) के रोगग्रस्त गोपशु प्रक्षेत्रों में से बीस दुधारू गायों से रक्त निकालकर श्वेत एककेन्द्रकी कोशिकाओं को अलग करके उनमें से आर. एन. ए. निकाल कर रीयल–टाइम पी. सी. आर. किया गया। इस अध्ययन में बारह कोशिकीय द्रव्यों (आई. एल.–2, आई. एल.–4, आई. एल.–6, आई. एल.–10, आई. एल.–12क्ष40, आई. एल.–16, आई. एल.–21, आई. एल.–27क्ष28, आई. एफ. एन.–अल्फा, आई. एफ. एन–बीटा, आई. एफ. एन–गामा, टी. एल. आर.–7) के एम.–आर. एन. ए. के बारे में जांच की गई। आई. एफ. एन.–गामा का एम.–आर. एन. ए. स्वस्थ दिखने वाले पशुओं में रोगी पशुओं की तुलना में ज्यादा पाया गया।

हैदराबाद (आंध्र प्रदेश) के आई. आई. एल. में खुरपका–मुँहपका टीकाकरण (एक–संयोजी खुरपका–मुँहपका टीके द्वारा), टीकाकरण–कृत्रिम विषाणु संक्रमण और कृत्रिम विषाणु संक्रमण के पश्चात् गोवंशीय पालतू पशुओं से रक्त एकत्रित कर श्वेत एककेन्द्रकी कोशिकाओं को निकाला गया। जिन्हें एक विशेष द्रव्य में घोलकर उचित स्थिति में आगे के अध्ययन के लिये मुक्तेश्वर (उत्तराखण्ड) प्रयोगशाला में लाया गया। उनका आर. एन. ए. निकालकर सी.–डी. एन. ए. बनाया गया और उसमें आई. एफ. एन.–गामा के एम.–आर. एन. ए. का अन्तर ज्ञात किया गया। टीकाकरण करने के पन्द्रह दिनों के पश्चात् आई. एफ. एन.–गामा का एम.–आर. एन. ए. ज्यादा पाया गया जो कृत्रिम विषाणु संक्रमण करने पर घट गया। इन पशुओं में टीकाकरण के पन्द्रह दिन के पश्चात प्रतिरक्षण क्षमता उच्चतम स्तर की थी।

उपरोक्त परिणामों को फिर से जांचने के लिये मुक्तेश्वर के गोवंशीय पशुओं, जिनमें टीकाकरण (बहु–संयोजी खुरपका–मुँहपका टीके द्वारा) हो चुका था, उनसे टीकाकरण के पूर्व और पश्चात् समस्त श्वेत रक्त कोशिकाओं और श्वेत एककेन्द्रकी कोशिकाओं को निकाला गया और पहले बतायी हुई विधि से अध्ययन करने पर घटा हुआ आइ. एफ. एन–गामा पाया गया (जबकि उस समय रोग प्रतिरक्षण क्षमता उच्चतम स्तर पर थी) जोकि पहले हुये अध्ययन से विपरीत था।

प्रस्तुत अध्ययन में 96 कोशिकीय द्रव्यों के एम.–आर. एन. ए. में से 64 का सी.–डी. एन. ए. क्लोन किया गया जिनमें से 61 का न्यूक्लीओटाइड अनुक्रम अधिकतम सही पाया गया।

कोशिकीय द्रव्यों की उत्पत्ति, विषाणु, पशु, टीके का शुद्ध होना, टीके के प्रकार (एक–संयोजी अथवा बहु–संयोजी टीका), वातावरण की स्थिति एवं अन्य कारणों पर निर्भर कर सकता है। खुरपका–मुँहपका रोग की प्रतिरक्षण क्षमता के बारे में जानने के लिए पुनः विस्तार से ऐसे अध्ययन किये जाने की आवश्यकता है।

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“Reading is to the mind what exercise is to the body”

…Richard Steele

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APPENDIX

“The shortest way to do many things
is to do only one thing at once”

…Samuel Smiles

APPENDIX

Commonly used solutions and buffers

Tris Borate EDTA buffer (TBE) 5x

Tris base – 60.55 g (500 mM)

Boric acid – 27.82 g (450 mM)

EDTA – 3.72 g (10 mM)

Adjust pH upto 8.4 with HCl/NaOH

Milli Q water – 1 litre

Ethidium bromide solution for staining of the nucleic acids (5 µg/µl)

Ethidium bromide – 10 mg

Water – 1 ml

6 x loading dye

Bromophenol blue (0.25 % w/v) – 0.025 g

Sucrose (40 % w/v) – 4 g

Water – Upto 10 ml

Used in cloning

Luria Bertani (LB) broth

Dissolve 5 tablets (1.1 g each) in 250 ml of MilliQ water

Autoclaved for 20 min at 121 ^{0}C at 15 lbs

Luria Bertani (LB) agar

Dissolve 5 tablets (1.6 g each) in 250 ml of MilliQ water

Autoclaved for 20 min at 121 ^{0}C at 15 lbs

Transformation storage solution

Luria Bertani broth 2X – 10 ml

Magnesium++ 2 M – 200 µl

Dimethyl sulfoxide – 1 ml

Polyethylene glycol (PEG) 8000 30 % - 7 ml

MilliQ water – 1.8 ml

Filtered through 0.2 µ filter

SOC medium

Hanahan's broth – 28 g

MilliQ water – Upto 1 litre

Autoclave and add 2M sterile $MgCl_2$ – 5 ml

1M sterile glucose – 20 ml

SOB medium (Hanahan's broth)

Hanahan's broth – 28 g

MilliQ water – 1 litre

Autoclave and add 2M sterile $MgCl_2$ – 5 ml

Used in LPBE

Coating buffer (pH 9.6)

Carbonate bicarbonate buffer capsule – 1

MilliQ water – 100 ml

Washing buffer (5 x)

Sodium dihydrogen phosphate.$2H_2O$ – 7.8 g

Sodium hydrogen phosphate.$2H_2O$ – 26.72 g

Sodium chloride – 584.5 g

Adjust the pH with 1 M Sodium hydroxide

Add MilliQ water to make 4 litre

Working washing buffer - 400 ml 5x wahing buffer + 1600 ml MilliQ water

Blocking buffer

Lactalbumin hydrolysate (LAH) – 3 g

Healthy rabbit serum – 5 ml

Healthy calf serum – 5 ml

Washing buffer (pH 7.4) to make 100 ml

Phosphate-Citrate buffer

Phosphate – citrate buffer tablet – 1

MilliQ water – 100 ml

Store at 4 ^{0}C for few days

Substrate solution

Phosphate-citrate buffer (pH 5) – 15 ml

Orthophenylenediamine dihydrochloride – 10 mg

Hydrogen peroxide (30 % w/v) – 8 µl

Stopping solution

Sulfuric acid – 95-97 % - 5.55 ml

MilliQ water – 94.45 ml

Dilution for coating serum

Type 'O' – 1:5000

Type 'A' – 1:4000

Type 'Asia 1' – 1:4000 , Diluent: Coating buffer

Dilution for tracing serum

Type 'O' – 1:6000

Type 'A' – 1:1000

Type 'Asia 1' – 1:4000 , Diluent: Blocking buffer

Dilution for antigen

Type 'O' – 1+5

Type 'A' – 1+2

Type 'Asia 1' – 1+3, Diluent: Phosphate Buffer Saline-Tween-20

Used in DIVA

Diluted 3AB3 protein antiogen for caoting

Take one vial of Lyophilized protein antigen

Add 5.5 ml carbonate-bicarbonate coating buffer, pH 9.5

Dissolve properly

Controls

Positive and negative control serum samples

Dssolve in 160 µl of distilled water, Store at 4 ^{0}C

***Escherichia coli* lysate**

Take one vial of *Escherichia coli* lysate

Add 35 µl of Phosphate Buffer Saline

Store at – 20 ^{0}C

Washing buffer (1 x)

Sodium dihydrogen phosphate.$2H_2O$ – 0.39 g (0.0025 M)

Disodium hydrogen phosphate.$2H_2O$ – 1.336 g (0.0075 M)

Sodium chloride – 29.325 g (2.93 % w/v)

Tween-20 – 500 µl (0.05% v/v)

Make the volume upto 1 litre with distilled water

Adjust pH with 1 N NaOH, if needed (7.2 to 7.4)

Coating (Carbonate-bicarbonate buffer), pH 9.5 (0.05 M)

Dissolve one Carbonate-bicarbonate buffer capsule in 100 ml distilled water.

Check pH and store at 4 ^{0}C.

Diluent buffer

Skimmed Milk Powder – 0.6 g (3% w/v)

Chicken serum – 2 ml (10% v/v)

E. coli lysate – 2.5 µl (0.01%)

Washing buffer upto 20 ml

Substrate (phosphate-citrate) buffer, pH 5.0 (0.05 M)

Dissolve one tablet of phosphate-citrate buffer in 100 ml distilled water

Store at 4 ^{0}C

Substrate solution (prepare before use)

Substrate buffer – 7.5 ml

OPD – 5 mg

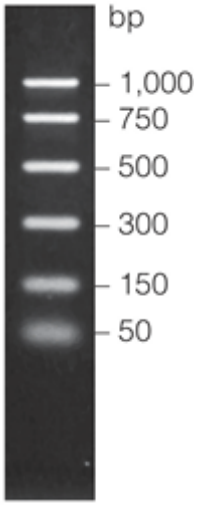
Hydrogen peroxide (30 %) 4 µl

Stop solution (1 M)

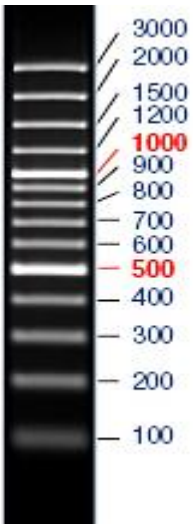
Sulphuric acid (95-97 %) – 5.55 ml

Make the volume to 100 ml with distilled water

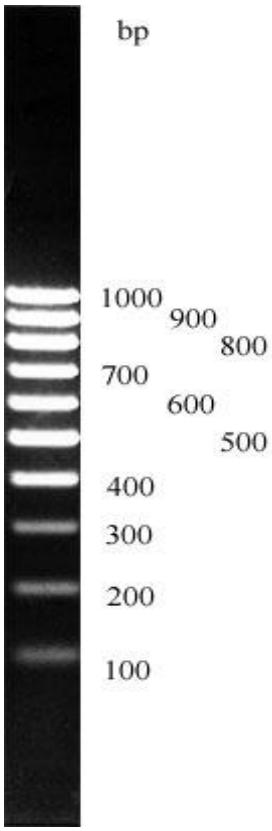
Markers



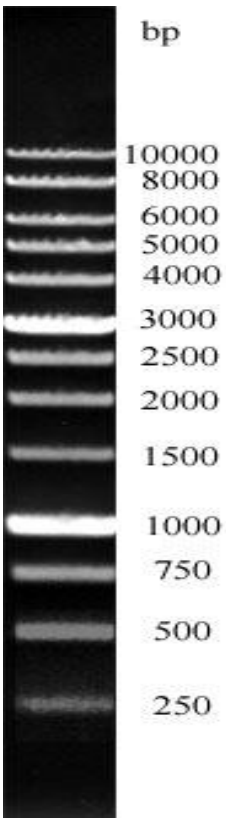
PCR Markers, Promega



100 bp Plus DNA Ladder, Fermentas



100 bp DNA Ladder, SibEnzyme



GenRuler™ 1 kb DNA Ladder, Fermentas/

1 kb DNA Ladder, SibEnzyme

VITA

“Of life’s two chief prizes beauty and truth,
I found one in the loving heart and another in the labourer’s hand”
…Khalil Jibran

VITA

Name	:	Surname Name Father's name **Audarya Sachin Digambar**
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