Mutational Analysis of residue V408 of LEDGF/p75

Keerthana B

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The Degree of Master of Technology

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Declaration

I declare that this written submission represents my ideas in my own words, and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the Institute and can also evoke penal action from the sources that have thus not been properly cited, or from whom proper permission has not been taken when needed.

KEERTHANA B.

(BO14MTECH11001)

Approval Sheet

This thesis entitled 'Mutational analysis of residue V408 of LEDGF/p75' by Keerthana B. is approved for the degree of Master of Technology from IIT Hyderabad.

Dr. Anindya Roy
Associate Professor
Department of Biotechnology, IIT Hyderabad
Internal Examiner

Dr. G. Satyanarayan
Associate Professor
Department of Chemistry, IIT Hyderabad
External Examiner

Dr. N. K. Raghavendra
Assistant Professor
Department of Biotechnology, IIT Hyderabad
Chairman/Adviser

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My foremost and immense gratitude to **GOD ALMIGHTY** without **HIS** love and help this task would not have been accomplished.

Dedicated to

JESUS CHRIST

Abstract

Lens Epithelium Derived Growth Factor (LEDGF/p75) is a human transcriptional co-activator and a dominant cellular binding partner of HIV-1 Integrase (IN). LEDGF/p75 tethers IN to the host chromatin aiding in formation of proviral DNA. This study involves generation of site directed mutations of Valine residue at position 408 and analyzing the effect of mutation upon interaction with IN. Previously characterized mutations L368A and D366A have been generated for serving as controls in pull-down analysis. The binding affinity of V408 and L368 mutants will be characterized by pull down analysis.

Nomenclature

LEDGF – Lens Epithelium Derived Growth Factor

IN - Integrase

IBD – Integrase Binding Domain

IPTG – Isopropyl β-D 1galactothiopyranoside

LB broth – Luria Bertani broth

Contents

Chapter 1

Introduction

1.1 LEDGF and Integrase Interaction

HIV-1 infects $CD4^+$ cells of the immune system such as T helper cells, monocytes, macrophages and dendritic cells (DC). HIV-1 interacts with several host proteins for a successful infection. For integration of viral DNA into host genome, LEDGF/p75, a cellular cofactor of HIV-1 integrase plays a very crucial role. LEDGF/p75 promotes viral integration in host cell by tethering the viral pre-integration complex (PIC) to the chromatin. A knock-down of LEDGF/p75 results in 10-fold decrease in levels of infection (Shun et al, 2007) indicating LEDGF/p75-IN interaction to be critical for infection and thus provides an attractive target for anti-viral therapy (Debyser et al, 2010).

LEDGF/p75 is a 60 kDa human protein with a chromatin-binding domain PWWP domain located at the N terminus (residues 1-93) and Integrase Binding domain (IBD) at the C-terminus (residues 347-429) (figure 1). IBD expressed alone has been shown to interact with IN in a manner similar to that of full length LEDGF/p75 (Cherepanov et al, 2005).

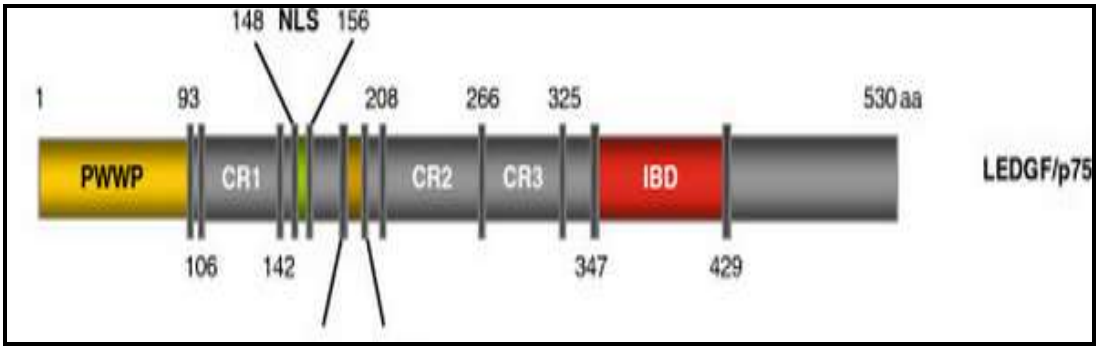

Figure 1. Domains of LEDGF/p75; Cartoon representation of LEDGF/p75 and HRP-2: PWWP-domain (PWWP), charged region 1–3 (CR1-3), nuclear localization signal (NLS), AT hook-like sequence (AT), and integrase binding domain (IBD). Adapted from Schrijvers et al. Retrovirology 2012 **9**:84 doi:10.1186/1742-4690-9-84

HIV-1 Integrase is a 32-kDa protein that is composed of three structural domains. IN is generated from a longer Gag-Pol poly peptide protein as a result of HIV-1 protease activity during maturation of the virion upon release from the infected cell. Figure 2 shows the domain organization of IN.

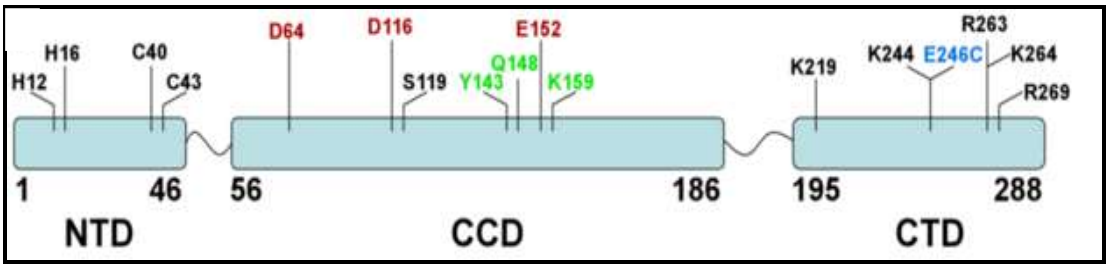

Figure 2. Schematic presentation of the three domain structure of HIV-1 IN. The NTD residues (H12, H14, C40 and C43) coordinate Zn and contribute to the functional multimerization. The CCD contains the catalytic DDE motif. This domain interacts with both viral and target DNA. A number of residues (Y143, Q148 and K159) selectively interact with terminal U5 bases, while S119 has been implicated in direct interactions with the target DNA. The CCD is also critical for the functional multimerization. The CTD is highly basic and non-specifically interacts with viral DNA. Adapted from Kvaratskhelia et al. Virus 2009 1(3), 713-736; doi:10.3390/v1030713

N-terminal domain (NTD) is a helical bundle stabilized by the coordination of a single zinc atom in the HHCC zinc finger motif. Catalytic Core Domain (CCD) belongs to a superfamily of DNA/RNA strand transferases/Nucleases and contains the active site residues, Asp-64, Asp-116, and Glu-152 collectively referred to as the DDE triad. CCD binds to IBD of LEDGF/p75. Carboxyl-terminal domain (CTD) is least conserved domain among divergent retroviruses and possesses a Src homology 3-like fold. All three domains play a role in DNA recognition and protein-protein interaction. IN is found to exist in solution as mono-, di-, tetra- and oligomeric forms. HIV-1 IN mediates 2 sequential nucleophilic reactions. First step termed as 3' processing, is the removal of 3' terminal GT nucleotides from both ends of HIV-1 cDNA, this step exposes the 3' –OH of terminal A nucleotide for attaching the phosphodiester bond at site of integration. Second step is integration, where the processed 3' termini make a nucleophilic attack on the opposing strands of human genome at characteristic 5 base pair interval.

1.2 Structural Basis for HIV-1 Integrase and LEDGF/p75 interaction

A crystal structure of IBD of LEDGF/p75 and CCD of IN was solved to reveal the interaction interface and the residues critical for this interaction (Cherepenov et al, 2005). The crystal structure is shown in figure 3. A dimer of CCD of IN was found to interact with two monomers of IBD, with IBD present at the opposite ends of CCD-CCD interface (shown in left panel). Biochemical analysis has revealed that amino acid residues Ile-365, Asp-366, and Phe-406, located in the interhelical loops of IBD play critical roles in interaction with IN. Mutation of V408 to alanine was shown to have only partial effect in IBD-CCD interaction. Residues A128, A129, W131, W132, E170, H171, T174 and M178 of CCD are shown to be forming the pocket for interaction of residues from IBD (shown in right panel). Interaction between NTD of IN and IBD increases the affinity of interaction.

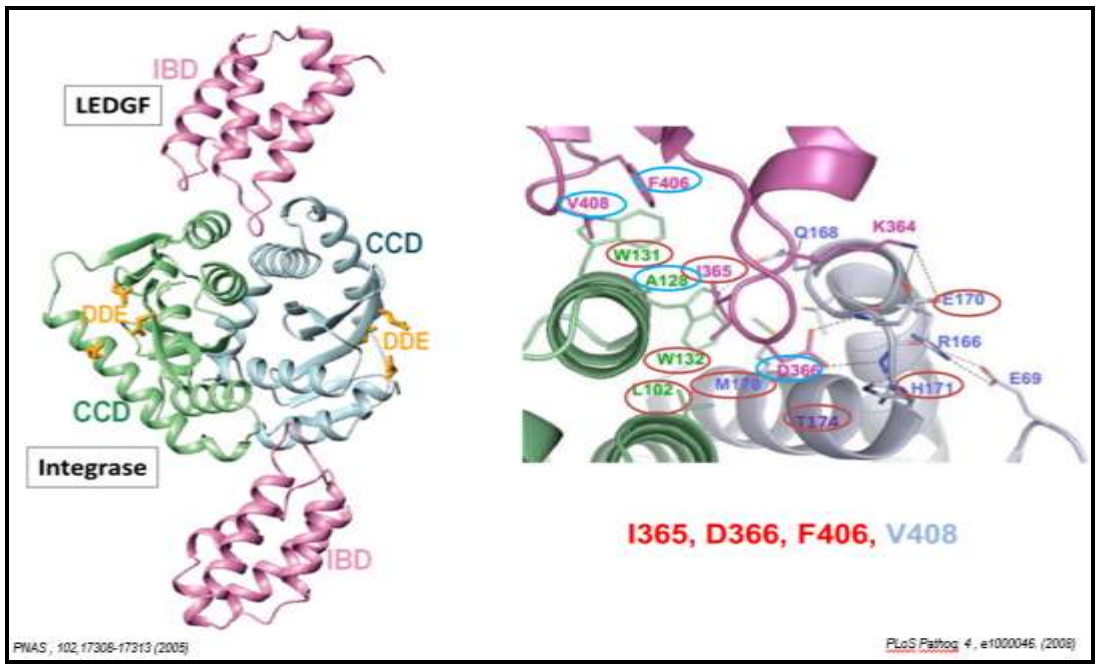

Figure 3. IBD-CCD interaction interface. Right panel shows interaction of two monomers of IBD with a dimer of CCD. The interface of each monomer of IBD with CCD dimer was found to be identical. The residues and interactions at the interface of IBD-IN are shown in right panel. Side chains of I365, D366, F406 and V408 of IBD and L102, A128, W131, E170, H171, T174 and M178 of CCD are highlighted. I365 of IBD makes hydrophobic interactions with CCD, while D366 makes salt bridge with main chain of E170 and H171.

1.3 Work performed in this study

Earlier studies have revealed that mutation of V408 to alanine results in a partial loss of interaction between IBD and CCD, unlike that of I365, D366 and F406 that resulted in a complete loss. In this study, following double mutations have been carried out to complement the ongoing analysis of the single mutants of V408 of IBD. Double mutants V408F/L368F, V408F/D366A and V408L/D366A have been generated by overlapping PCR protocol. The work flow is shown schematically in figure 4. In addition, single mutants L368A and D366A have been generated to serve as controls in the pull down analysis of mutants. L368A has no effect on IBD-CCD interaction, while D366A results in a complete loss of interaction (Cherepanov et al, 2005).The mutants of IBD generated in this study have been labeled as follows: pRA52 (L368A); pRA53 (V408F/L368F); pRA54 (D366A); pRA59 (V408F/D366A); pRA60 (V408L/D366A) and pRA71 (V408M/D366A)

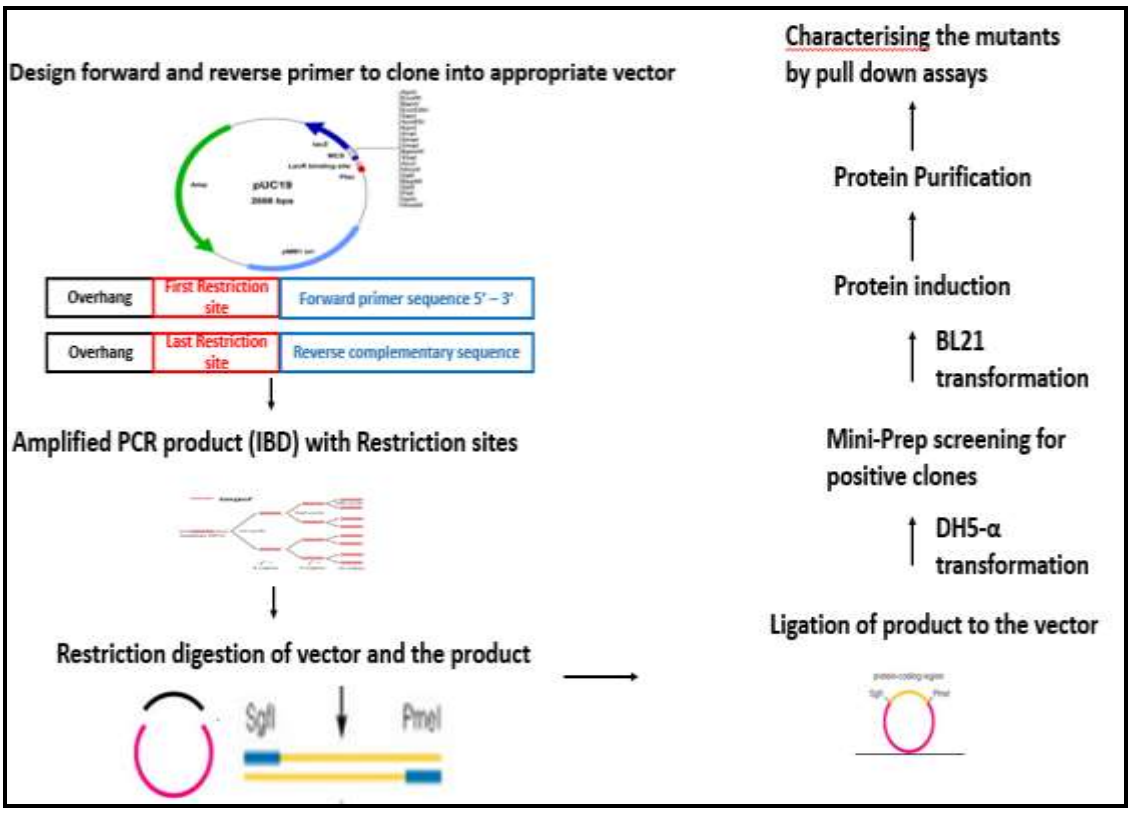

Figure 4. Work flow followed for generation of mutants of IBD using overlapping PCR method

Chapter 2

Results and Discussion

2.1 Generation of IBD L368A mutant (pRA52)

Mini-Prep screening of ligation colonies

The pGEX4T2 vector and the PCR product of IBD L368A were digested with BamHI and SalI separately. The digested vector pGEX4T2 and the IBD L368A mutant gene were ligated together by T4 DNA ligase and transformed into DH5-alpha E.coli cells. The transformed cells were plated on LB plates (with ampicillin at concentration 50ug/ml). Single colonies were grown on the plate. 24 single colonies from ligation plate were inoculated into 3 ml LB tubes (with ampicillin). The cultures were allowed to grow overnight. The plasmids were isolated from all the 24 cultures by Mini-Plasmid isolation by alkaline lysis method. The isolated plasmids were loaded on a 0.6% agarose gel. Of the 24 colonies, 7 colonies (Col 1, 3, 5, 6, 7, 8, 18) were moving slower than that of control pGEX vector indicating the presence of an insert.

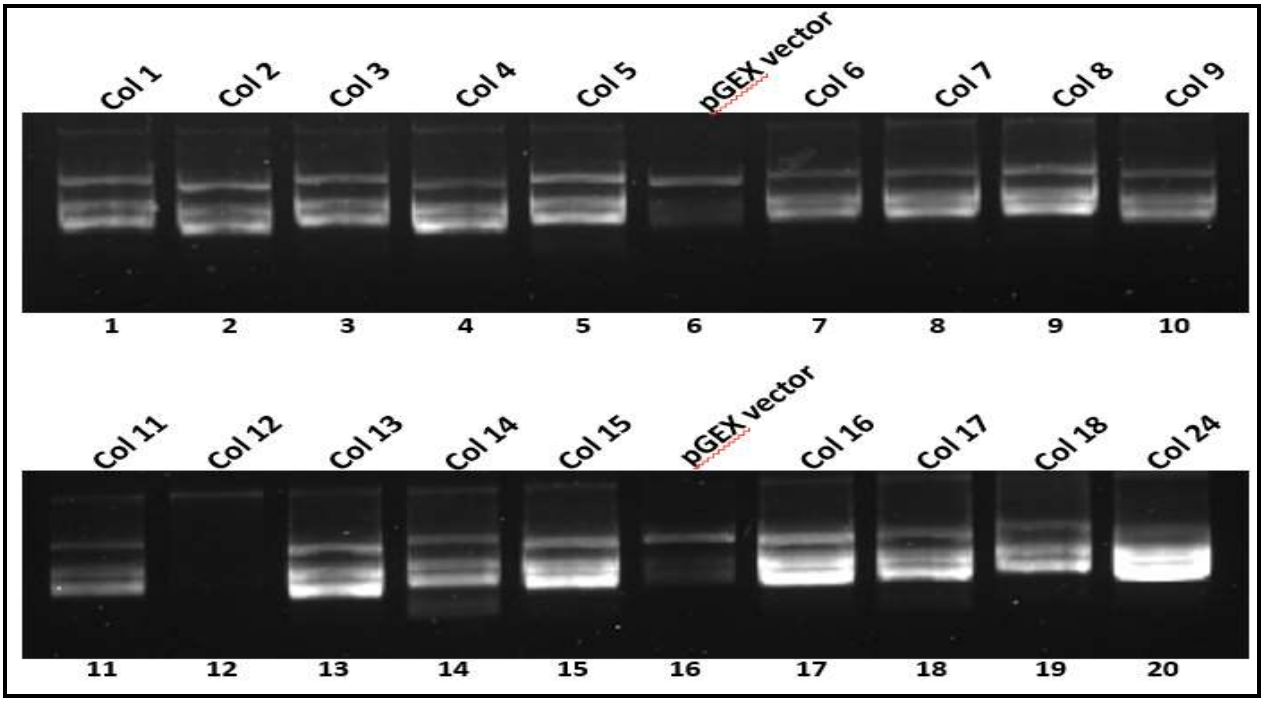

Figure 5. Mini-Prep plasmid isolation of pGEX ligated with IBD L368A; pGEX4T2 vector was loaded as control in Lane 6 and 16. The supercoiled front of col 1, 3, 5, 6, 7, 8 and 18 (Lanes 1, 3, 5, 7, 8, 9 and 19 respectively) are running slower than that of control pGEX4T2 vector.

Restriction Digestion

BamHI-SalI digestion of pRA52 was done to check the insert release. An expected insert of size 389 bps was observed in a 0.6% gel (figure 6, left panel). This was followed by HpaI digestion to check the L368A mutation (figure 6, right panel).

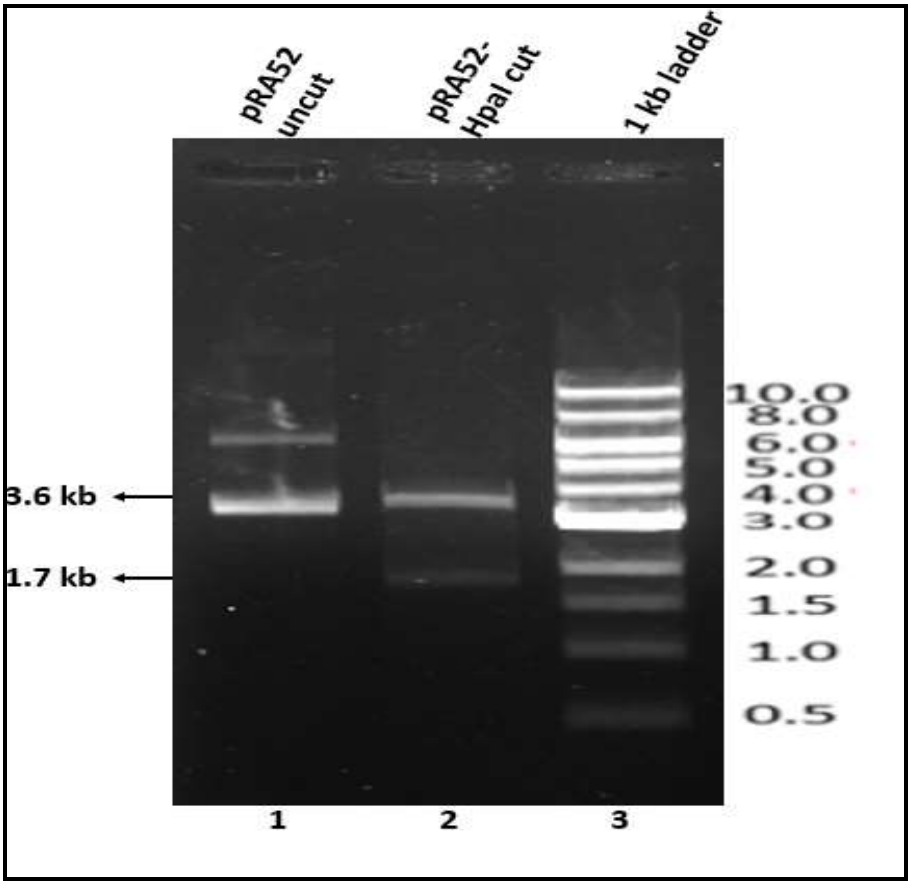

Figure 4. HpaI digestion of pRA52 (IBD L368A); Expected bands of sizes 3.6 kb and 1.7 kb was observed (lane 2)

The expected bands of size 3.6 kb and 1.7 kb was observed when pRA52 was digested with HpaI (Lane 2), indicating L368A mutation. In absence of mutation, only linearization of the plasmid will be observed upon HpaI digestion, as seen with wild type IBD (pCPGST 75-81) (figure 9, lane 7).

Induction of the protein

The midi-Prep DNA was transformed to BL21 (DE3) pLysS competent cells. 2 Colonies were picked in 3ml broth to check the protein induction. The cultures were allowed to grow till 0.6 OD and were induced with 1M IPTG for 4 hours at 28^0C.

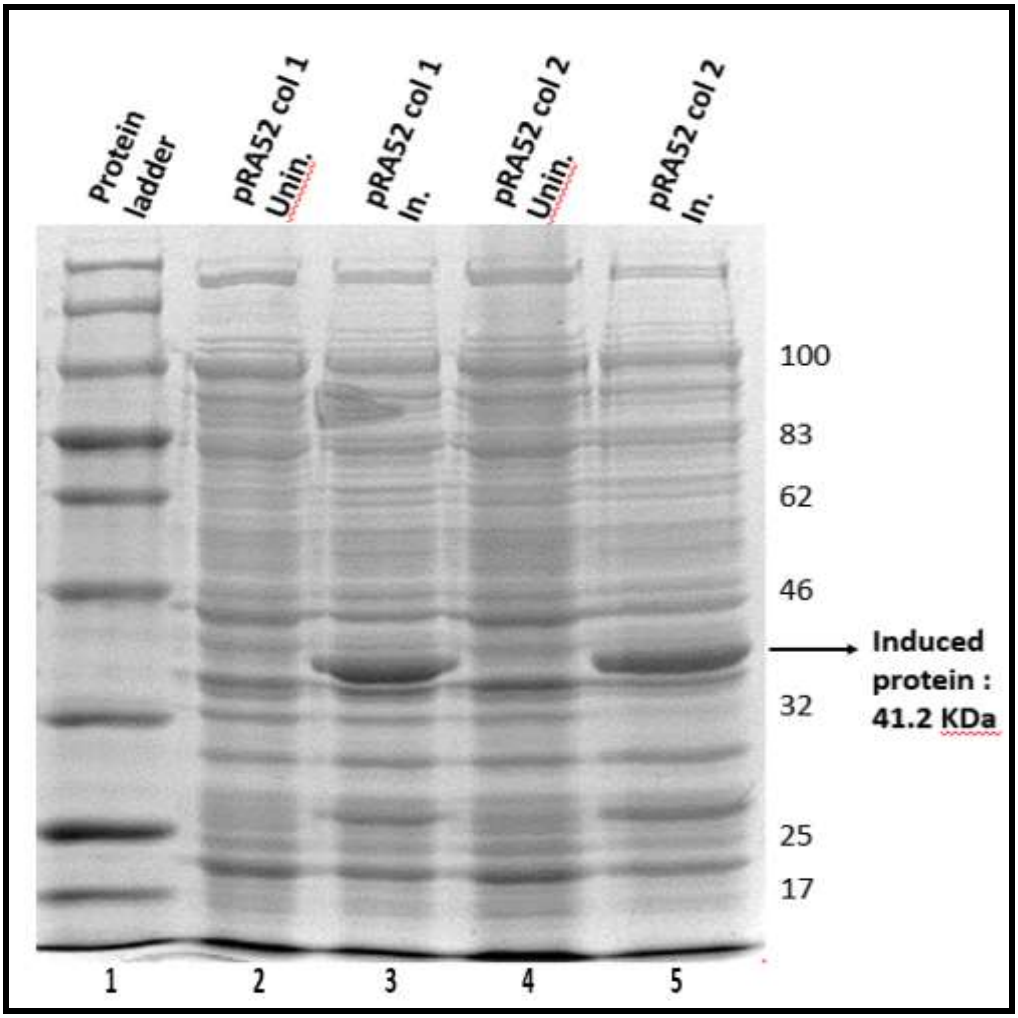

Figure 5. Induction of L368A IBD in BL21 cells. Induction was observed at an expected size of 41.2 kDa in lane 3 and lane 5

After observing the induction in 3ml culture, 125 ml Induction of col 1 of pRA52 (IBD L368A) was done under the same conditions. The culture was induced at 0.6 OD with 1M IPTG for 4 hours at 28^0C and the induction was checked on gel.

2.2 Generation of IBD V408F L368F mutant (pRA53)

Mini-prep screening of ligation colonies

The PCR and ligation of the mutant PCR product were performed as done for pRA52. Of the 36 colonies, 2 colonies (Col 14 and 31) were moving slower than that of control pGEX vector (figure 8).

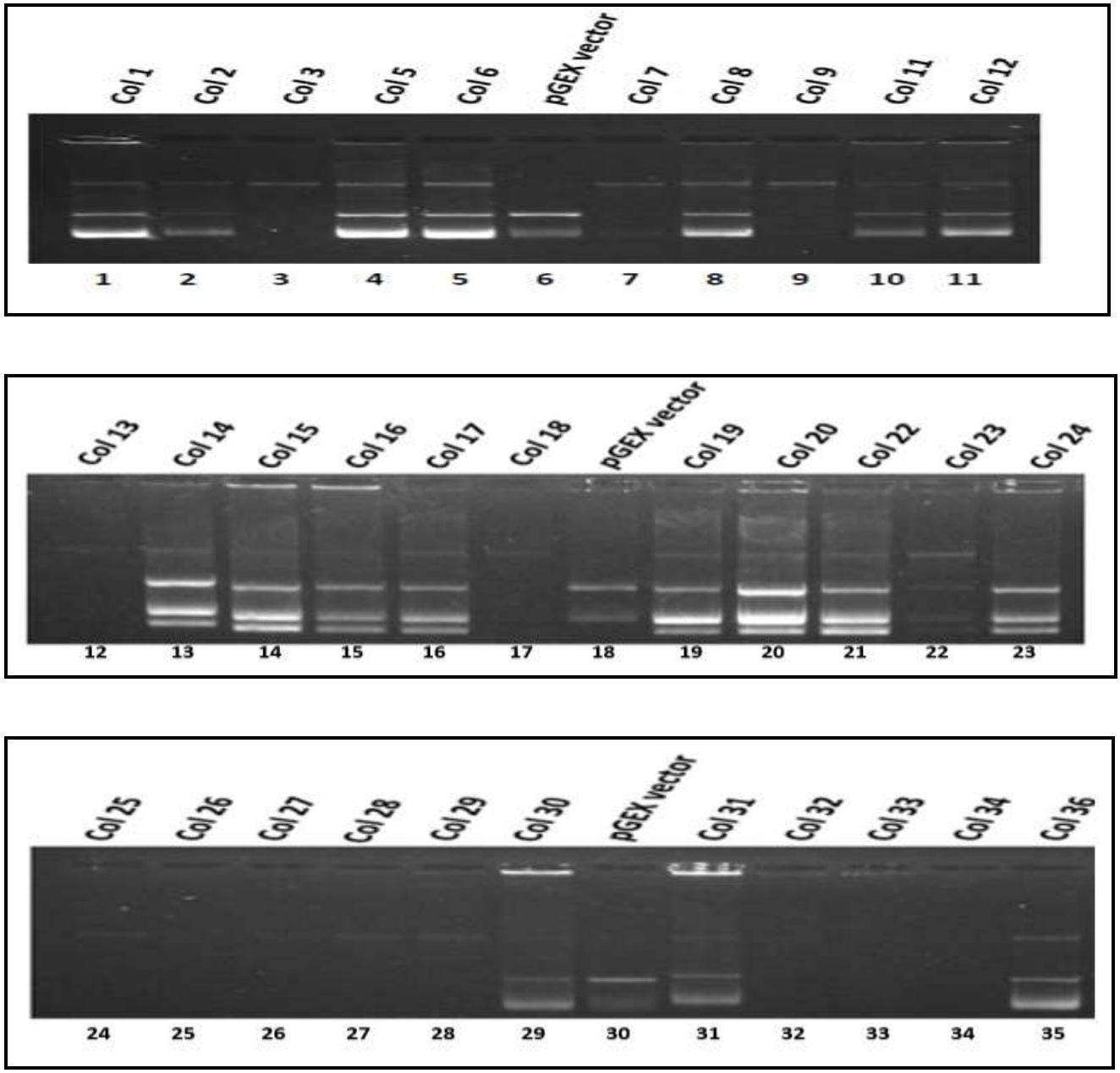

Figure 8. Mini-Prep plasmid isolation of pGEX ligated with IBD V408F L368F; pGEX4T2 vector was loaded as control in Lane 6, 18 and 30. The supercoiled front of col 14 and 31 (Lanes 13 and 31 respectively) are running slower than that of control pGEX vector

Restriction Digestion

BamHI-SalI digestion of pRA53 was done and an insert of 389 bps was observed as expected in a 0.6% gel. This was followed by HpaI digestion to check the L368F mutation and DraI digestion to check V408F mutation along with HpaI and DraI digestion of control

plasmid pCPGST [75-81]. The expected sizes of 3.6 kb and 1.7 kb was observed for HpaI digestion in lane 3 indicating L368F mutation whereas DraI digestion of pRA53 yields expected bands of sizes of 3.35 kb, 841 bps and 691 bps was observed (Lane 4).

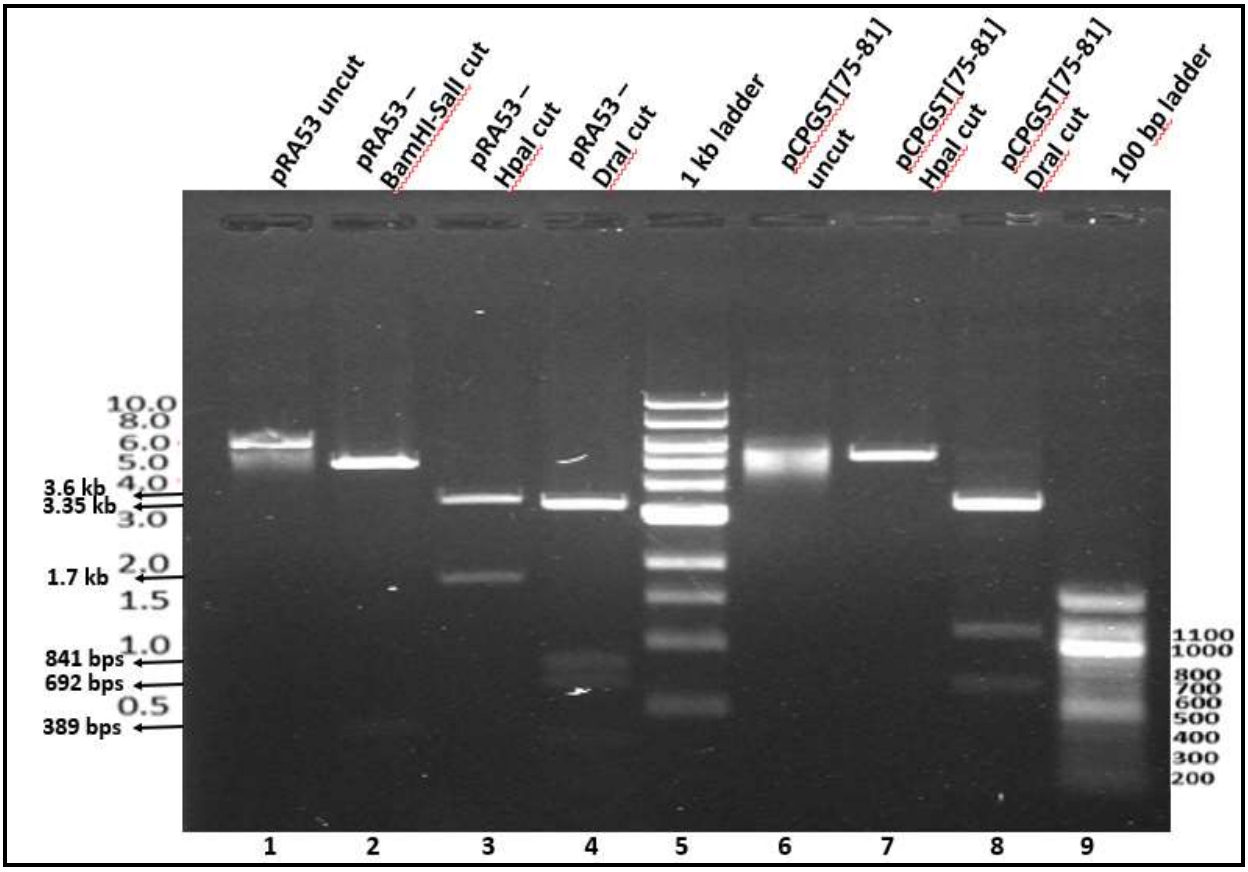

Figure 9. BamHI-SalI, HpaI and DraI digestion of pRA53; An expected insert release of 389 bps was observed (lane 2). HpaI digestion of pRA53 was done to check L368F mutation along with control pCPGST [75-81]. Expected bands of size 3.6 kb and 1.7 kb was observed in pRA53 (lane 3) whereas control vector got linearized as there is no HpaI site in wt IBD but only in the vector (lane 7). DraI digestion of pRA53 yielded expected band sizes of 3.35 kb, 841 bps and 692 bps in Lane 4 whereas DraI digestion of control pCPGST[75-81] yields bands of sizes 1.07 kb, 3.35 kb and 692 bps (lane 8).

Induction of the protein

The midi-Prep DNA was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth

and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 10) and the remaining induced culture was pelleted for further purification of protein.

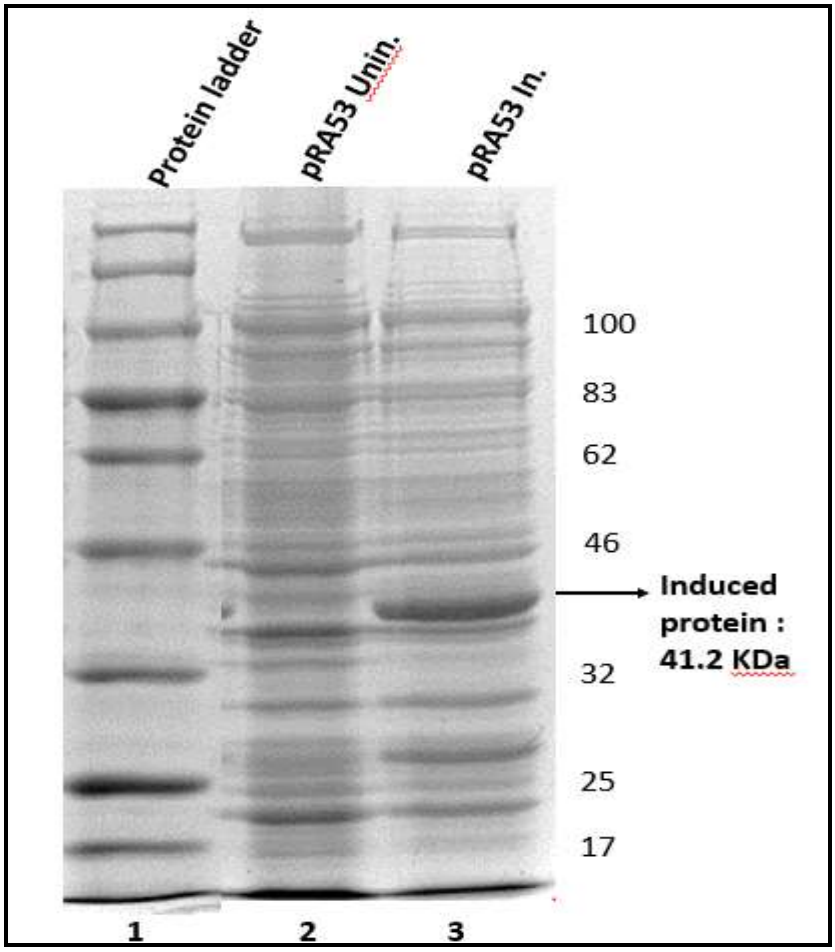

Figure 10. Induction of pRA53 (IBD V408F L368F); An induction of expected protein size of 41.2 kDa was observed in Lane 3

2.3 Generation of pRA54 (IBD D366A)

Mini-Prep screening of ligation colonies

The PCR and ligation of the mutant PCR product were performed as done for pRA52. Of the 12 colonies, 5 colonies (Col 4, 6, 7, 9 and 10) were moving slower than that of control pGEX vector (figure 11).

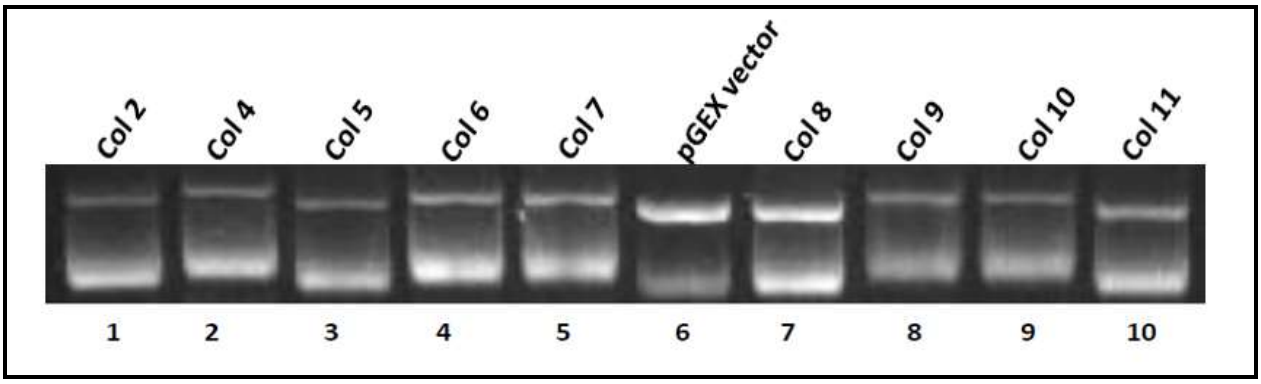

Figure 11. Mini-Prep plasmid isolation of pRA54 (IBD D366A) by alkaline lysis method; control pGEX vector was loaded in Lane 6 and the supercoiled front of 5 colonies (col 4, 6, 7, 9 and 10) in lanes 2, 4, 5, 8 and 9 are running slower than the supercoiled front of the vector

Restriction digestion

BamHI-SalI digestion of pRA54 was done to check the insert release of 389 bps.

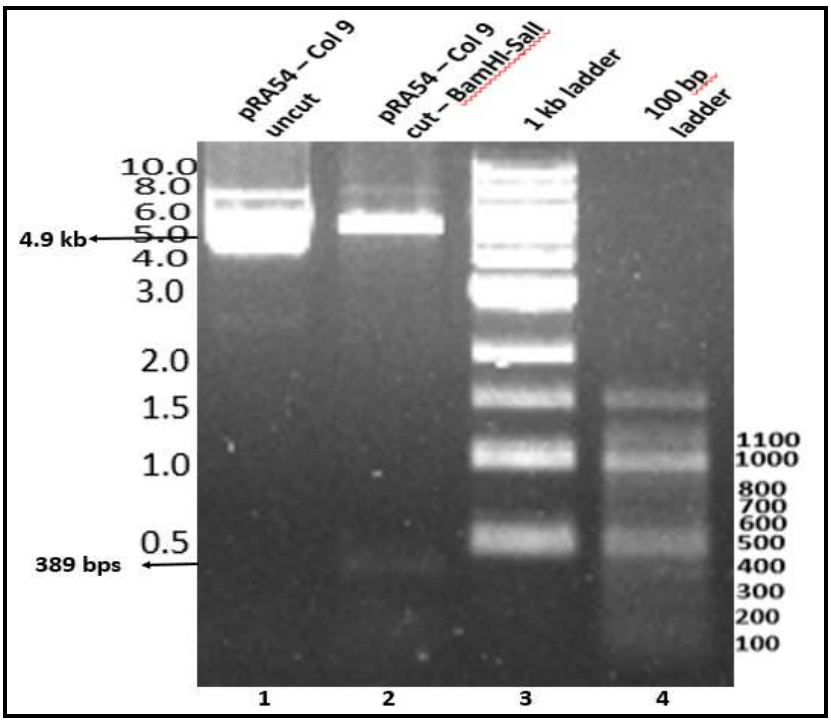

Figure 12. BamHI-SalI digestion of pRA54 (IBD D366A); An expected insert release of 389 bps was observed (Lane 2)

XbaI digesion was done along with control pCPGST[75-81]. IBD with D366A mutation gets linearized with XbaI digestion, whereas pGEX with wt IBD does not have an XbaI site

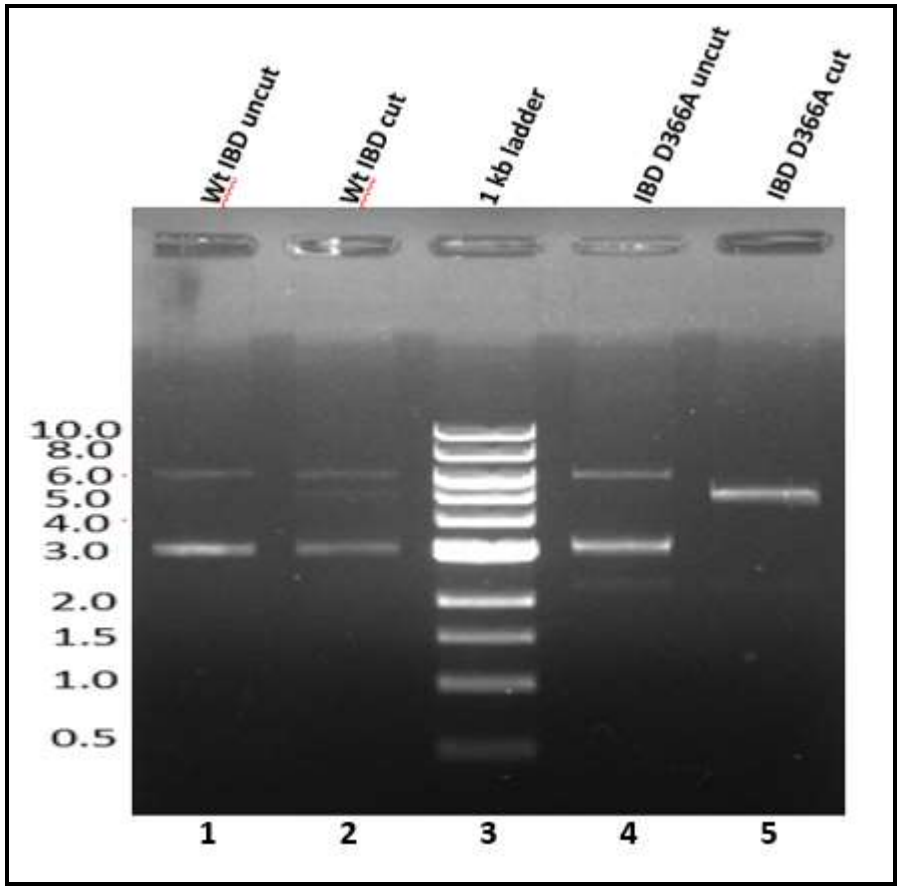

Figure 13. XbaI digestion of pRA54 (IBD D366A); XbaI digestion of pRA54 results in linearization of the plasmid at 5.3 kb (lane 5) whereas there is no XbaI site in control pCPGST [75-81] in lane 2

Induction of the protein

The midi-Prep DNA was transformed to BL21 (DE3) pLysS competent cells. 2 colonies were picked for 3 ml induction check. Following Induction in 3 ml, a 125 ml induction was performed. Induction was confirmed on SDS-PAGE and the cell pellet was stored at -80’C for further use.

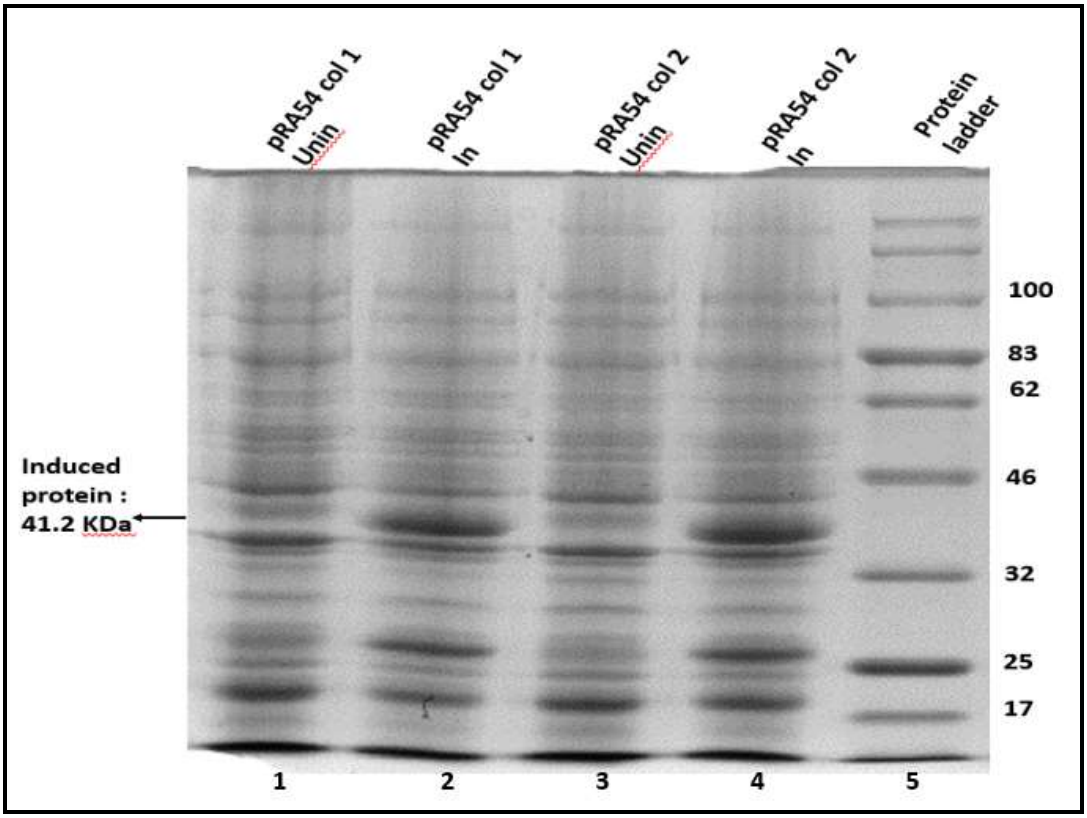

Figure 14. Induction check of pRA54; An expected induction at 42.1kDa was observed in both colony 1 [lane 2] and colony 2 [lane 4]

2.4 Generation of IBD V408F D366A mutant

Mini-Prep screening of ligation colonies

The PCR and ligation of the mutant PCR product were performed as done for pRA52. Of the 12 colonies, 2 colonies (Col 25 and 29) were moving slower than that of control pGEX vector and along with the positive control pCPGST [75-81] indicating the ligation of IBD V408F D366A mutant with pGEX vector (figure 15).

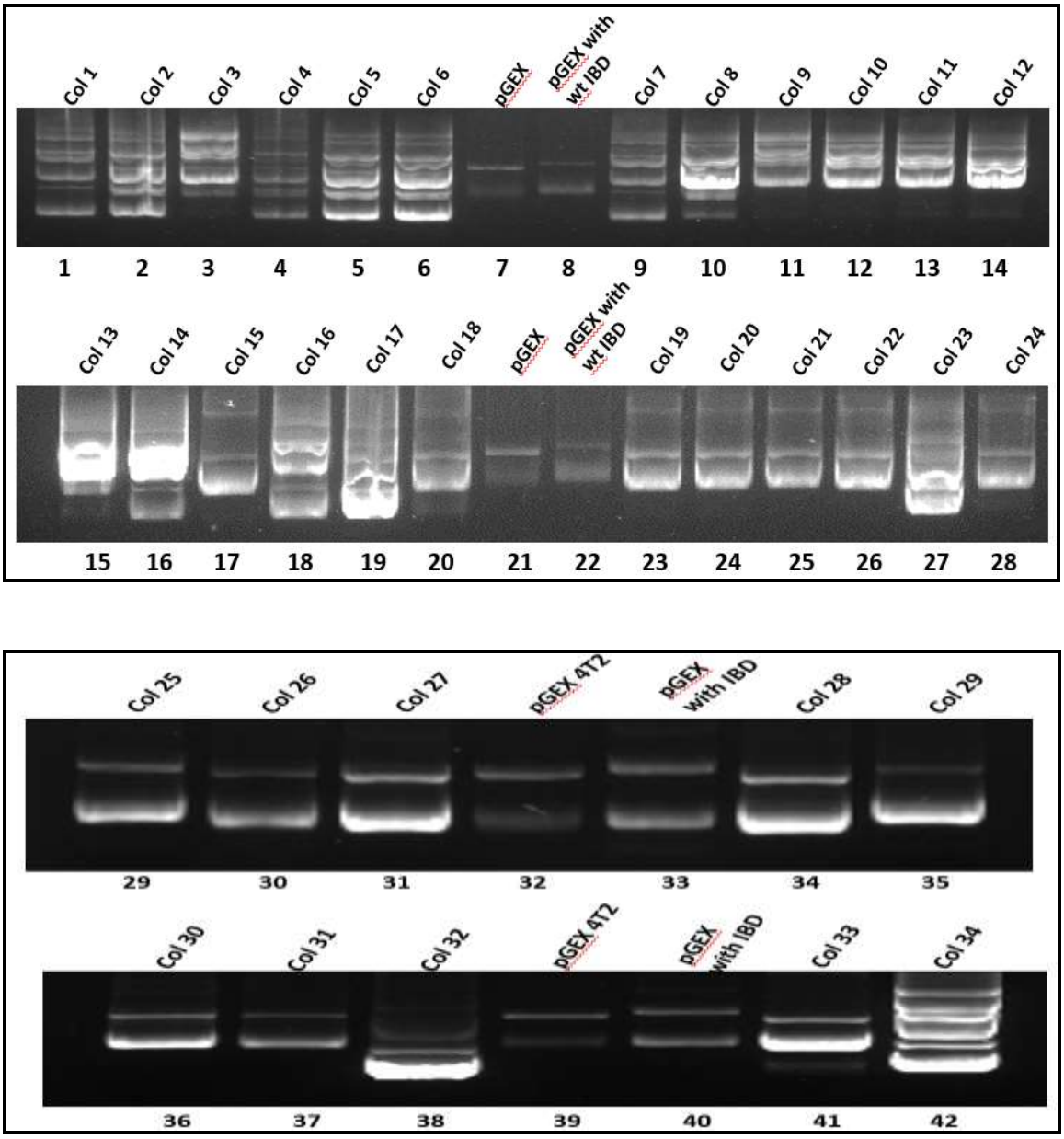

Figure 15. Mini-Prep plasmid isolation of pRA59 (IBD V408F D366A) by alkaline lysis method; control pGEX vector was loaded in Lane 7, 21,32 and 39 along with positive control pCPGST [75-81]. The supercoiled front of 5 colonies (col 4, 6, 7, 9 and 10) in lanes 2, 4, 5, 8 and 9 respectively are running slower than the supercoiled front of pGEX vector and along with that of positive control pCPGST [75-81]

Restriction digestion

Midi-Prep DNA (colony 29) was digested with BamHI-SalI to check the insert release. An expected insert release of 389 bps was seen in a 0.6% gel. This was followed by XbaI

digestion to check the D366A mutation and DraI digestion to check V408F mutation along with XbaI and DraI digestion of control plasmid pCPGST [75-81]. The expected linearization of 5.3 kb was observed for XbaI digestion in lane 4 indicating D366A mutation whereas DraI digestion of pRA59 shows expected bands of sizes of 3.35 kb, 841 bps and 691 bps was observed (Lane 3).

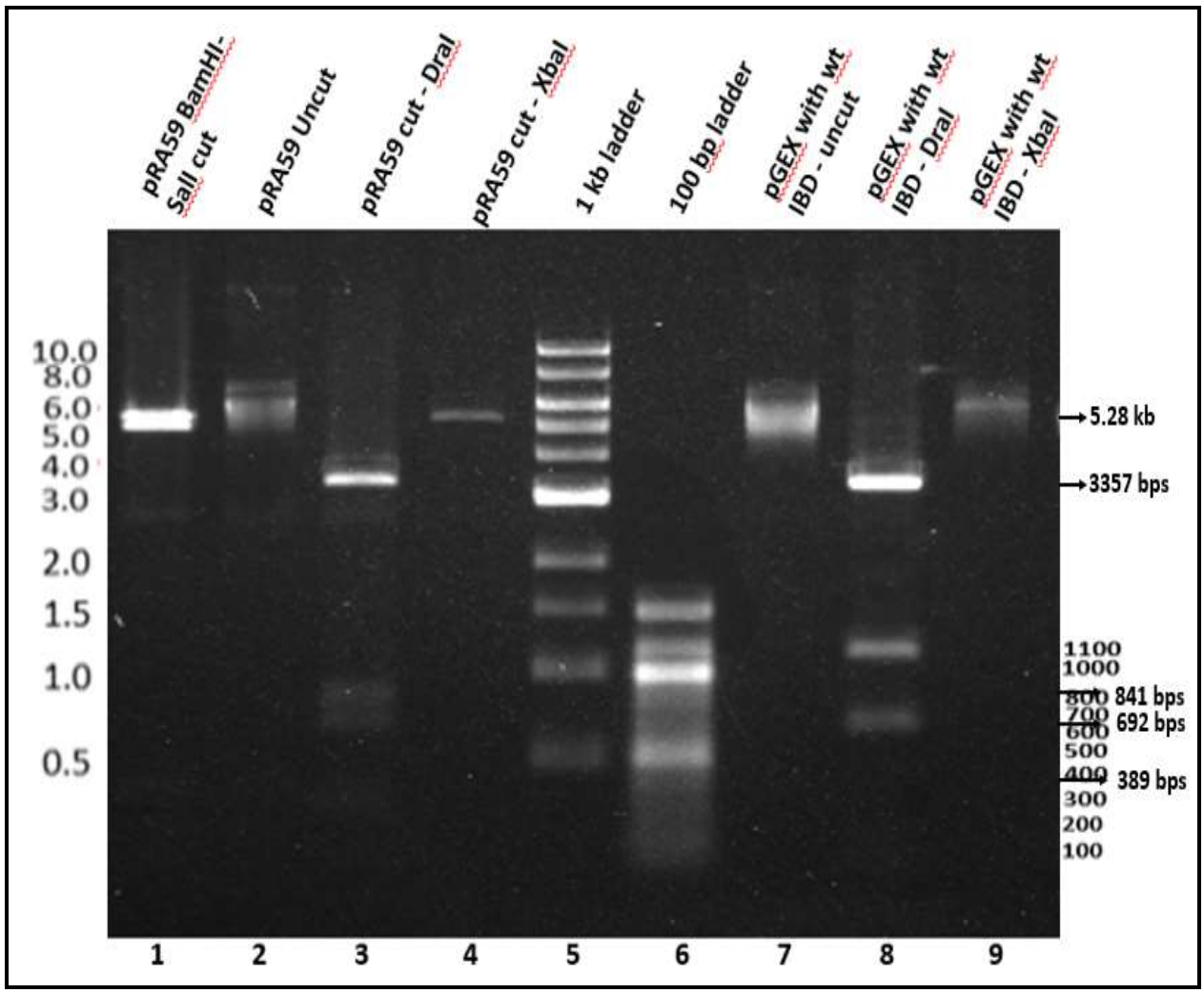

Figure 16. Restriction Digestion of pRA59 (IBD V408F D366A) with BamHI-SalI, DraI and XbaI; An expected insert release of 389 bps was observed when pRA53 was cut with BamHI-SalI in Lane 1. DraI digestion of pRA59 yielded expected band sizes of 3.35 kb, 841 bps and 692 bps in Lane 3 indicating V408F mutation whereas DraI digestion of control pCPGST[75-81] yields bands of sizes 1.07 kb, 3.35 kb and 692 bps in lane 8. XbaI digestion of pRA59 results in linearization of the plasmid at 5.3 kb (lane 4).

Induction of the protein

The midi-Prep DNA was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 10) and the remaining induced culture was pelleted for further purification of protein.

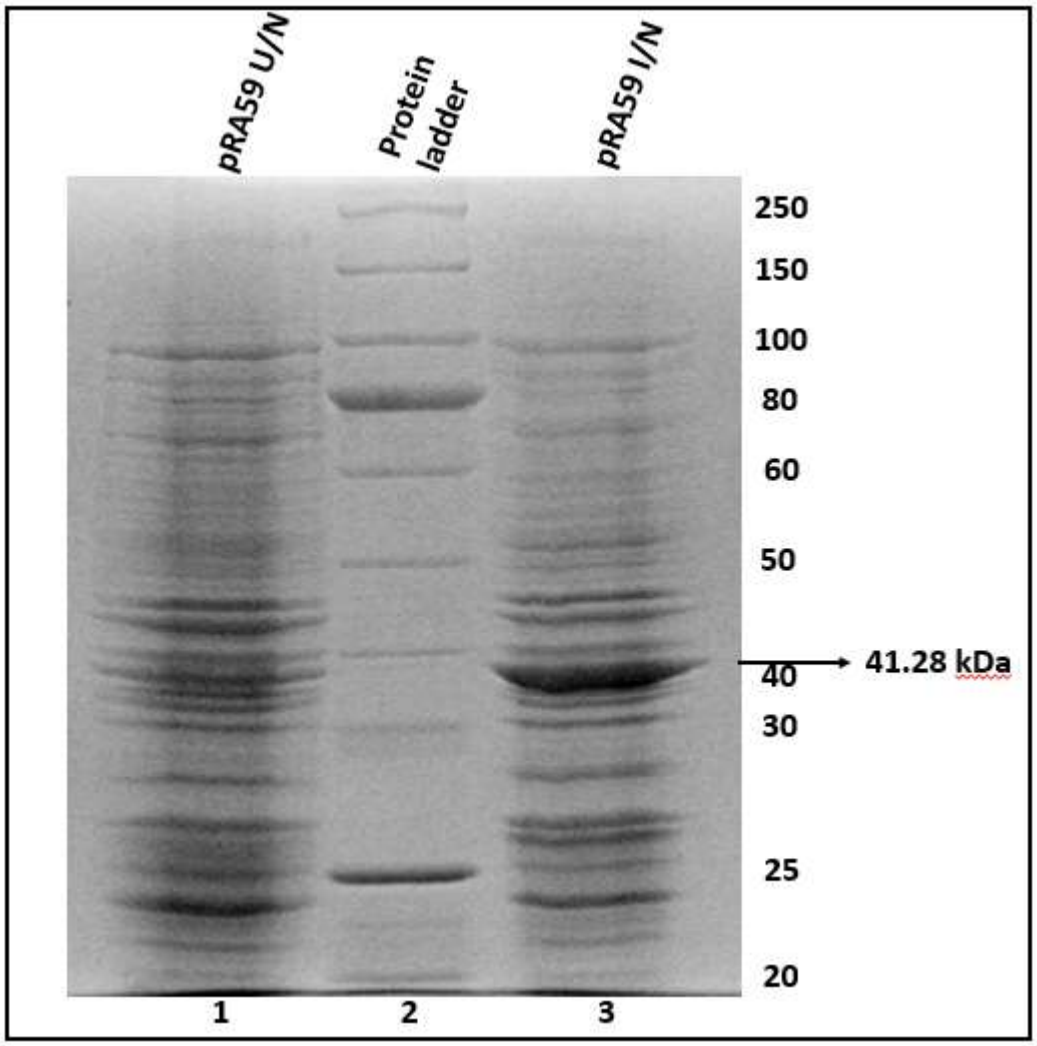

Figure 17. Induction of pRA59 (IBD V408F D366A); An induction of expected protein size of 41.2 kDa was observed (Lane 3)

2.5 Generation of IBD V408L/D366A mutant

Mini-Prep screening of ligation colonies

The PCR and ligation of the mutant PCR product were performed as done for pRA52. Of the 48 colonies, 2 colonies (Col 17 and 34) were moving slower than that of control pGEX vector and along with the positive control pCPGST [75-81] (figure 18)

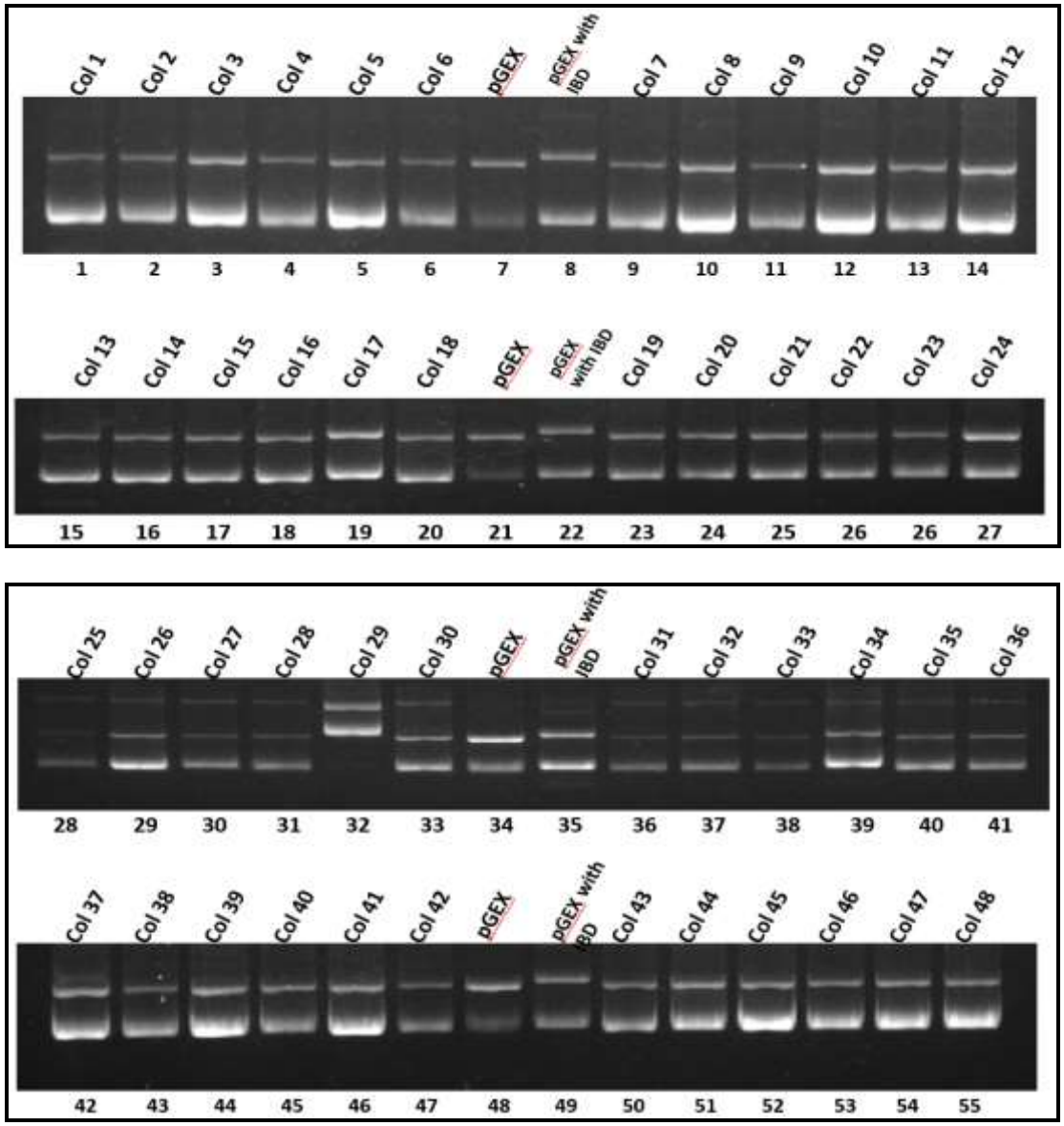

Figure 18. Mini-Prep plasmid isolation of pRA60 (IBD V408L D366A) by alkaline lysis method; control pGEX vector was loaded in Lane 7, 21,34 and 48 along with positive control pCPGST[75-81 in lane 8, 22, 35 and 49. The supercoiled front of 2 colonies, col 17 and 34 (in lane 19 and 39) moves slower than that of control pGEX4T2 vector and along with the positive control pCPGST[75-81]

Restriction digestion

Midi-Prep DNA (colony 34) was digested with BamHI-SalI to check the insert release. An expected insert release of 389 bps was seen in a 0.6% gel (figure 18, lane 6). This was followed by XbaI digestion to check the D366A mutation and DraI digestion to check V408L mutation along with XbaI and DraI digestion of control plasmid pCPGST [75-81]. The expected linearization of 5.3 kb was observed for XbaI digestion in lane 4 indicating D366A mutation whereas DraI digestion of pRA60 shows expected bands of sizes of 3.35 kb, 841 bps and 691 bps was observed in Lane 3.

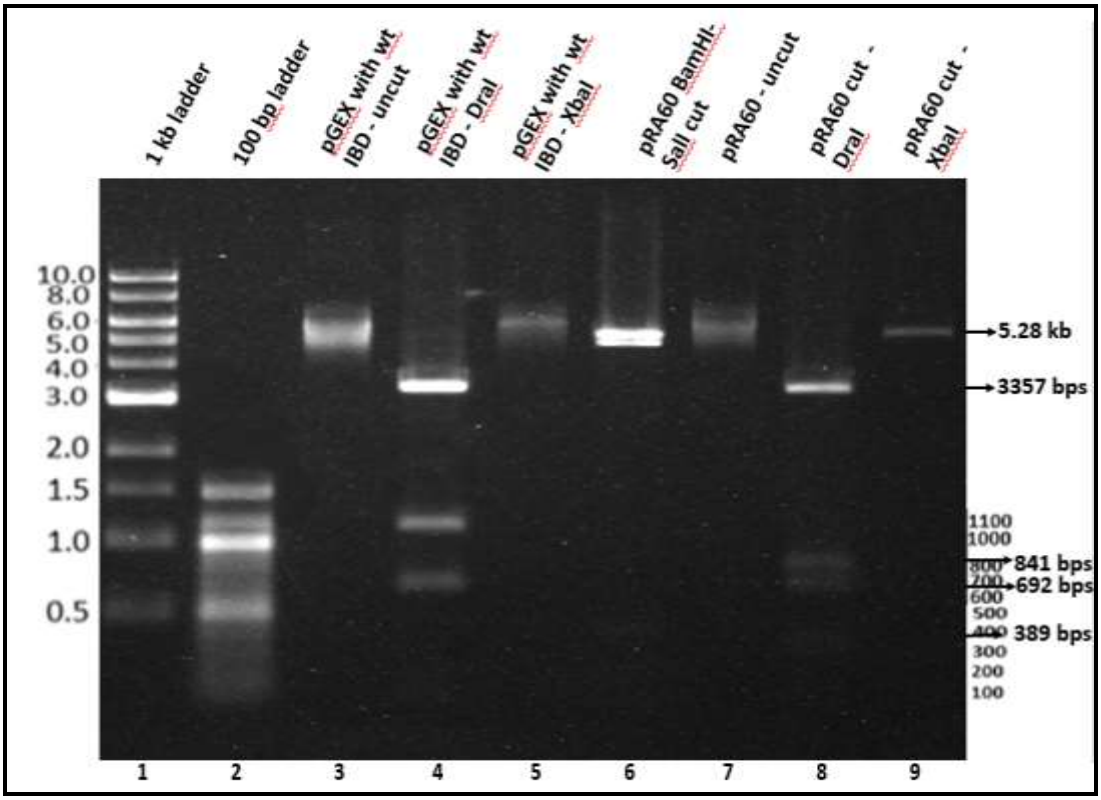

Figure 19. Restriction digestion of pRA60 with BamHI-SalI, DraI and XbaI; An expected insert release of 389 bps was observed when pRA60 was cut with BamHI-SalI in Lane 6. DraI digestion of pRA60 yielded expected band sizes of 3.35 kb, 841 bps and 692 bps in Lane 3 indicating V408L mutation whereas DraI digestion of control pCPGST[75-81] yields bands of sizes 1.07 kb, 3.35 kb and 692 bps in lane 4. XbaI digestion of pRA60 results in linearization of the plasmid at 5.3 kb (lane 9) indicating D366A mutation.

Induction of the protein

The midi-Prep DNA was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 10) and the remaining induced culture was pelleted for further purification of protein.

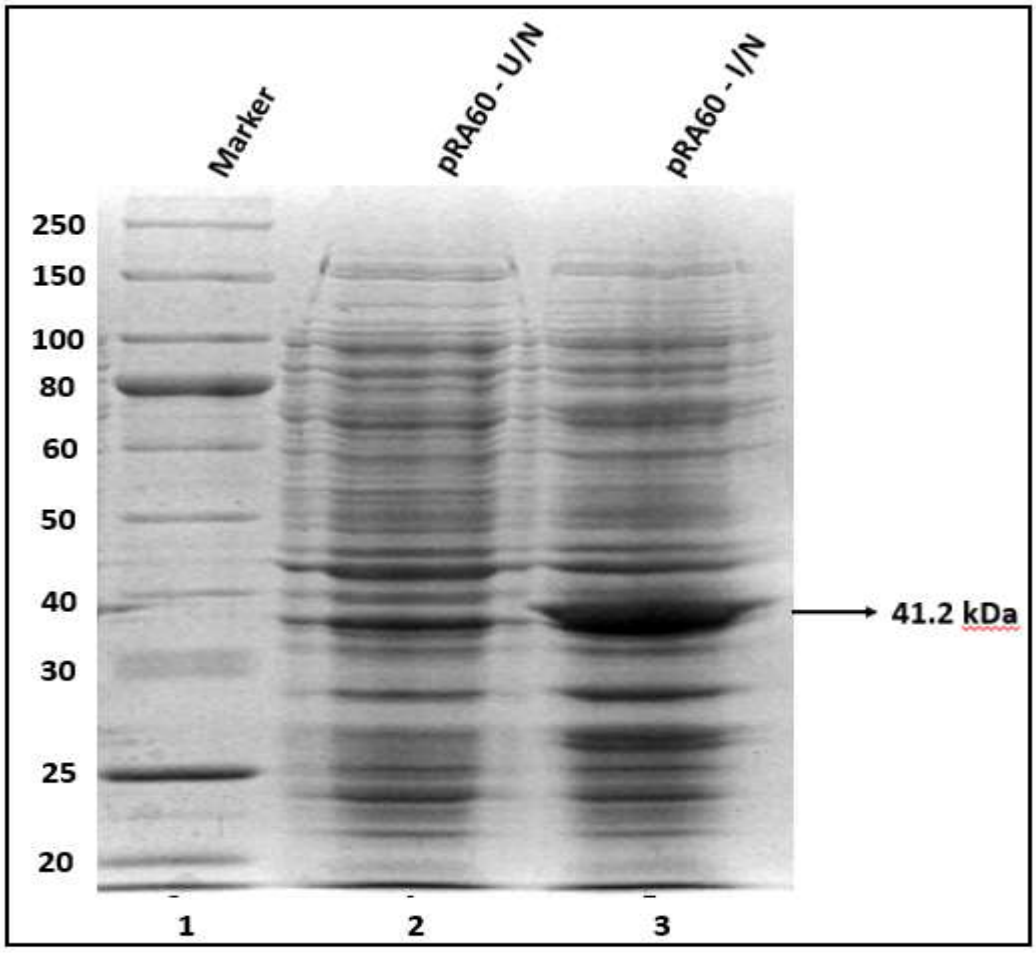

Figure 20. Induction of pRA60 (IBD V408L D366A); An induction of expected protein size of 41.2 kDa was observed (Lane 3)

2.6 Generation of IBD V408M/D366A mutant (pRA71)

Overlap PCR to generate IBD V408M/D366A double mutant

Using pRA35 (IBD V408M) as the template and using pGEX vector specific primers (NR10, NR66) along with IBD D366A mutant specific primers (NR 96, NR97), two rounds of PCR was done. In the first round, two amplicons of 144 bps and 425 bps were generated using NR10 vector specific forward primer with NR97 IBD D366A gene specific reverse

primer and NR96 IBD D366A gene specific forward primer with NR66 vector specific reverse primer respectively. In the second round the two products were used as templates along with NR10/66 gene specific primers to get a product of 543 bps.

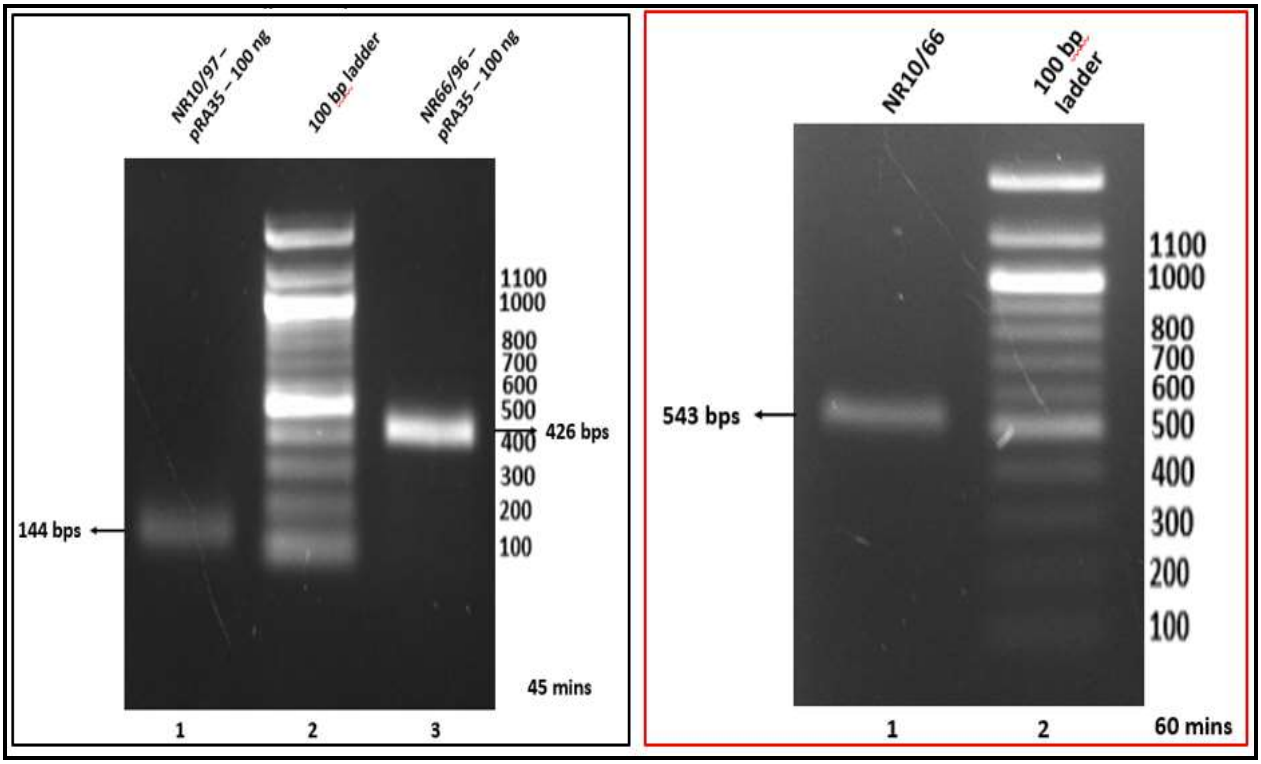

Figure 21. Overlap PCR method to generate IBD V408M D366A. Left panel shows the amplification of pRA35 (IBD V408M) as template and using NR10 vector specific forward primer with NR97 IBD D366A gene specific reverse primer (Lane 2) and NR96 IBD D366A gene specific forward primer with NR66 vector specific reverse primer. Expected products of 144 bps (Lane 1) and 425 bps (Lane 3) were observed. Right panel shows the amplified 543 bp product using the above two products as template with NR 10/66 primers

Mini-Prep screening of ligation colonies

The PCR and ligation of the mutant PCR product were performed as done for pRA52. Of the 48 colonies, 2 colonies (Col 17 and 34) were moving slower than that of control pGEX vector and along with the positive control pCPGST [75-81] (figure 18)

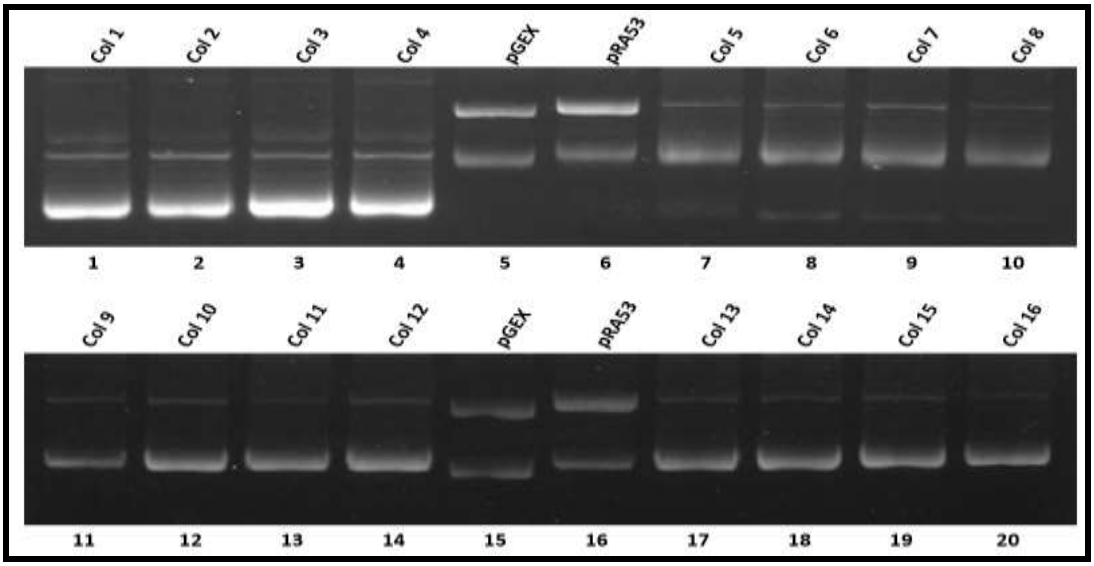

Figure 6. Mini-Prep plasmid isolation of pRA71 (IBD V408M D366A) by alkaline lysis method; control pGEX vector was loaded in Lane 5 and 15 along with positive control pRA53 (IBD V408F L368F) in lane 6 and 16. The supercoiled front of 12 colonies, col 5-16 moves slower than that of control pGEX4T2 vector and along with the positive control pRA53.

Restriction digestion

Midi-Prep DNA (colony 34) was digested with BamHI-SalI to check the insert release. An expected insert release of 389 bps was seen in a 0.6% gel (figure 18, lane 6). This was followed by XbaI digestion to check the D366A mutation and DraI digestion to check V408L mutation along with XbaI and DraI digestion of control plasmid pCPGST[75-81]. The expected linearization of 5.3 kb was observed for XbaI digestion in lane 4 indicating D366A mutation whereas DraI digestion of pRA60 shows expected bands of sizes of 3.35 kb, 841 bps and 691 bps was observed in Lane 3.

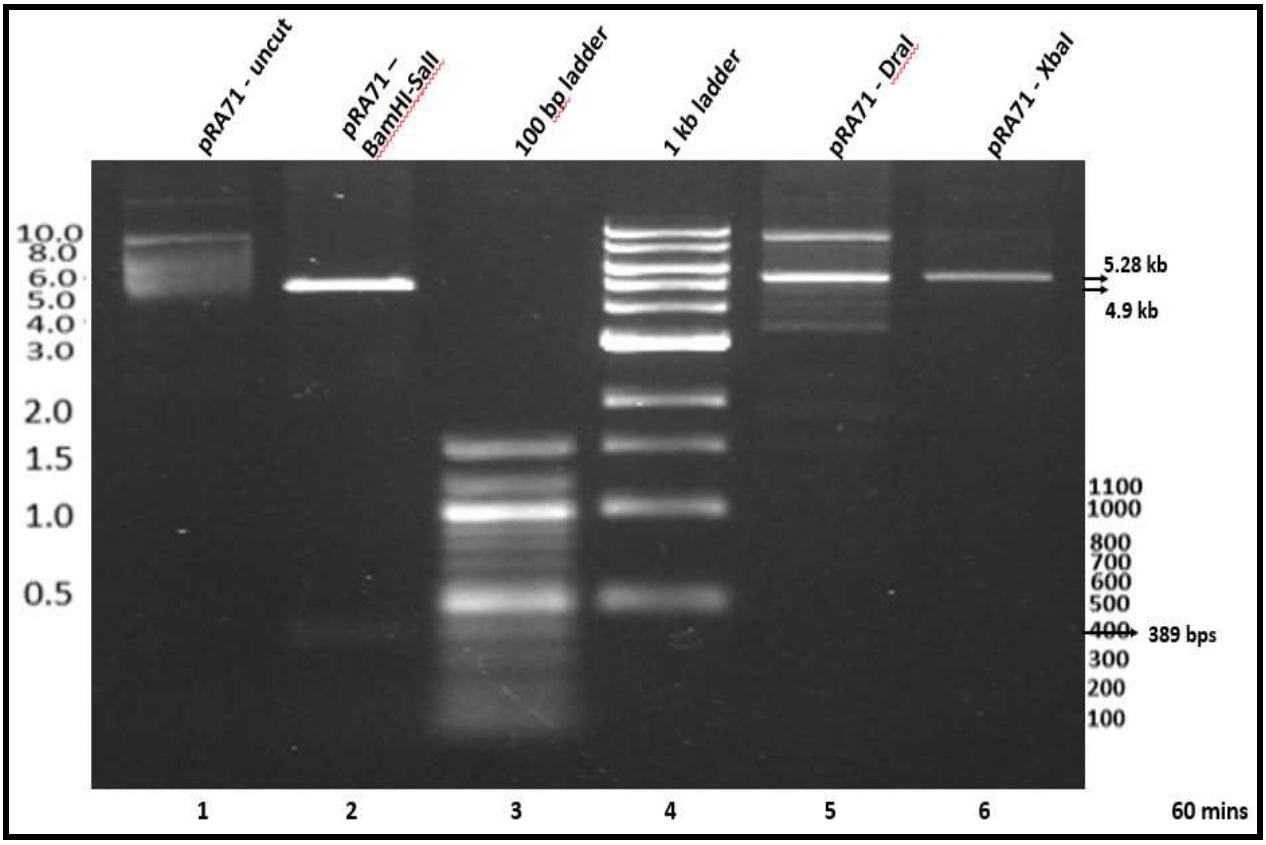

Figure 23. Restriction digestion of pRA71 with BamHI-SalI and XbaI; An expected insert release of 389 bps was observed when pRA71 was cut with BamHI-SalI (Lane 2). XbaI digestion of pRA71 results in linearization of the plasmid at 5.3 kb (lane 6) indicating D366A mutation

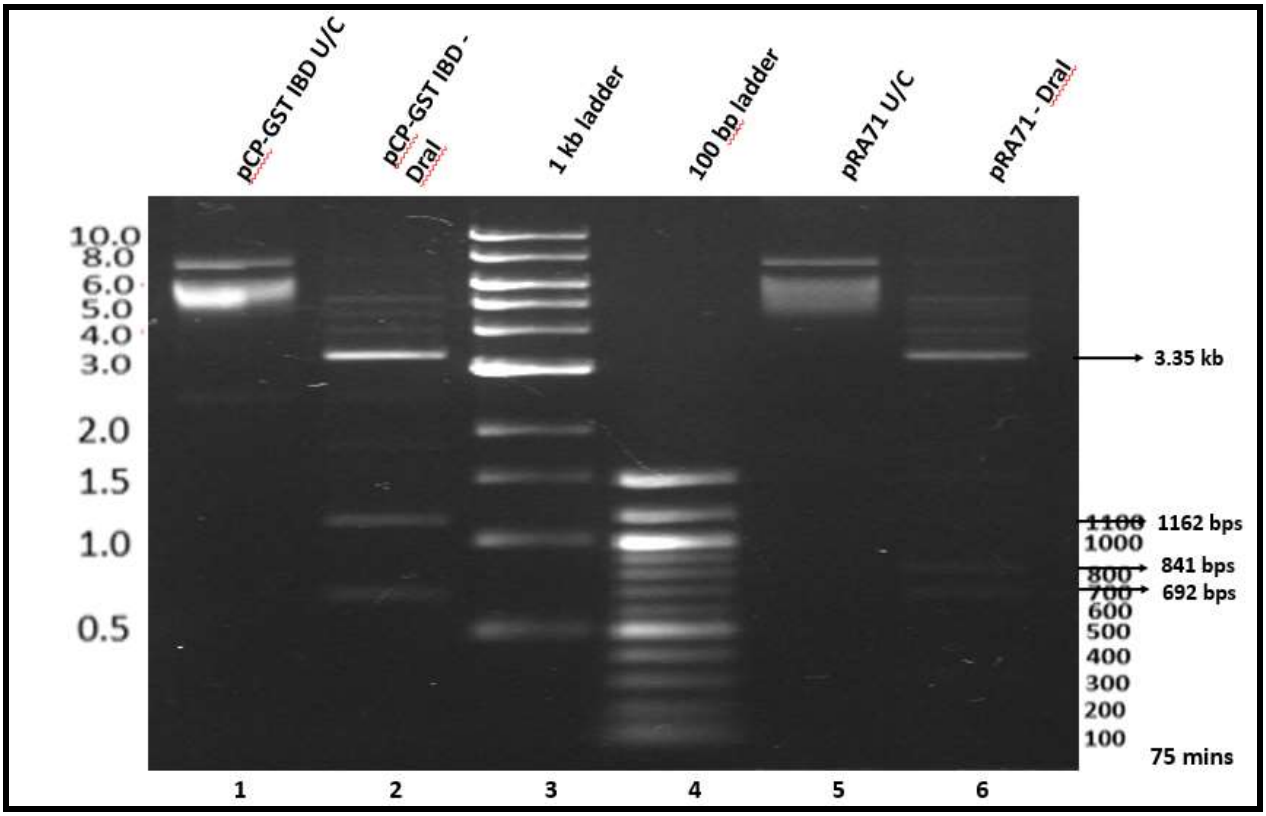

Figure 7. Restriction digestion of pRA71 with DraI; DraI digestion of pRA71 yielded expected band sizes of 3.35 kb, 841 bps and 692 bps (Lane 6) indicating V408M mutation whereas DraI digestion of control pCPGST[75-81] yields bands of sizes 1.07 kb, 3.35 kb and 692 bps (Lane 2).

Induction of the protein

The midi-Prep DNA was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 10) and the remaining induced culture was pelleted for further purification of protein.

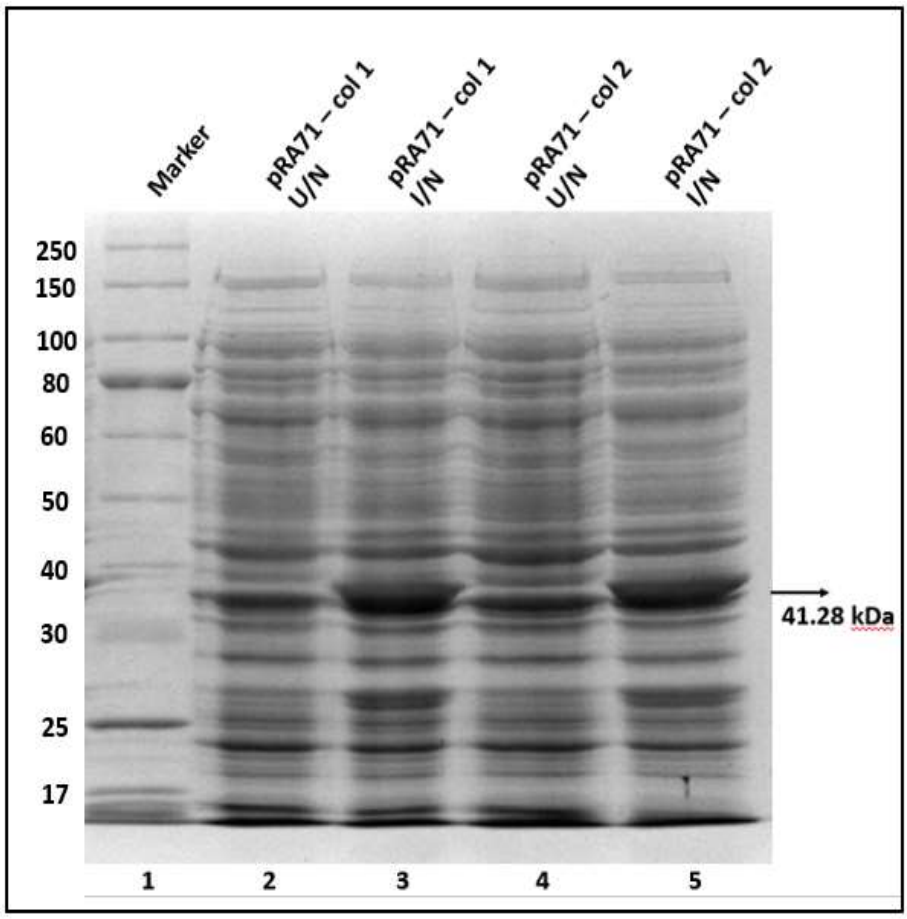

Figure 25. Induction of pRA71 (IBD V408M D366A); An induction of expected protein size of 41.2 kDa was observed (Lane 3)

2.7 Generation of IBD L368M/D366A mutant (pRA72)

Mega-primer method to generate IBD L368M/D366A double mutant

Using pRA54 (IBD D366A) as template and using NR10 vector specific forward primer with NR97 IBD D366A gene specific reverse primer, a product of 144 bps was obtained which was digested with XbaI to get the mega-primer. Using the mega-primer and NR66 vector specific reverse primer, with pRA31 (IBD L368M) as template, amplification was done to get IBD L368M D366A double mutant PCR product of 543 bps.

As amplification was very less, further amplification was done using IBD L368M D366A PCR product as the template with NR 10/66 primers.

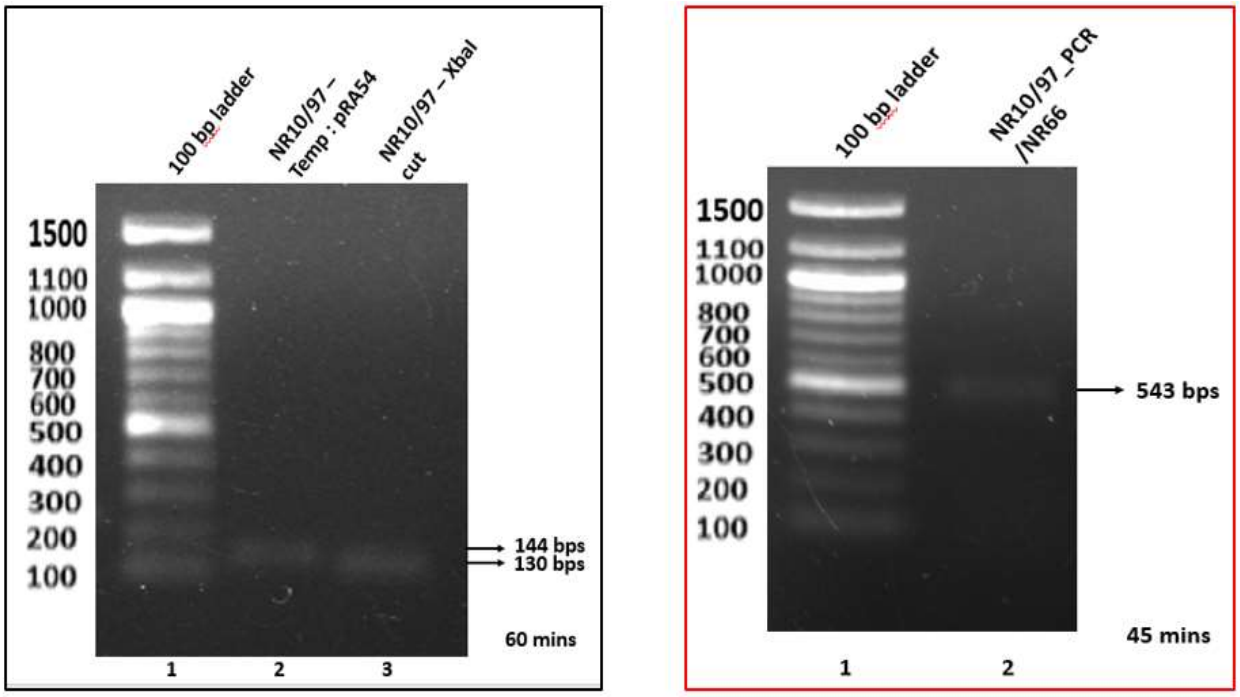

Figure 26. Mega primer method to generate IBD L368M D366A. Left panel shows the amplification of pRA54 (IBD D366A) as template and using NR10 vector specific forward primer with NR97 IBD D366A gene specific reverse primer (Lane 2) and XbaI digestion of the same (Megaprimer) (Lane 3). Right panel shows the amplified 543 bp product using mega-primer and NR66 vector specific reverse primer, with pRA31 (IBD L368M) as template.

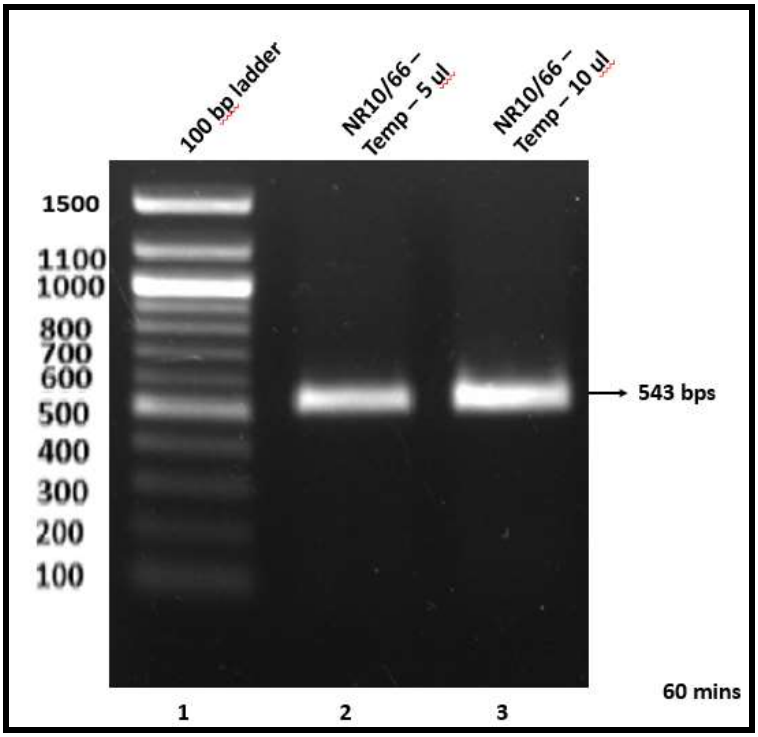

Figure 27. PCR amplification of IBD L368M D366A using NR10/66 primers; An expected 543 bps product was observed (Lane 2 and 3) with 5 and 10 ul of mega-primer generated IBD L368M/D366A as template with NR10/66 primers

Mini-Prep Screening of ligation colonies

The ligation of the mutant PCR product were performed as done for pRA52. Of the 48 colonies, 2 colonies (Col 17 and 34) were moving slower than that of control pGEX vector and along with the positive control pCPGST [75-81] (figure 18)

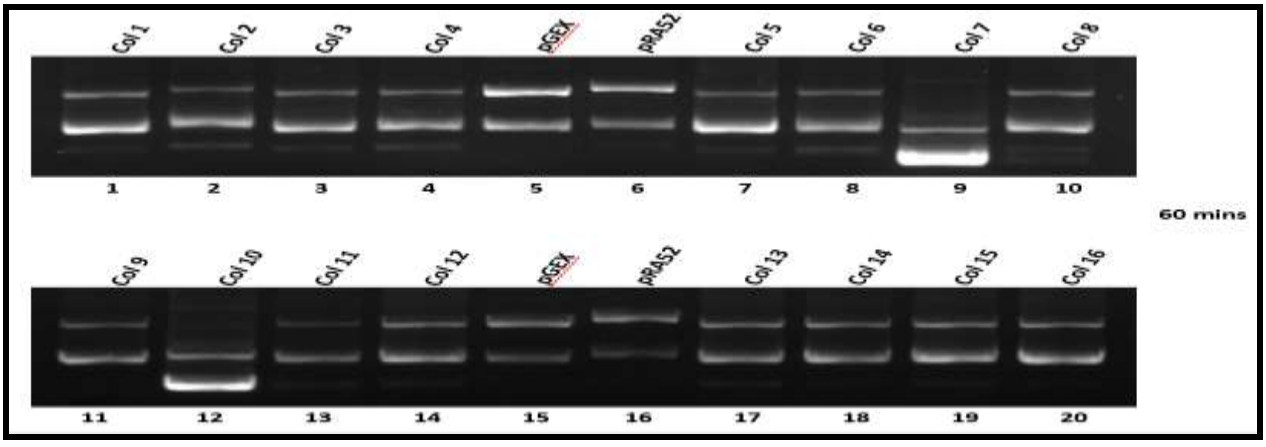

Figure 28. Mini-Prep plasmid isolation of pRA72 (IBD L368M D366A) by alkaline lysis method;

Control pGEX vector was loaded in Lane 5 and 15 along with positive control pRA52 (IBD L368A) in lane 6 and 16. The supercoiled front of 1 colony, col 2 moves slower than that of control pGEX4T2 vector and along with the positive control pRA52. *Restriction digestion*

Midi-Prep DNA (colony 2) was digested with BamHI-SalI to check the insert release. An expected insert release of 389 bps was seen in a 0.6% gel (figure 18, lane 6). This was followed by XbaI digestion (the XbaI site would be lost because of digestion with XbaI in the mega-primer and use of pRA31 as the template) and HpaI digestion to check L368M mutation along with XbaI and HpaI digestion of pRA54 (IBD D366A) and control plasmid pCPGST[75-81] respectively. . HpaI digestion of pRA72 results in expected band sizes of 1.7 kb and 3.4 kb (Lane 3), while linearization of the plasmid at 5.3 kb was observed in control vector pCP-GST IBD [75-81] (Lane 8)

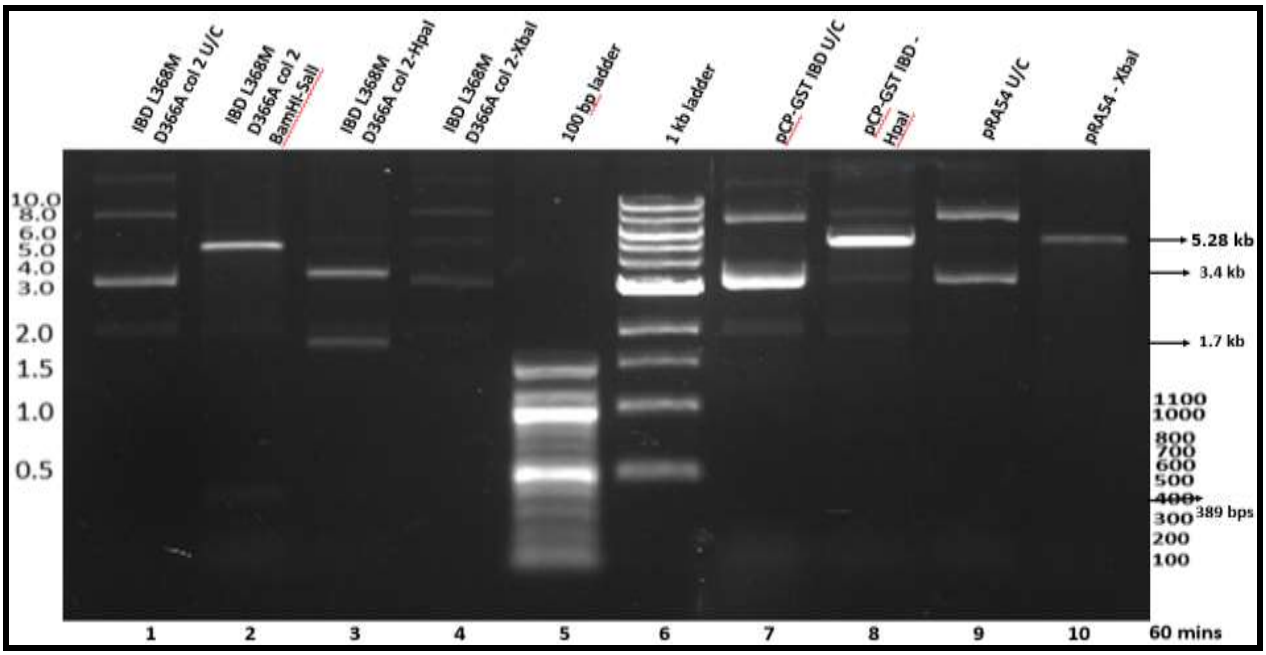

Figure 29. Restriction digestion of pRA72 with BamHI-SalI, HpaI and XbaI; An expected insert release of 389 bps was observed when pRA72 was cut with BamHI-SalI (Lane 2). XbaI digestion of pRA72 results in no cut as expected (Lane 4) (while linearization of the plasmid at 5.3 kb was observed control vector pRa54 (IBD D366A) (lane 10) indicating D366A mutation. HpaI digestion of pRA72 results in expected band

sizes of 1.7 kb and 3.4 kb (Lane 3), while linearization of the plasmid at 5.3 kb was observed in control vector pCP-GST IBD [75-81] (Lane 8)

Induction of the protein

The midi-Prep DNA (col 2) was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 10) and the remaining induced culture was pelleted for further purification of protein.

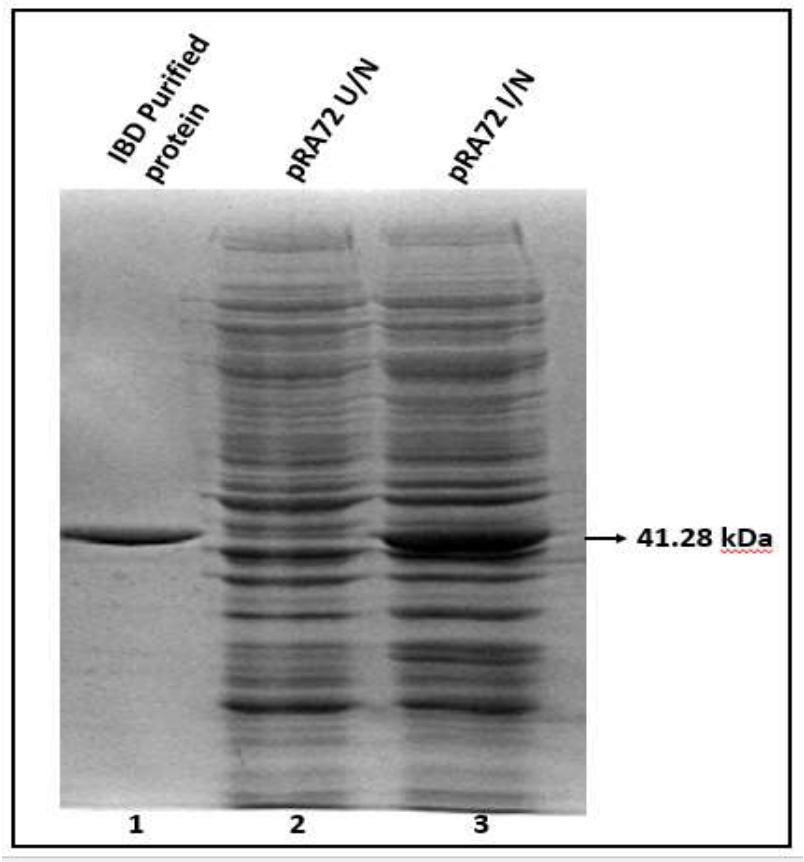

Figure 30. Induction of pRA72 (IBD L368M D366A); An induction of expected protein size of 41.2 kDa was observed (Lane 3).

2.8 Generation of IBD L368F/D366A mutant (pRA73)

Mega-primer method to generate IBD L368F/D366A double mutant

As done for IBD L368M/D366A pRA72 double mutant, using the mega-primer (generated for pRA72 – IBD L368M D366A) and NR66 vector specific reverse primer, and with

pRA32 (IBD L368F) as template, amplification was done to get IBD L368F D366A double mutant PCR product of 543 bps. As amplification was very less, further amplification was done using IBD L368M D366A PCR product as the template with NR 10/66 primers.

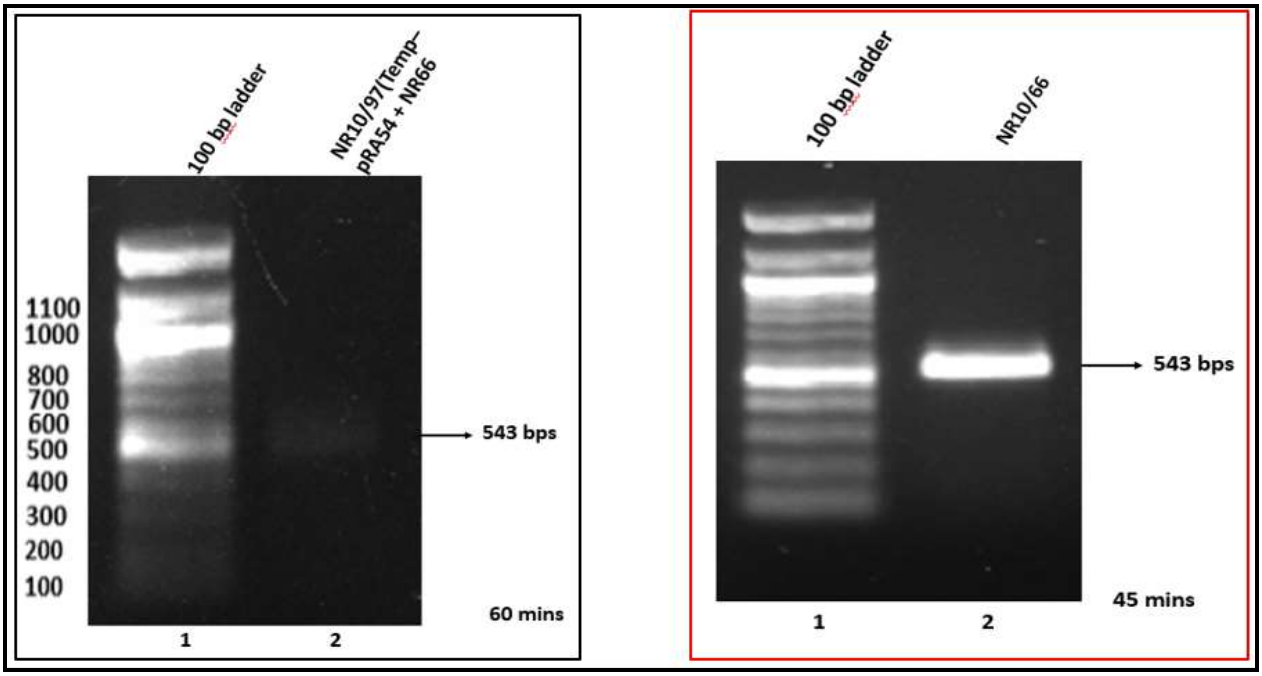

Figure 31. Mega primer method to generate IBD L368F D366A. Left panel shows the amplified 543 bp product using mega-primer and NR66 vector specific reverse primer, with pRA32 (IBD L368M) as template. Right panel shows an expected 543 bps product (Lane 3) with mega-primer generated IBD L368F/D366A as template and NR10/66 primers.

Mini-Prep screening of ligation colonies

The ligation of the mutant PCR product were performed as done for pRA52. Of the 6 colonies, all the 6 colonies were moving slower than that of control pGEX vector and along with the positive control pRA52 (IBD L368A) (figure 32)

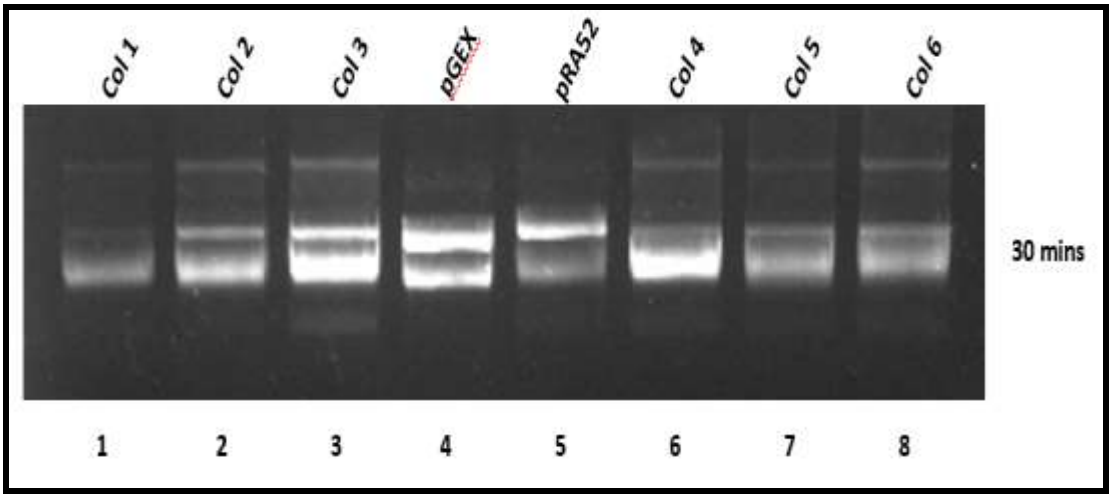

Figure 8. Mini-Prep plasmid isolation of pRA73 (IBD L368F D366A) by alkaline lysis method; control pGEX vector was loaded in Lane 4 along with positive control pRA52 (IBD L368A) in lane 5. The supercoiled front of all the colonies (Col 1-6) moves slower than that of control pGEX4T2 vector and along with the positive control pRA52.

Restriction digestion

Mini-Prep DNA (colony 2, 3) were digested with BamHI-SalI to check the insert release. An expected insert release of 389 bps was seen in a 0.6% gel (figure 18, lane 6).

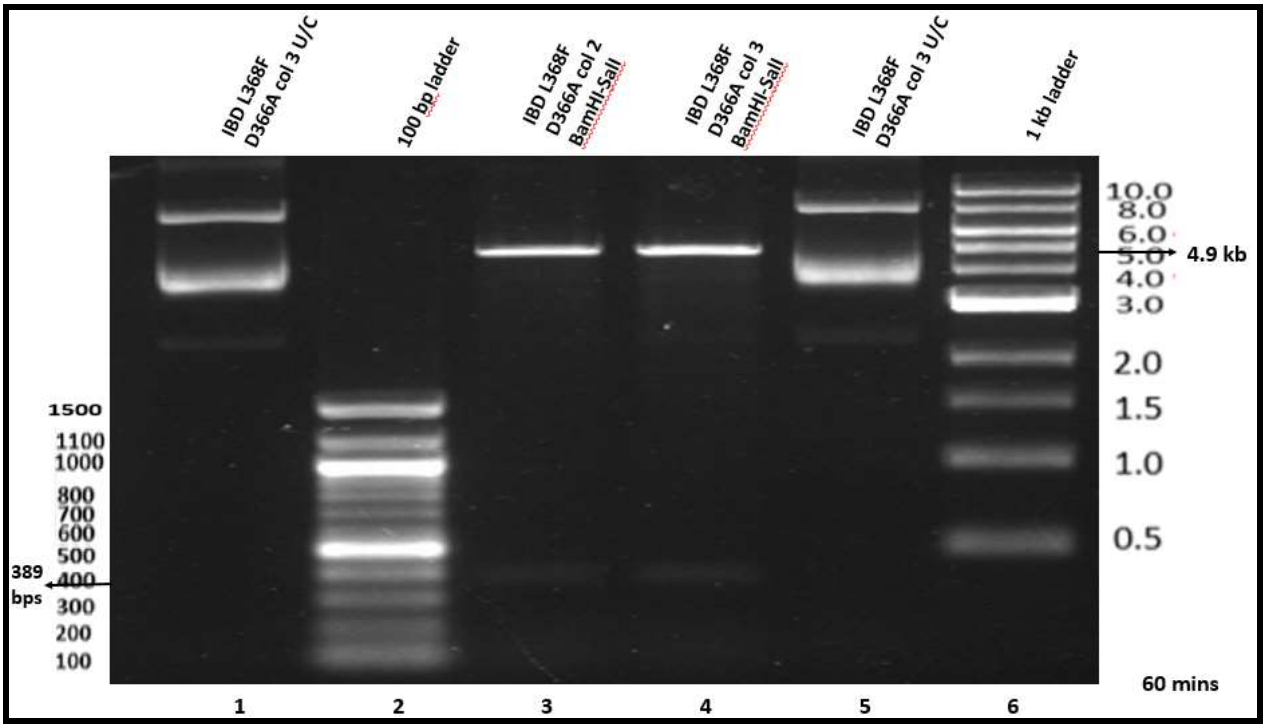

Figure 33. Restriction digestion of pRA73 with BamHI-SalI; An expected insert release of 389 bps was observed when pRA72 was cut with BamHI-SalI (Lane 2).

This was followed by XbaI digestion (the XbaI site would be lost because of digestion with XbaI in the mega-primer and use of pRA32 as the template) of pRA73 col 2 and of control vector pRA54 (IBD D366A). No cut was observed in XbaI digested col 2 of IBD L368F D366A as expected whereas an expected linearization of 5.3 kb was observed in pRA54 (IBD D366A).

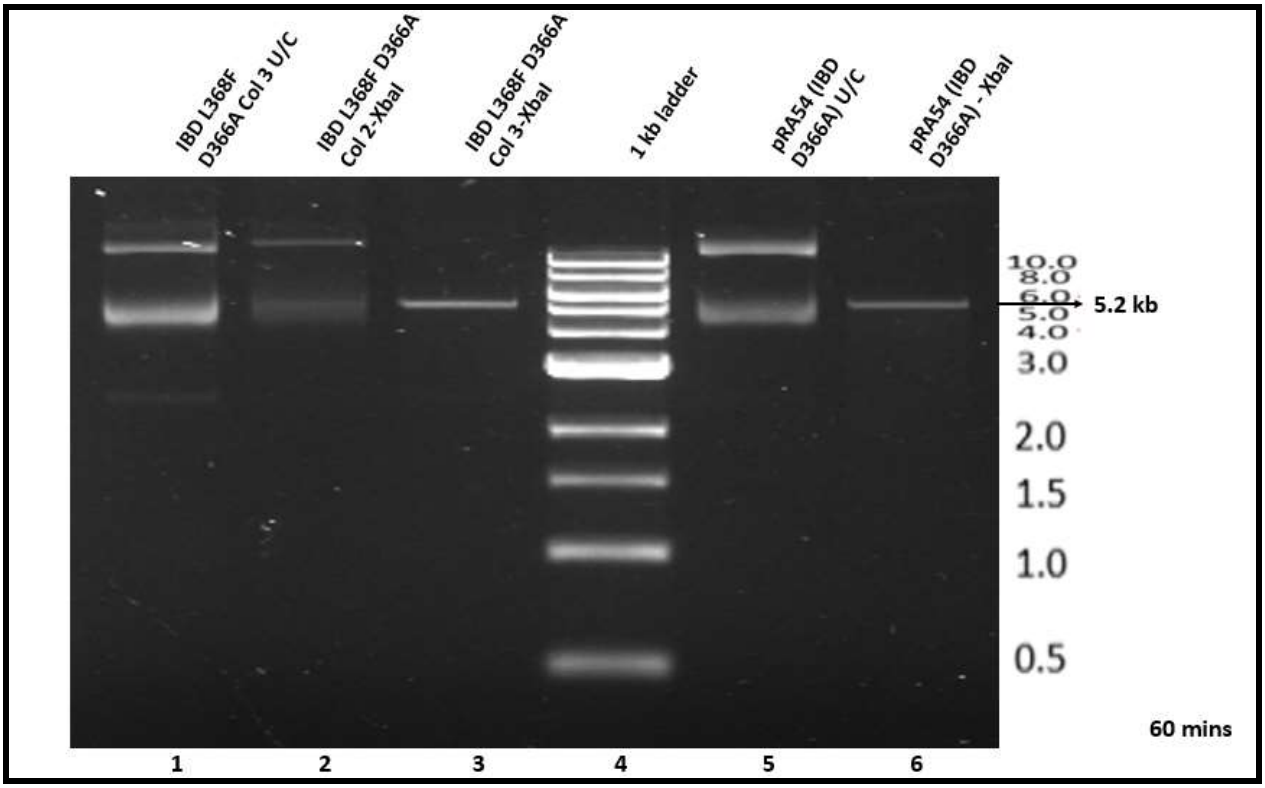

Figure 34. Restriction digestion of pRA73 with XbaI; XbaI digestion of pRA73 results in no cut as expected (Lane 2) (while linearization of the plasmid at 5.3 kb was observed control vector pRA54 (IBD D366A) (lane 6) indicating D366A mutation

HpaI digestion was done to check L368F mutation along with control plasmid pCPGST[75-81]. HpaI digestion of pRA73 results in expected band sizes of 1.7 kb and 3.4 kb (Lane 3), while linearization of the plasmid at 5.3 kb was observed in control vector pCP-GST IBD [75-81] (Lane 8)

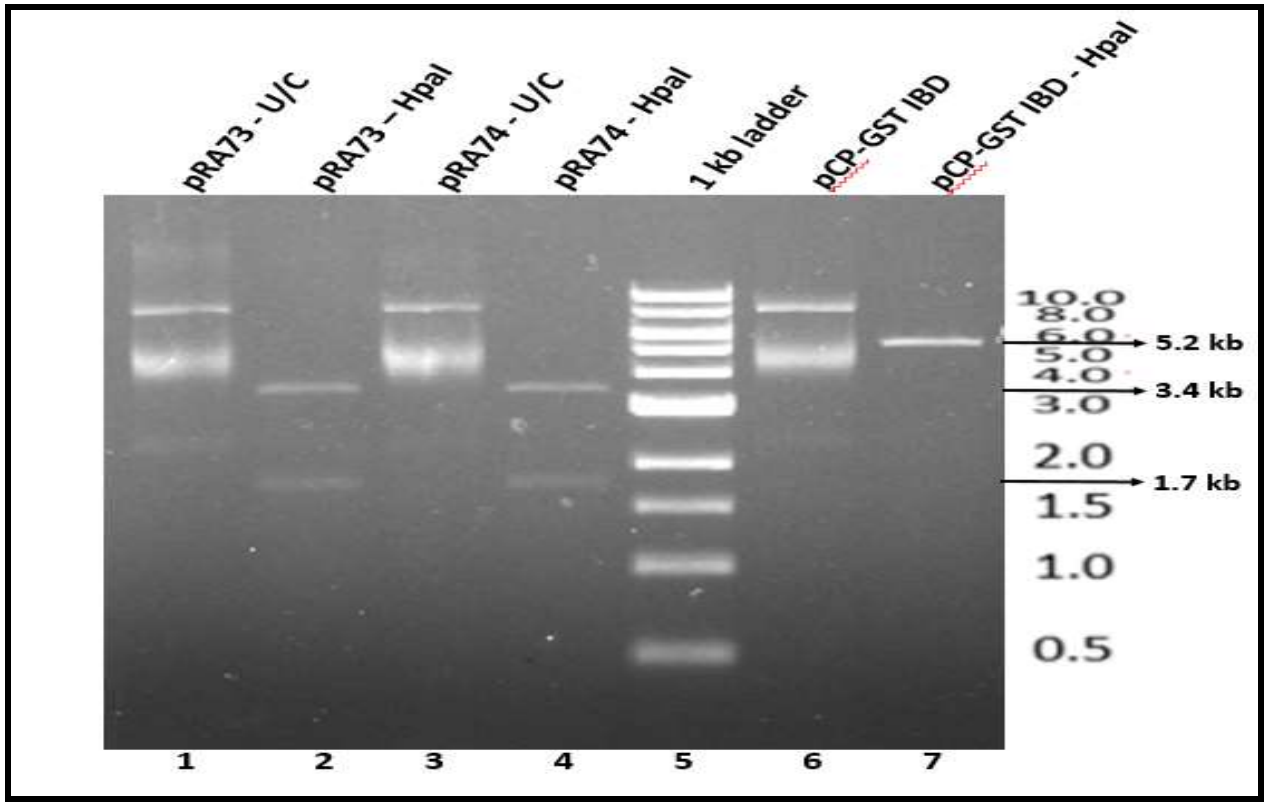

Figure 35. Restriction digestion of pRA73 with HpaI. HpaI digestion of pRA73 results in expected band sizes of 1.7 kb and 3.4 kb (Lane 2), while linearization of the plasmid at 5.3 kb was observed in control vector pCP-GST IBD [75-81] (Lane 7)

Induction of the protein

The midi-Prep DNA (col 2) was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 10) and the remaining induced culture was pelleted for further purification of protein.

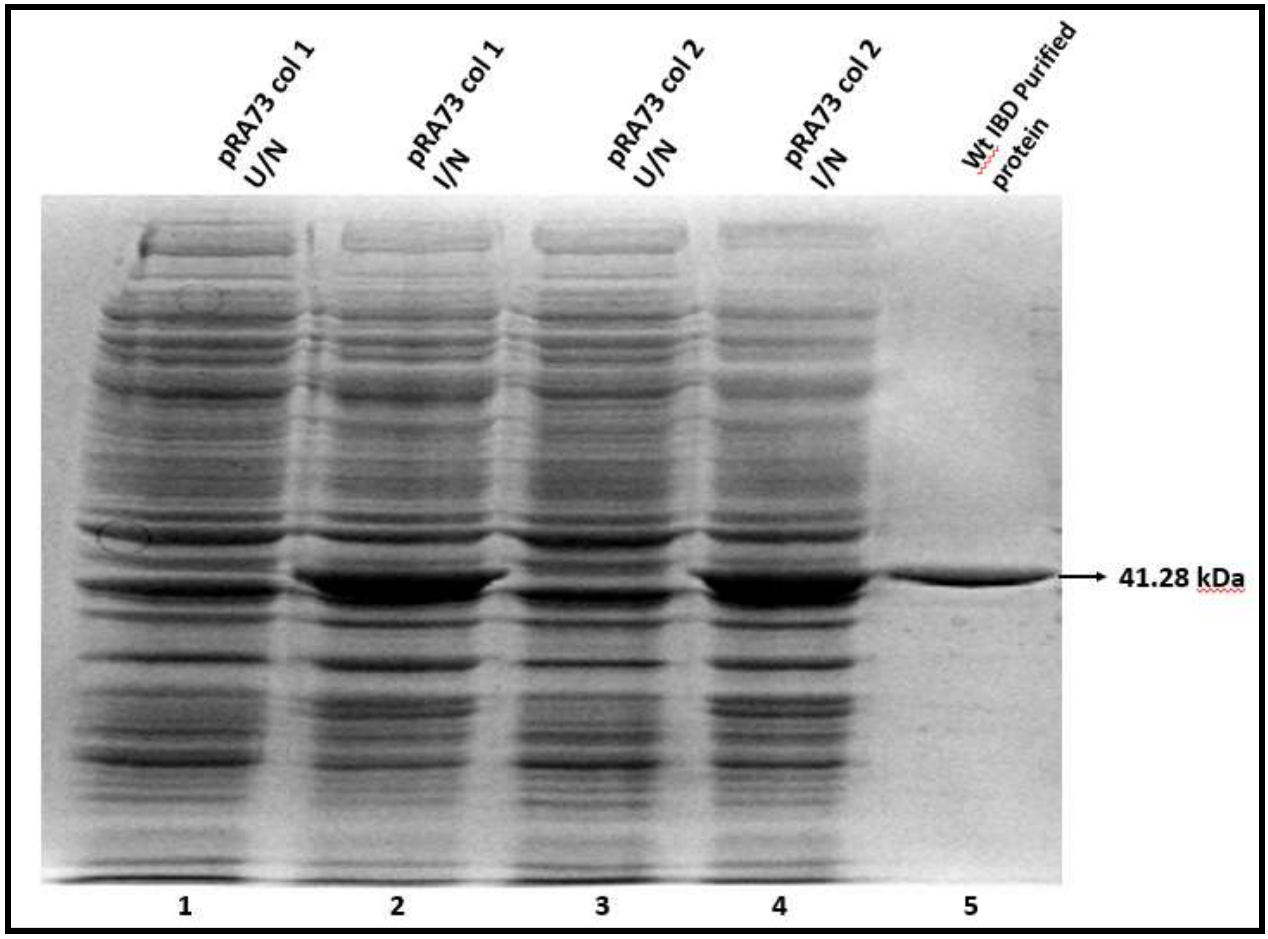

Figure 36. Induction of pRA73 (IBD L368F D366A); An induction of expected protein size of 41.2 kDa, running along with marker purified wt IBD protein was observed (Lane 2, 4).

2.9 Generation of IBD L368I/D366A mutant (pRA74)

Mega-primer method to generate IBD L368F/D366A double mutant

As done for IBD L368M/D366A pRA72 double mutant, using the mega-primer (generated for pRA72 – IBD L368M D366A) and NR66 vector specific reverse primer, and with pRA33 (IBD L368I) as template, amplification was done to get IBD L368I D366A double mutant PCR product of 543 bps. As amplification was very less, further amplification was done using IBD L368I D366A PCR product as the template with NR 10/66 primers.

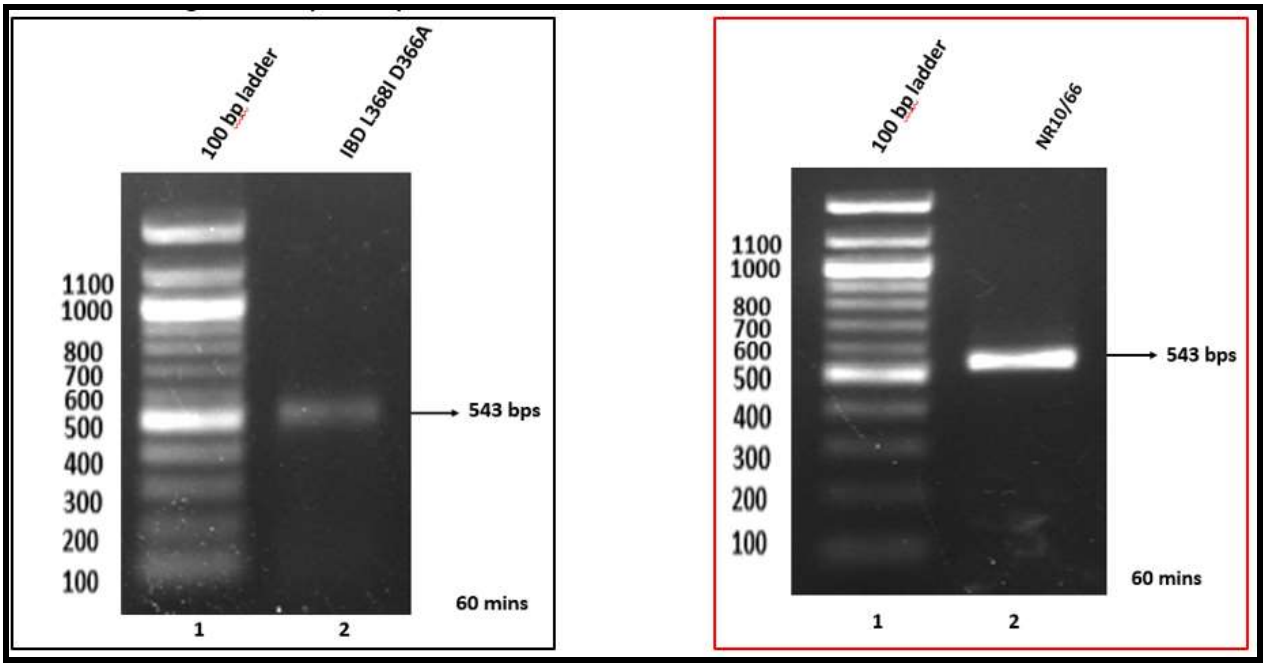

Figure 37. Mega primer method to generate IBD L368F D366A. Left panel shows the amplified 543 bp product using mega-primer and NR66 vector specific reverse primer, with pRA33 (IBD L368I) as template. Right panel shows an expected 543 bps product (Lane 3) with mega-primer generated IBD L368I/D366A as template and NR10/66 primers.

Mini-Prep screening of ligation colonies

The ligation of the mutant PCR product were performed as done for pRA52. Of the 6 colonies, all the 6 colonies were moving slower than that of control pGEX vector and along with the positive control pRA52 (IBD L368A) (figure 38)

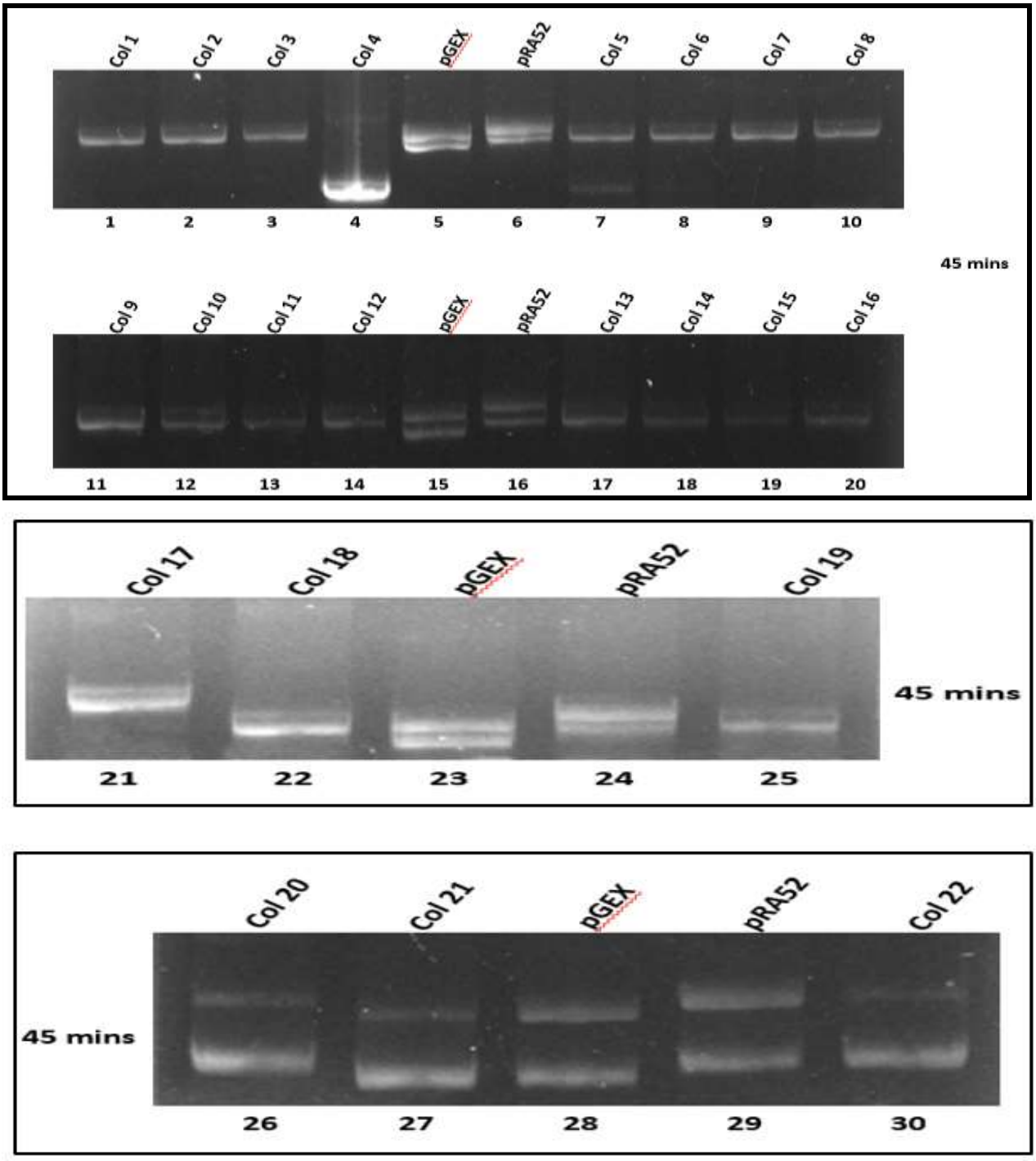

Figure 38. Mini-Prep plasmid isolation of pRA74 (IBD L368I D366A) by alkaline lysis method; control pGEX vector was loaded (Lane 5, 15) along with positive control pRA52 (IBD L368A) (Lane 6, 16). The supercoiled front of all the colonies (Col 1-22) except col 4 (lane 4) and col 21 (lane 27) moves slower than that of control pGEX4T2 vector and along with the positive control pRA52.

Restriction digestion

Midi-Prep DNA (colony 21) were digested with BamHI-SalI to check the insert release. An expected insert release of 389 bps was seen in a 0.6% gel (figure 39, lane 2).

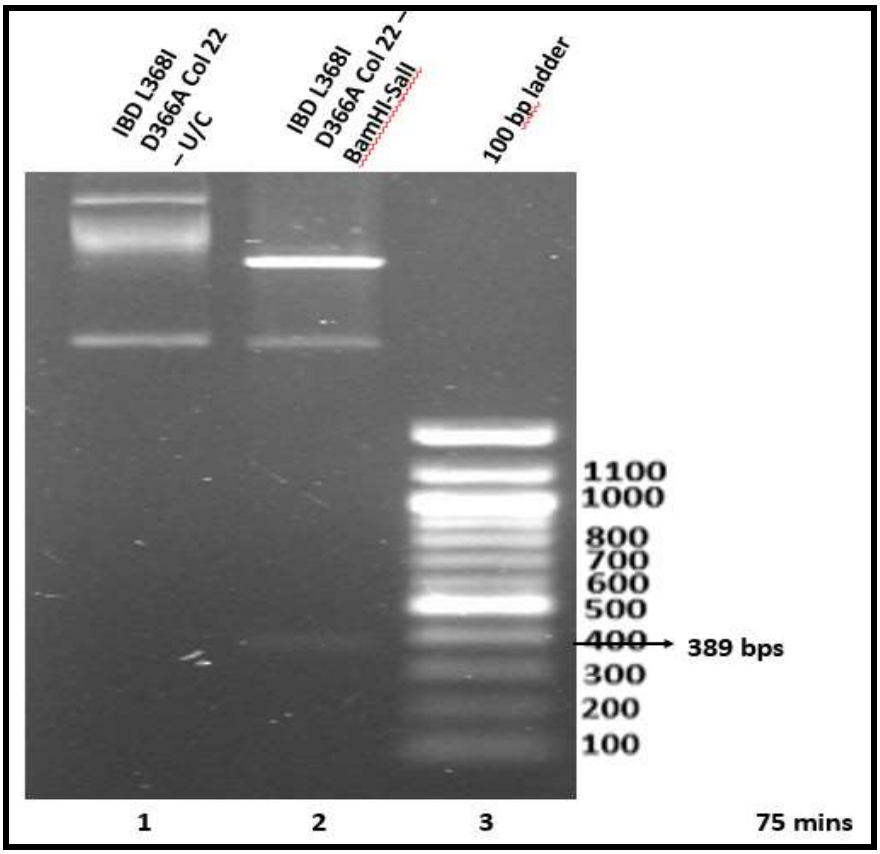

Figure 39. Restriction digestion of pRA73 with BamHI-SalI; An expected insert release of 389 bps was observed when pRA74 was cut with BamHI-SalI (Lane 2).

This was followed by XbaI digestion (the XbaI site would be lost because of digestion with XbaI in the mega-primer and use of pRA32 as the template) of pRA74 col 22 and of control vector pRA54 (IBD D366A). No cut was observed in XbaI digested col 2 of IBD L368I D366A as expected whereas an expected linearization of 5.3 kb was observed in pRA54 (IBD D366A).

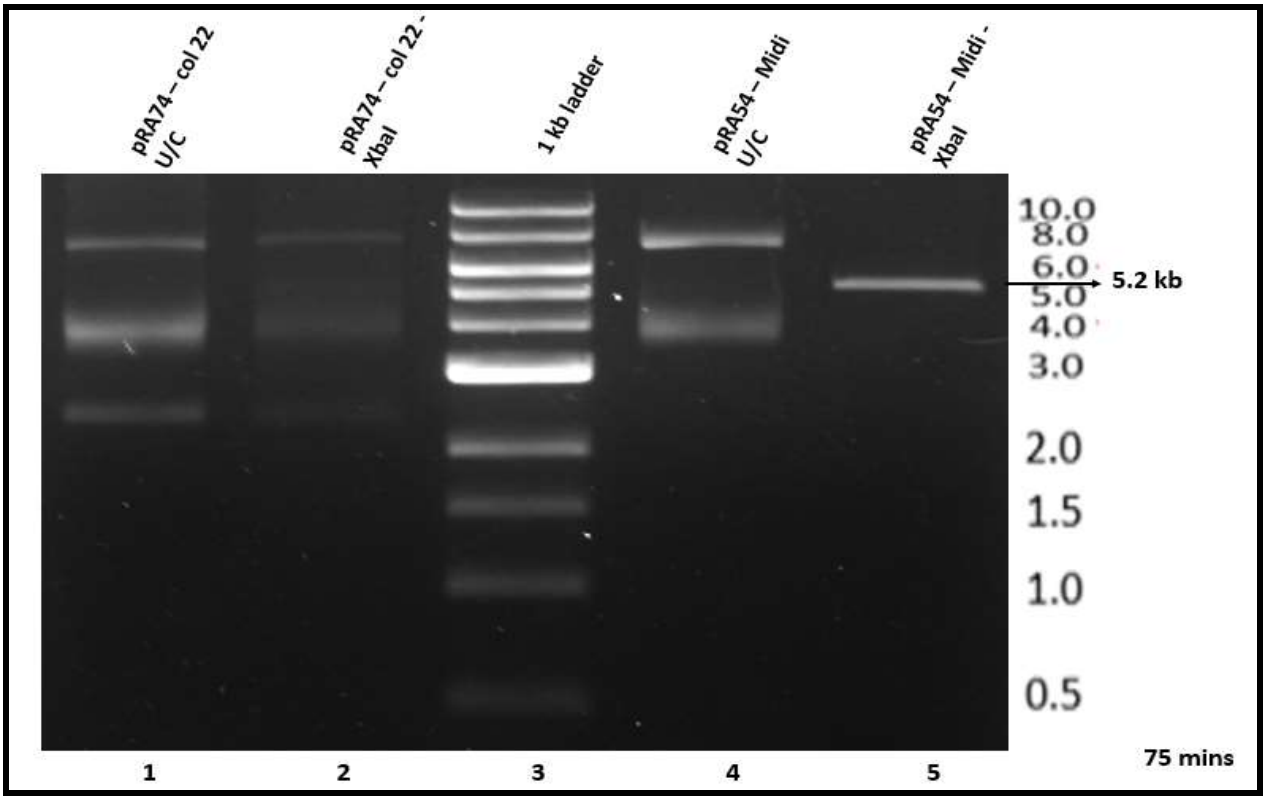

Figure 90. Restriction digestion of pRA73 with XbaI; XbaI digestion of pRA73 results in no cut as expected (Lane 2) (while linearization of the plasmid at 5.3 kb was observed control vector pRA54 (IBD D366A) (lane 6) indicating D366A mutation.

HpaI digestion was done to check L368I mutation along with control plasmid pCPGST[75-81]. HpaI digestion of pRA74 results in expected band sizes of 1.7 kb and 3.4 kb (Fig. 41, Lane 2) indicating L368I mutation, while linearization of the plasmid at 5.3 kb was observed in control vector pCP-GST IBD [75-81] (Fig. 41, Lane 7)

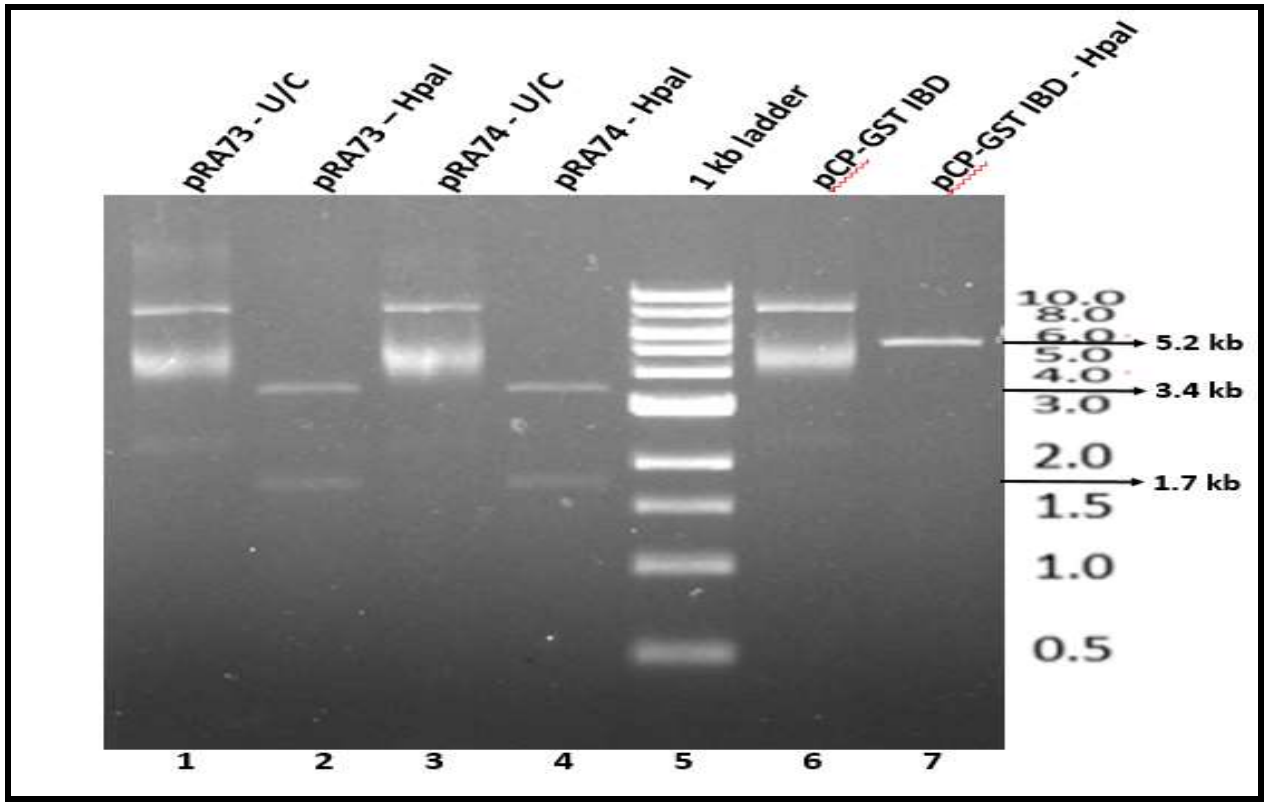

Figure 101. Restriction digestion of pRA74 with HpaI. HpaI digestion of pRA73 results in expected band sizes of 1.7 kb and 3.4 kb (Lane 2), while linearization of the plasmid at 5.3 kb was observed in control vector pCP-GST IBD [75-81] (Lane 7)

Induction of the protein

The midi-Prep DNA (col 22) was transformed to BL21 (DE3) pLysS competent cells. After observing Induction in 3ml LB broth, 5% of pre-inoculum was added to fresh 125 ml broth and the culture was allowed to grow till 0.6 OD and induced for 4 hours with 1M IPTG at 28^0C. 125 ml Induction was checked on gel using an aliquot (figure 42, Lane 3) and the remaining induced culture was pelleted for further purification of protein.

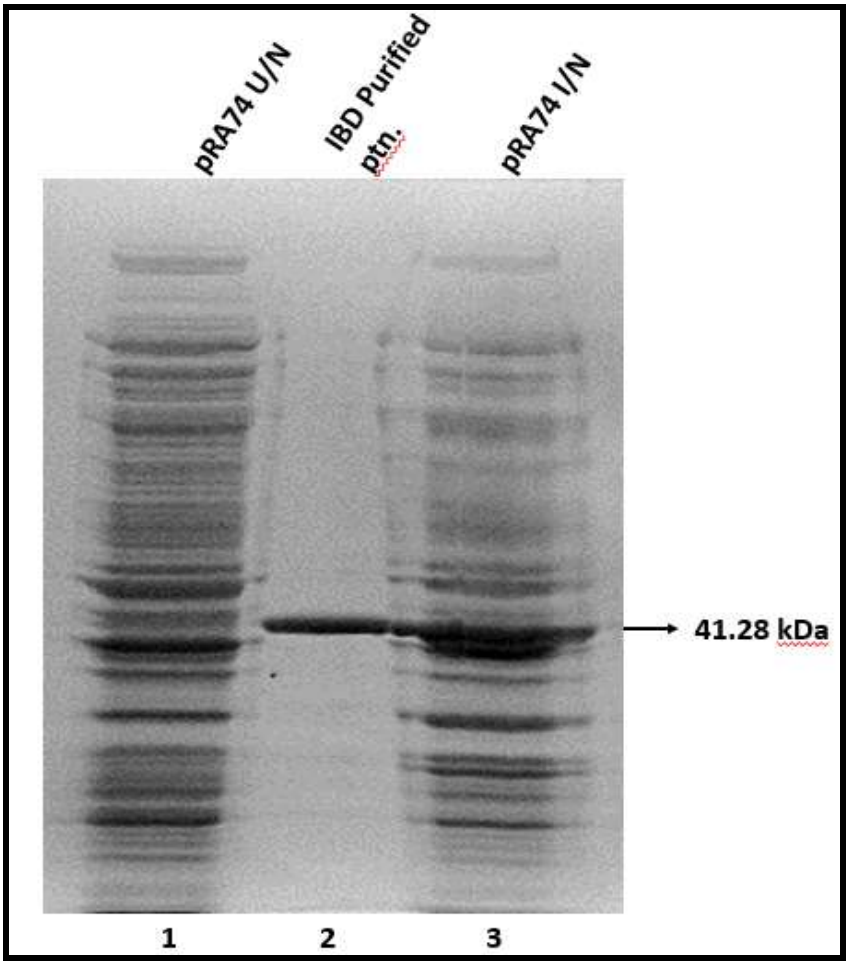

Figure 11. Induction of pRA74 (IBD L368I D366A); An induction of expected protein size of 41.2 kDa, running along with marker purified wt IBD protein was observed (Lane 2, 4).

Chapter 3

Methodology

3.1 PCR amplification of mutant IBD gene

For a 25 µl of PCR reaction, 50-100 ng of template was used. Phusion polymerase (10 units) was used as Taq polymerase lack proof reading ability. 1µM working concentration of forward and reverse primer was used along with 1X working concentration of Phusion buffer and 200 µM working concentration of each dNTPs.

Thus for a 100 µl reaction,

Template – concentration (1-30 ng)

10X pfu buffer – 10 µl

5 µM forward primer – 10 µl

5 µM reverse primer – 10 µl

2 mM dNTPs – 10 µl

Pfu DNA polymerase – 1 µl (0.02U/µl)

Water – upto 100 µl

The mutant IBD was amplified using pCP-GST IBD [75-81] as the template along with the primers, buffers, enzyme of the required concentration as mentioned above.

3.2 DH5-alpha Transformation of ligation plasmids:

The chemical competent cells were thawed in ice. The ligated products of the insert : vector molar ratio 4 : 1 were added to the competent cells and they were mixed by tapping. The cells were incubated in ice for 5 minutes. The cells were then incubated at 42°C for 90 seconds. The cells were again incubated in ice for 7 minutes. 1ml of fresh LB was added and the cells were incubated at 37°C for 1 hour. The cells were then pelleted down by spinning at 5000 rpm for 5 minutes. The clear supernatant was removed and the cells were plated on plates with ampicillin of conc. 50 µg/ml. The plasmids pRA52, pRA53, pRA54, pRA59, pRA60, pRA71, pRA72, pRA73 and pRA74 were transformed to the chemical competent cells by the above procedure

3.3 BL21 Transformation of ligation plasmids

The BL21 competent cells were thawed in ice. The midi-prep plasmid DNA were added to the competent cells and they were mixed by tapping. The cells were incubated in ice for 5 minutes. The cells were then incubated at 42°C for 90 seconds. The cells were again incubated in ice for 7 minutes. 1ml of fresh LB was added and the cells were incubated at 37°C for 1 hour. After recovery, 100 µl of the cells were plated on plates with ampicillin of conc. 50 µg/ml. The plasmids pRA52, pRA53, pRA54, pRA59, pRA60, pRA71, pRA72, pRA73 and pRA74 were transformed to BL21 cells by the above procedure

3.4 Purification of plasmids from transformed cells

Overnight culture of DH5-α strains of E.coli (transformed with pRA59..) were pelleted down at 14000 rpm for 2 minute. 100 µl of alkaline lysis solution I (Resuspension buffer) was added and the cells were vortexed until no cell clumps were seen. 200 µl of alkaline lysis solution II (lysis buffer) was added and the tube was mixed gently by inverting 3-4 times. The solution was incubated at room temperature for about 5 minutes until the lysate appeared clear. 150µl of alkaline lysis solution III (neutralization buffer) was added and the solution was mixed thoroughly by inverting the tubes 6-8 times. The tubes were incubated in ice for 10 minutes and then centrifuged at 14000 rpm for 7 minutes at 4^0C. The supernatant was decanted to a new tube and Phenol : Chloroform in the ratio of 1:1 was added. The mixture was spinned at 14K/4^0C/5 mins and the aqueous phase was proceeded for ethanol precipitation at -80^0C by adding one tenth of the salt and 2X of absolute ethanol. After 15 mins at -80^0C, the mixture was spinned at 14K/4^0C/10 mins and the supernatant was discarded. The pellet was washed with 70 % ethanol and air dried at 37^0C. The final pellet was dissolved in TE buffer and loaded on gel

3.5 Restriction digestion

Bam HI – SalI restriction digestion of IBD mutants ligated with pGEX vector

The PCR generated IBD mutants (L368A, D366A, V408F/L368F, V408L/D366A, V408F/D366A, V408M/D366A, L368M/D366A, L368F/D366A, L368I/D366A) were cut with BamHI and SalI enzymes which are present in either side of the IBD gene. Normally, 5 units of restriction enzymes are incubated at the appropriate reaction temperature for appropriate time in a mix containing 1 µg of template. 5 µg of the above vector was restricted with 10 U of BamHI and SalI by incubating the mix at 37°C for 14-16 hours.

In a 20 µl reaction:

PCR generated IBD mutants – 8 ug

10 X NEB 3 buffer – 2 µl

10 X BSA – 2 µl

Enzyme – BamHI – 10 units

SalI – 10 units

After 16 hour incubation at 37°C, the digested mixture was loaded on gel to observe insert release.

All the amplified mutants and pGEX vector also were digested the same way. After ligation of pGEX (BamHI-SalI digested) vector with IBD mutant (BamHI-SalI digested), the ligated plasmid was transformed to DH5-alpha cells and plasmid isolation was done using alkaline lysis method and the plasmids were observed on gel.

The plasmids which were running along with the positive control (pCP-GST IBD [75-81]), were again subjected to BamHI-SalI digestion as mentioned above to check for insert release.

HpaI digestion of IBD L368 mutants

The pGEX ligated with L368 IBD mutants (L368A, V408F/L368F, L368M/D366A, L368F/D366A, L368I/D366A) were cut with HpaI (the presence of which indicates the L368 mutation). Normally, 5 units of restriction enzymes are incubated at the appropriate reaction temperature for appropriate time in a mix containing 1 µg of template. 4 µg of the above vector was restricted with 10 U of HpaI by incubating the mix at 37°C for 14-16 hours. In a 20 µl reaction

Template (pGEX with IBD L368 mutants) – 4 µg

10 X NEB Buffer 4 – 2 µl

Enzyme – HpaI – 10 units

The volume is made up to 20 µl with water. After incubation at 37^0C for 16 hours, the digested mixture was loaded on gel to observe cut at two sites (3.4 kb and 1.7 kb), whereas wt IBD shows only one cut on HpaI digestion.

DraI digestion of IBD V408 mutants

The pGEX ligated with V408 IBD mutants (V408F/D366A, V408F/L368F, V408L/D366A, V408M/D366A) were cut with DraI (the presence of which indicates the V408 mutation). Normally, 5 units of restriction enzymes are incubated at the appropriate reaction temperature for appropriate time in a mix containing 1 µg of template. 8 µg of the above vector was restricted with 20 U of DraI by incubating the mix at 37°C for 14-16 hours. In a 20 µl reaction :

Template (pGEX with IBD L368 mutants) – 4 µg

10 X NEB Buffer 4 – 2 µl

Enzyme – DraI – 20 units

The volume is made up to 20 µl with water. After incubation at 37^0C for 16 hours, the digested mixture was loaded on gel to observe restricted bands of sizes 3.35 kb, 841 bps and 692 bps indicating V408M mutation whereas DraI digestion of control pCPGST[75-81] yields bands of sizes 1.07 kb, 3.35 kb and 692 bps.

XbaI digestion of IBD D366A mutants

The pGEX ligated with D366A IBD mutants (V408F/D366A, D366A, V408L/D366A, V408M/D366A, L368M/D366A, L368F/D366A, L368I/D366A) were cut with XbaI (which indicates the D366A mutation). Normally, 5 units of restriction enzymes are incubated at the appropriate reaction temperature for appropriate time in a mix containing 1 µg of template. 2 µg of the above vector was restricted with 5 U of XbaI by incubating the mix at 37°C for 14-16 hours. In a 20 µl reaction:

Template (pGEX with IBD D366A mutants) – 2 µg

10 X NEB Buffer 4 – 2 µl

Enzyme – DraI – 20 units

The volume is made up to 20 µl with water. After incubation at 37^0C for 2 hours, the digested mixture was loaded on gel to observe restricted bands of size of 5.28 kb indicating D366A mutation and D366A double mutation with V408 residue, whereas no cut with XbaI symbolize D366A double mutation with L368 residue.

3.6 Protein Induction

5% of overnight fresh pre-inoculum was added to fresh LB broth and the culture was allowed to grow till 0.6 OD. The culture was then induced with 1 mM IPTG and kept at 28^0C for 4 hours. 3 ml induction was checked first followed by 125 ml induction. The 125 ml induced culture was pelleted and kept at -80^0C for further purification.

Chapter 4

Conclusion

The double mutants of V408 and L368 with D366A were generated and the proteins were induced. The induced proteins can be purified and the same can be characterized by pull down assays

References

[1] Cherepanov P, Sun ZY, Rahman S, Maertens G, Wagner G, Engelman A (2005). "Solution structure of the HIV-1 integrase-binding domain in LEDGF/p75". Nat. Struct. Mol. Biol. 12 (6): 526–32.P. Aguiar, C. S. Adjiman, and N. P. Brandon. Anode-supported intermediate temperature direct internal reforming solid oxide fuel cell . I : model-based steady-state performance. J. Power Sources 138, (2004) 120–136.

[2] Shun MC, Raghavendra NK, Vandegraaff N, Daigle JE, Hughes S, Kellam P, Cherepanov P, Engelman A (2007)."LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration". Genes Dev. 21 (14): 1767–78.

[3] Burton DR, Ahmed R, Barouch DH, Butera ST, Crotty S, Godzik A, Kaufmann DE, McElrath MJ, Nussenzweig MC, Pulendran B, Scanlan CN, Schief WR,Silvestri G, Streeck H, Walker BD, Walker LM, Ward AB, Wilson IA, Wyatt R (2012) "A Blueprint for HIV Vaccine Discovery". Cell Host Micorbe. 12(4):396-407

[4] Alan Engelman[1,*] and Peter Cherepanov, (2008) "The Lentiviral Integrase Binding Protein LEDGF/p75 and HIV-1 Replication", PLoS Pathog. Mar; 4(3):