EXPRESSION & PURIFICATION OF Cas12 (LbCpf1) AND ITS APPLICATION IN DETECTION OF TOMATO LEAF CURL NEW DELHI VIRUS-POTATO

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Thesis submitted in the partial fulfilment of the requirements for the degree of

Masters of Science in Biotechnology

By

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SELF-DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation thesis entitled **"Expression & Purification of Cas12 (Lbcpf1) And Its Application In Detection of Tomato Leaf Curl New Delhi Virus-Potato"** submitted at Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India, is an authentic record of my work carried out under the supervision of **Dr. Anil Kant** (Associate Professor) at *Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Himachal Pradesh-173234, India* and in collaboration with **Dr. Sundaresha S.** at *Division of Crop Improvement, Central Potato Research Institute, Bemloe, Shimla, Himachal Pradesh-171001, India*.

I have not submitted this work elsewhere for any other degree or diploma.

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CERTIFICATE

This is to certify that the work reported in the M.Sc. dissertation thesis entitled "**Expression & Purification of Cas12 (Lbcpf1) And Its Application in Detection of Tomato Leaf Curl New Delhi Virus-Potato**" submitted by Divyansh Bhadiar (207818) at *Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India,* is a bonafide record of his original work and has not been submitted elsewhere for any other degree or diploma programme.

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All may not be mentioned, but no one is forgotten.

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List of Abbreviations

i. AFT - Affinity Fusion Tags
ii. APS - Ammonium Per Sulfate
iii. BLAST - Basic Local Alignment Search Tool
iv. Cas protein – CRISPR Associated protein
v. CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
vi. gRNA – guide RNA
vii. GST - Glutathione-S-Transferase
viii. IPTG - Isopropyl-β-D-1-ThioGalactopyranoside
ix. IMAC - Immobilized Metal Affinity Chromatography
x. kbp - Kilo base pairkDa - Kilo Dalton
xi. MBP - Maltose Binding Protein
xii. MCS - Multiple Cloning site
xiii. MS - Mass Spectrometry
xiv. MSA - Multiple Sequence Alignment
xv. NA – Nucleic acid
xvi. NCBI - National Centre for Biotechnology Information
xvii. NEB - New England Biolabs
xviii. Ni-NTA - Nickel-Nitriloacetic acid
xix. ORFs - Open Reading Frames
xx. RDT - Recombinant DNA Technology
xxi. RPP - Recombinant Protein Purification
xxii. TEMED - Tetramethyl Ethylenediamine
xxiii. ToLCNDV – Tomato Leaf Curl New Delhi Virus

Abstract

Tomato Leaf Curl New Delhi Virus (ToLCNDV) was found to infect tomato and other members of Solanaceous crops. But lately it has been reported to infect a range of plant species at various geographical locations. There are many reports indicating spread of ToLCNDV to other vegetable and fiber crops, inflicting severe economic losses. Symptoms of ToLCNDV on different host plants include yellow mosaic, leaf curling, vein swelling and plant stunting, which overlap with many other viruses or disease conditions and thus detection is not possible on the basis of symptoms. So, it becomes very essential to develop fast, specific and efficient diagnosis techniques for detection of such a devastating and rapidly spreading plant virus. Conventional approaches such as antigen-antibody interaction, AFLP and PCR, etc. having some limitations could be overcome by 'CRISPR' based detection of targeted nucleic acids. CRISPR renowned for its precise and sensitivity as targeted genome editing (cis-cleavage) tool. Prior to the recognition of targeted genome by CRISPR-Cas complex, its 'Collateral activity' (trans-cleavage) gets activated. Which could be utilised as attomolar level sensitive, novel nucleic acid detection platform with the help of random ssDNA (reporter molecules) labelled at one end with fluorophore and other with quencher. Such an approach will be more sensitive, specific, faster and efficient to perform compared to available diagnostic methods. In this study, we're interested in designing of specific gRNAs (*in silico*) against ToLCNDV-Potato alongside with expression & purification of Cas12a (LbCpf1) through IMAC technique then check their efficacy in CRISPR based diagnosis of ToLCNDV from potato samples.

CHAPTER 1 – INTRODUCTION

India is an agricultural land and it's the primary source of income for >70% of rural Indians, employs around 52% of the workforce [1] and contributing 20.2 % to the GDP (Gross Domestic Product) of the country in financial year 2020-2021 [2], [3]. India is one of prime producer and exporter of pulses, wheat, milk, jute, cotton, rice, spices, fruits and vegetables, etc. contributing a lot to the economy of country [4]–[7]. But modern emergence of various Plant viruses causing huge loss of these economically important and regular use crops. One of such viruses are Tomato Leaf Curl New Delhi Virus (ToLCNDV); Begomoviral [8], [9], aka; Potato Apical Leaf Curl Virus (PALCV) initially found affecting only Tomato plants (or other Solanaceous crops) but lastly spread their horizon to different hosts, at various geographical locations and became a large taxonomy now [10]–[14]. Potato which is the most consumable vegetable not just in India but in World and that we require in our daily households are one of their target host plants among 42 other plant species [15]. India is among top 10 producers for Potato export in world ranking and having a market of billions of rupees [16]–[18], contributing a lot to the economy of the country which is getting suffered due to the yield loss by infection of viruses like ToLCNDV [19]. As symptoms on ToLCNDV are common like leaf curling, shortening of leaves, stunted growth, etc. may arise due to several other factors [20]. So, it becomes very essential to develop fast, specific and efficient diagnosis technique for detection of such plant viruses. Which is not enough through conventional approaches like symptomatology, antigen-antibody interaction & PCR. Some lacks specificity, accuracy, more complex, require skilful personnel expensive or heavy instruments and are time consuming. These all could be overcome by a single 'CRISPR based approach for nucleic acid detection' [21], [22]. As CRISPR is already well known for its preciseness and specificity in genome editing (cis-cleavage), similar the case with their collateral activity (trans-cleavage) that could be used for diagnosis [23]–[26]. Which is comparatively more precise, faster, easy to perform hence efficient. So, in this study we're going to learn about isolation, extraction of the viral genome from the infected Potato plant, its Pre-amplification and then CRISPR based nucleic acid detection along with CRISPR-Cas components preparation by *in silico* selection & designing of primers & gRNAs specifically for ToLCNDV and purification of recombinant LbCpf1 (LbCas12a) protein via IMAC (Immobilised Metal Affinity Chromatography).

However, there are various approaches for recombinant protein production through blend of different expression and purification techniques [27], [28]. By utilising any of the expression system i.e.; expression host cells or expression vectors commercially available in the market depending on the type of gene or protein to be expressed or purified respectively and the

conditions. For example; some proteins produced more efficiently or effectively in Prokaryotic (E coli) expression system while other requires Mammalian expression system to get expressed (especially eukaryotic proteins). It's been also observed in practise that some proteins found to be more convenient with one expression system than others, for that conditions or protocol need to be standardise to get best results. The most common expression vectors are pET vectors, pCMV vectors, pHEK293 vectors, pQE-40 vectors, pRSET vectors, etc. and expression host cells are BL21 (DE3), NiCo21 (DE3) cells, Rosetta (DE3) cells, M15 cells, etc.

On successive expression of recombinant proteins, the purification is most commonly done through affinity fusion tags (i.e.; Affinity chromatography). Affinity fusion tags (AFT) are small biomolecules tagged alongside with recombinant proteins in expression vectors, having strong affinity to bind specifically with another molecules in column matrix and get purified. There are number of AFT available in market today such as; Proteinase A, GST (Glutathione-S-Transferase), MBP (Maltose Binding Protein) tag, Poly Histidine tags, etc. having affinity to different ligands or molecules immobilised in column matrix and hence manage to purify recombinant proteins [29]–[31]. A short sequence of protease cleavage site could be inserted in between AFT & recombinant proteins sequence in expression vectors for removal of these tags at later applications. But most commonly it's been observed that AFT at C or N terminus end of desired proteins doesn't interferes with the working or applications of that protein. So, on purification of recombinant LbCpf1 (Cas12a) protein their collateral activity could be employed in diagnosis of ToLCNDV infecting potato plant with aid of specific crRNAs and non-specific labelled ssDNA's (reporter molecules).

Following are the objectives of my dissertation work:

I. *In silico* designing of primers and gRNAs respectively targeting specifically to the selected regions of ToLCNDV-Potato genome.

II. Expression of recombinant LbCpf1 gene within pRSET-A vector in BL21 (DE3) host cells.

III. Extraction and Purification of 6X-His tagged recombinant LbCpf1 protein in NI^{2+}-NTA agarose column (IMAC) and analysis of purified samples in SDS PAGE.

IV. CRISPR based detection of ToLCNDV infecting potato plant using above prepared CRISPR-Cas components.

CHAPTER 2 - REVIEW OF LITERATURE

2.1 Recombinant Protein Expression & Purification

After the birth of Recombinant DNA Technology (RDT) through immense efforts of some American biochemists; P Berg, H Boyer, S Cohen and few others in early 1970's [32]–[34], it opened a broad scope for the new innovations in the fields of Genetic engineering, RDT & Molecular biology. One of such are production of Recombinant proteins having wide range of applications from agriculture to pharmaceuticals. This noble work was pioneered by production of first recombinant product hormone somatostatin followed by human insulin in *E coli* [35], [36] . These developments lead protein industry to new heights and advanced the field of Science in RDT. To date there are hundreds of recombinant proteins approved to the market and been in use[37]. Where the upstream processing of recombinant proteins was forwarding to new discoveries and advancements, the downstream processing or extraction and purification of these recombinant proteins were tedious and time consuming required more improvements and development of new methodologies for downstream recombinant protein purification. The use of AFT fulfilled that requirement reduced the time and efforts, and increased the yield of final product on downstreaming chromatography of Recombinant Protein Purification (RPP). This work of RPP using AFT was first practised by Uhlén et al. who employed staphylococcal protein A as AFT having affinity with IgG Sepharose [38]. And now there are several affinity fusion strategies are developed for RPP such as; Protein A, GST (Glutathione-S-Transferase), FLAG peptide, MBP (Maltose Binding Protein) tag, Poly Histidine tags, etc. [31], [39].

2.2 Immobilised Metal-Affinity Chromatography

Immobilised Metal-Affinity Chromatography (IMAC) is one of such approaches used to purify recombinant proteins pioneered by Porath et al. [40]. This technique consists of three main components transition metal ions (d-block elements) such as; Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, matrix to which metal ions are immobilised such as; Iminodiacetic Acid (IDA), Nickle-Nitrilotriacetic acid (Ni-NTA), Cobalt-Carboxylmethylaspartate (Co-CMA), etc. coupled with resins mostly like Sepharose and Agarose[41]–[43]. Among all these Ni-NTA is most commonly adopted for RPP in modern era as NTA having four coordination sites (tetradentate) compared to three coordination sites (tridentate) in IDA which weakly binds to metal ions resulting into lower yields or impure products [44]. Transitional ion Ni^{2+} having six coordination sites out of these four gets chelated to NTA and remaining two form bonds with affinity tags (100-1000X than IDA), similar the case with Co-CMA **(Figure 2.**1**)**. But it was observed that Ni-NTA having

better affinity to Poly-Histidine tags than Co-CMA due to some unspecified reasons [44], [45]. And last but not least the Affinity fusion tags (AFT) encoded on plasmid expression vectors in fusion with our desired protein having affinity to bind these matrix chelated metal ions. Most common and efficient AFT in IMAC is Poly-Histidine residues as electron donor groups on imidazole ring of amino acid histidine form strong coordination bonds with immobilised-chelated metal ions [41], [46]–[49]. Poly-Histidine tags having the arrangement of 6-12 consecutive histidine residues for efficient binding with metal ions. Out of which 6X Histidine residues are standardized by many as AFT for best RPP results and in practise most commonly [42], [44], [50], [51]

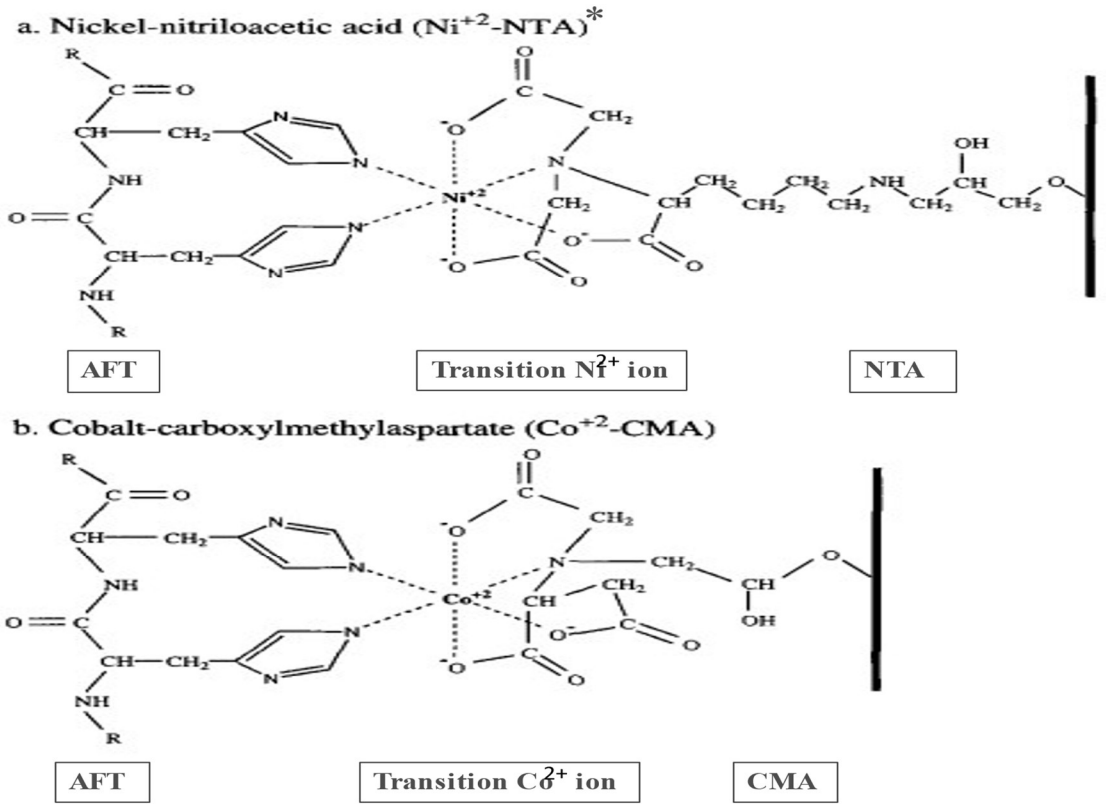


Figure 2.1: Interaction of Matrices with transition Metal ions then to Imidazole of Poly His-tag. (a) Ni-NTA-AFT interaction. (b) Co-CMA-AFT interaction.
(AFT) – Affinity fusion tags. (Ni^{2+}-NTA)* - Nickel-Nitrilotriacetic acid. (Co-CMA) - Cobalt-Carboxylmethylaspartate [46].

Following efficient binding of Ni^{2+} NTA to 6X-His tagged recombinant protein the subsequent washing and elution could be done using buffers with low pH (around 6.3 for washing and 4.3 to 5.9 for elution) or most commonly using resuspension or column buffers with varying Imidazole concentration (around 20-30mM for washing and about 250mM for elution) [44], [52] as this external imidazole competes with imidazole ring structures of histidine residues for

binding to Ni^{2+}-NTA matrix **(Figure 2.2)**. These buffer compositions can vary in accordance with type of His-tag (i.e.; 6X to 12X) or Proteins under purification [41]. Sometimes even low concentrations of EDTA could be employed for elution step, as a chelating agent it strips down tagged recombinant proteins alongside with Ni^{2+} ions, it's one among the drawbacks of this method to separate Ni^{2+} ion from purified proteins later [53]. Over successive purification affinity tags could be removed from the desired recombinant proteins by utilising Proteases like Enterokinase or Factor Xa, etc. That makes cut on their respective cleavage site integrated in between 6X His tag and recombinant protein [41], [44]. However, this step is not that necessarily required as these AFT rarely interferes with purified protein's activity. Unless or until the protein need to be get sequenced or physical and structural characterisation of protein need to be done (via XRD or MS) [50], [54].

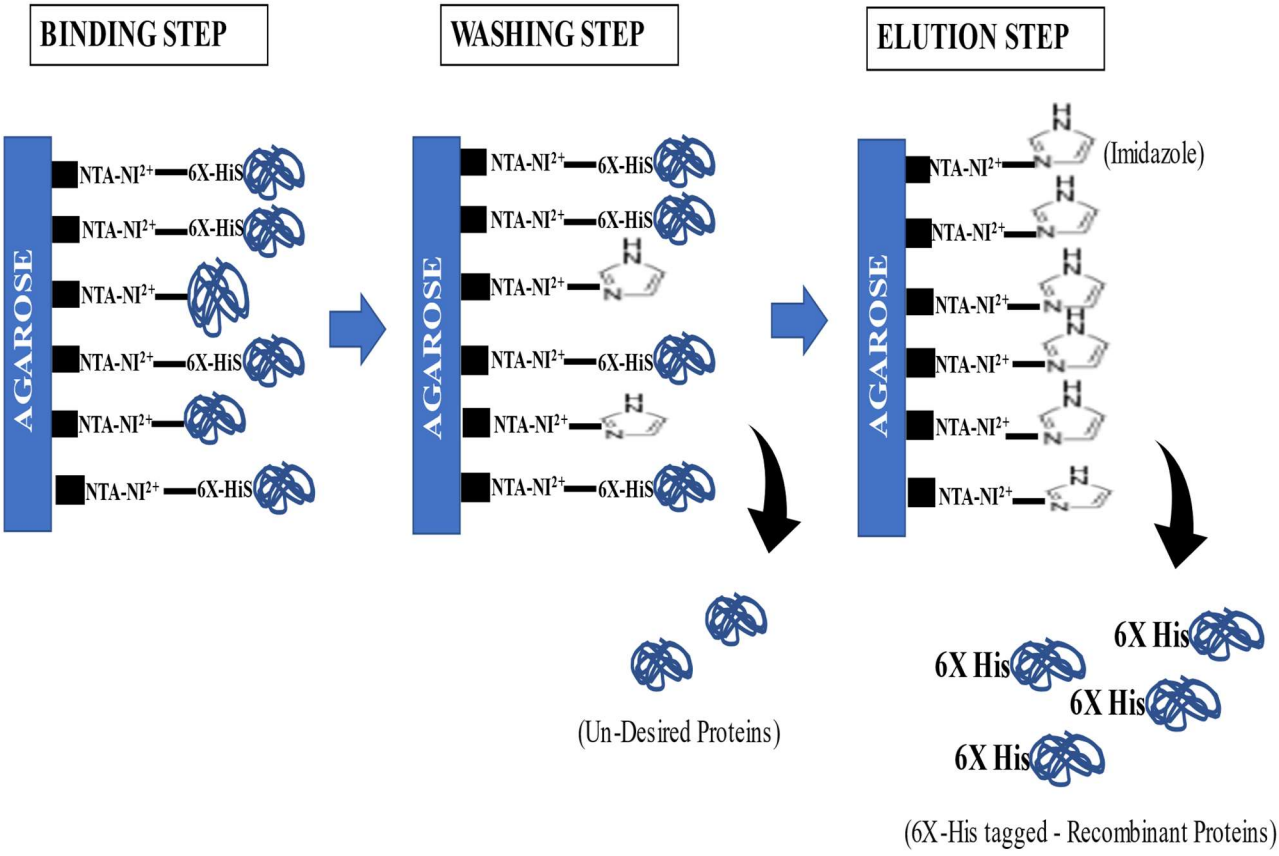


Figure 2.2: Illustration of Ni-NTA – 6X-His tagged (IMAC) based purification of recombinant proteins. In washing step only 6X-His tagged recombinant proteins retained on the column, untagged or un wanted proteins get washed away. In elution step all the proteins get eluted at high imidazole concentration in buffer.

2.3 Tomato Leaf Curl New Delhi Virus (ToLCNDV)

Tomato Leaf curl New Delhi virus is one of the types of leaf curl virus consist of bipartite, ssDNA under genus Begomovirus and Geminiviridae family, first discovered in New Delhi, India at 1995 thus named so, but initially called ToLCV India [55], [56]. Since then, it is spreading throughout resulted into huge taxonomy, classified on the basis of geographical regions like; ToLCV Bangalore, ToLCV Pakistan, ToLCV Australia and ToLCV New Delhi, etc. [57], [58] and then many of these been observed affecting different crops than Tomato and classified on that basis also like; ToLCNDV Chili pepper, ToLCNDV Bitter gourd, ToLCNDV Jatropha, ToLCNDV Okra, ToLCNDV pumpkin, etc. [13], [56] Even same ToLCNDV is spreading to the other nearby regions like Lucknow, Aurangabad, Kanpur, Pakistan, etc. according to the data collected from Taxonomy browser of NCBI (Taxonomy id: 223347).

Bemisia tabaci (whitefly) are generally responsible for spread of this begomovirus and act as vector insect. Reportedly on feeding of 15-30 minutes on infected plant they acquire this virus from them and the virus retain there for 10-12 days and at that period of time it spread to uninfected plants [59]. This viral is spreading either direct contact of vectors or through seedlings is not clearly known till now.

The virus-host mechanism here is based on the role of various genes present in DNA-A & DNA-B **(Figure 2.3)**. DNA-A have essential genes for coating, replication, transcription, etc. and DNA-B have genes for movement protein (BC1) and nuclear shuttle protein (BV1) mainly responsible for spread of the virus and pathogenicity **(Table 4. 1)**.

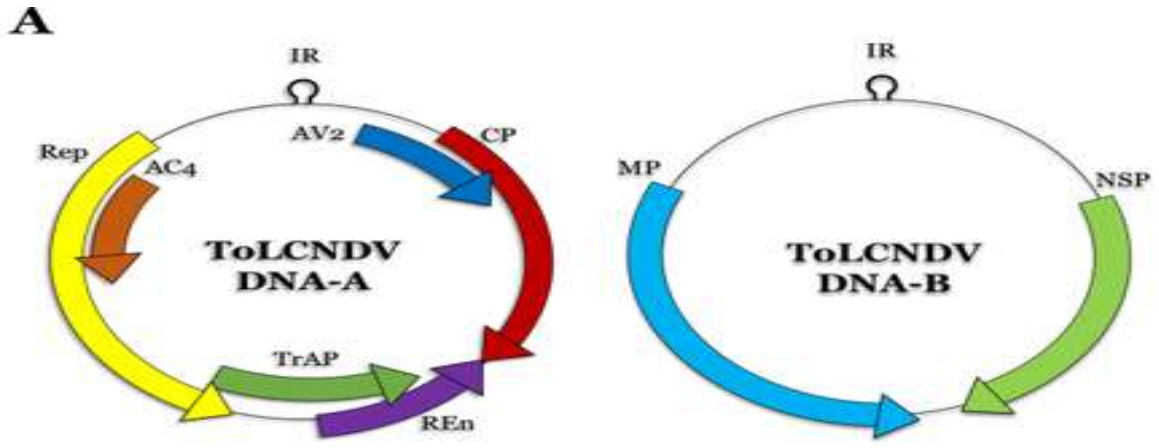


Figure 2.3: DNA-A & DNA-B components of ToLCNDV bipartite genome [60]**.**

ToLCNDV from three isolates of potato was agro-infected *to N. benthamiana* and *S. tuberosum* by Jeevalatha et al. They observed distinct characteristic symptoms in all infected plants. [61]. This research opened the door for further studies of symptomatology on infection with ToLCNDV. Before them, some researchers also did a study by expressing BV1 gene (nuclear shuttle protein) through Potato virus X in *N. benthamiana* and observed leaf curl and hypersensitive response, hence suggested that BV1 gene may be responsible for leaf curl (symptom) and generating immune response in host [62].

Initially this virus only affecting Tomato followed by *Solanaceae* family in India or nearby Asia-Pacific regions, but now the similar kind of infection have been found into wide range of other plants too like cucumber, chili, melon, pumpkin, Hibiscus cannabinus, papaya, Brinjal, Potato, and in total around 44 plant species or 445 plant species by begomovirus at different geographical regions across globe **(Figure 2.4)**. Despite, this virus dominantly affecting Solanaceae and Cucurbitaceae, related family especially at European - Mediterranean region that EPPO declared this virus in their 'Alert list' [56].

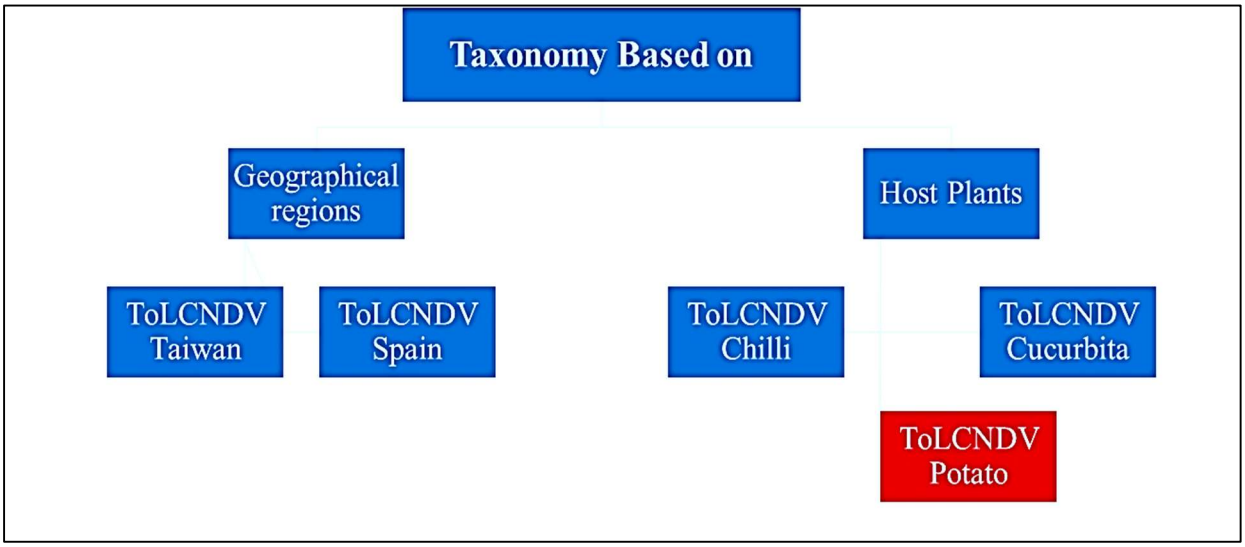


Figure 2.4: Taxonomical representation of few of the ToLCNDV variants.

The disease or symptoms of virus like; leaf curl, rumple leaves and striking mosaic on potato plant first time observed in INDIA during 1999 [63], the researcher also stated association of begomovirus with the help of Immunospecific electron microscopy. Later, it was proved by nucleotide sequencing that strain causing these symptoms in potato are emerged or related to ToLCNDV [15]. Since then, it's been observed infecting various crops of potato at different geographical location like Kanpur, Modi Puram, Faizabad, Pune, even in several places of Pakistan [12], [64]. This exploitation of potato crops resulted to great loss for the country like

India whose 15-17% economy is based on agriculture and associated sector[1], [2], [65]. India is among top 10 producers of Potato in the world and export potatoes to around 30 countries valued over 5.8 billion Indian rupees a/c to data in fiscal year 2020. Today, Potato is the most important vegetable and third most important crop in terms for food consumption a/c to recent report by International Potato Centre (CPI). Potato is very essential part of our daily cooking and rich in various vitamins, minerals and fibres. And hundreds of millions of people in developing countries depend on potatoes for their survival including India. So, protection of this crop from ToLCNDV (and other viruses) is in so much of necessity.

2.4 Why Diagnosis is required & Conventional techniques for Detection

For the treatment of any disease the very first step is to find out the cause of that disease, what are the factors responsible for that infection/disease, their interaction, mechanism of causing infection, study all this thoroughly even at molecular level. So, in order to tackle from this threat of ToLCNDV to Potato crops we need to diagnose and confirm it first. The conventional methods for ToLCNDV diagnosis in plant till now are:

1. Symptomatology – On infection with virus there are several recognisable changes in physical properties of plants such as; leaf curling, leaf margins darkening (or yellowish), thickening of veins, leaf size reduced, sometimes cause late ripening of fruits and flowers, stunted growth due to internode shortening [60]. But these symptoms can vary from host to host and main physiological reason behind these physical properties is not clear yet. And also viral infection is not the only reason for such symptoms in plants it could be caused due to deficiency of mineral ions such as; Mg, N, Fe (for leaf yellowing), lack of water or food supply to leaves or plant part at top and infection of other viruses than ToLCNDV, causes; leaf curl, leaf size shortening and stunted plant growth, etc. [58]–[60]. So, it wouldn't be advisable to diagnose ToLCNDV by just on basis of these symptoms.
2. When viral infection or disease is susceptible then best approach is molecular level diagnosis. Such as;
    a) Enzyme Linked Immuno-Sorbent Assay (ELISA)
    b) Southern Blot Hybridisation
    c) Gold standard Polymerase chain reaction (PCR)
    d) Polymerase chain reaction -Restriction Fragment Length Polymorphism (PCR-RFLP)

As done by Muniyappa, et al. the sequence comparison of ToLCV-BAN4 with ToLCV India (now ToLCNDV) with the help of techniques like; Triple antibody sandwich ELISA, Polymerase chain reaction and by Southern blot analysis either individually or in combinations [58].

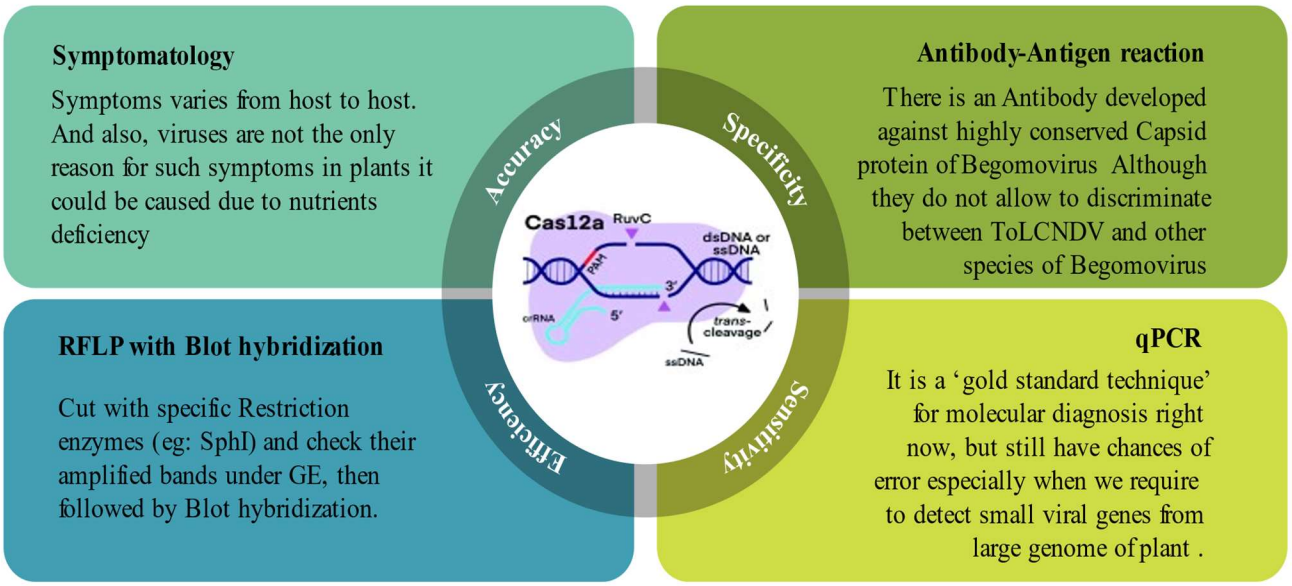


Figure 2.5: Conventional Techniques for Diagnosis v/s CRISPR. Summing up limitations of traditional techniques and how CRISPR based diagnosis overcome that.

But these molecular level diagnosis techniques even possess some limitations too such as; Antibodies in ELISA are for detection of begomovirus as whole and not specific for ToLCNDV. But this genus consists of 445 viral species and all are different in their physiological effects on host hence require specific treatment and so is diagnosis. In PCR, also there are high chances of off-target effects and give false positive results. Especially when diagnosing a small bit of viral genome (DNA) from a very large amount of plant genome, chances of getting false results are increased. And detection solely based on DNA polymerase is problematic since this enzyme is also error prone. [66], [67]. Similarly, the Southern blot hybridisation (analysis) not alone capable of viral diagnosis from plant host, requires assistance of some other techniques like PCR, gel electrophoresis. And even undergoing through two subsequent detection protocols still diagnosis is not that sensitive [58]. Hence, we require any technique which is more efficient, specific, sensitive, have lesser error rates, faster and give more accurate results. **(Figure 2.5)**

2.5 CRISPR in Nucleic acid detection

Due to the recent advancement in genome modification and genetic engineering applications via CRISPR-Cas (Cluster Regularly Inter-Spaced Palindromic Repeats – CRISPR Associated protein) it became the name which no longer require any introduction for researchers. It's naturally acts as defence mechanism for bacteria and archaea against phage's which was introduced as RNA guided Endonuclease first time in 2012 (i.e; CRISPR-Cas9) [68]. Since then, it is completely dominating the world of genome editing by replacing techniques like Zinc Finger Nucleases (ZFN), Mega nucleases, Transcription Activator Like Effector Nucleases (TALENs) which are all protein-based endonucleases because RNA based endonuclease is more precise and easier for delivery [69]–[71]. This innovation of CRISPR as very efficient genome editing tool leads Jennifer A Doudna and Emmanuelle Charpentier to win Nobel Prize in Chemistry, 2020. The principle of CRISPR-Cas dependent genome editing is based on its two components; CRISPR-RNA (crRNA) or guide RNA (gRNA) and Cas endonuclease. Where crRNA complementary to target region have directed repeats for Cas protein binding and guides Cas protein to target the region where Cas endonuclease recognises PAM site (Protospacer Adjacent Motif) on non-targeted strand and ended up on making cut (i.e.; cis cleavage) [68].

Sometimes later, CRISPR Cas12a (aka: Cpf1) [72] & CRISPR Cas13 (aka; C2c2) were also discovered to have amazing set of applications of their own. In contrast to CRISPR/Cas9, which was an RNA-based DNA endonuclease, Cas13 uniquely targets ssRNA [73]. And additionally having "collateral activity" (trans-cleavage) developed as an efficient and very precise Nucleic acid detection tool named SHERLOCK [25]. Similarly, Cas12a also capable of performing both the activities; genome editing with staggered end cuts on DNA [72] and collateral cleavage activity for diagnosis or nucleic acid detection [23] later used for developing Cas12 based nucleic acid detection tools named HOLMES [24] and DETECTR [26]. This technology widely been used on later for precise, fast, specific and very sensitive nucleic acid detection tool, even for diagnosis of current global pandemic SARS-CoV2 by many [74]–[78] and shows potential to be developed as Point-of-care-diagnosis (POCD) for nucleic acid [77], [79], [80]. In order to embrace CRISPR for nucleic acid detection we make use of collateral activity of Cas endonucleases (Cas12, Cas13, Cas14) which possesses an additional cleavage domain, that make cuts on random, non-specific/complementary ssDNA (in case of Cas12 & Cas14) or ssRNA (in case of Cas13) present in reaction (i.e.; Trans-cleavage) [24], [25] **(Figure 2.6)**. Hence, they utilised these ssDNA or ssRNA as reporter molecules by labelling them with

fluorophore at one end and quencher on other which on cleavage (due to collateral activity of Cas endonucleases) get separated and give fluorescent signals. **(Table 2.1)**

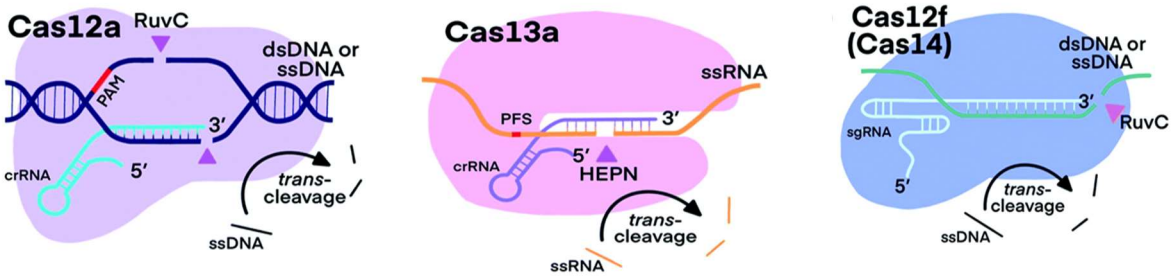


Figure 2.6: Showing "collateral-trans cleavage activity" of Cas12, Cas13 & Cas14 for precise nucleic acid detection [81].

Table 2.1: Different variants of Cas endonuclease with their affinity for nucleic acids as target or reporter molecules.

Cas-variants	Efficient detection of	Reporter molecules
Cas12	dsDNA/ssDNA	ssDNA
Cas13	ssRNA/dsRNA	ssRNA
Cas14	ssDNA	ssDNA

CHAPTER 3 - MATERIAL & METHODOLOGY

3.1 Materials

3.1.1 Computational Tools & Software's

Computational tools	Parent website	Their Role
Taxonomy Browser	NCBI (National Centre for Biotechnology Information)	It is the Taxonomy database contains classification and nomenclature of approximately 10% of all the identified species of life on earth
BLAST (Basic Local Alignment Search Tool)	NCBI	BLAST searches for similarities between biological sequences. It compares the nucleotide or protein sequences to biological sequence databases.
Primer-BLAST	NCBI	For designing specific primers for our template sequence in PCR, using algorithms of Primer3 and BLAST
MSA (Multiple Sequence Alignment) Tool	EMBL-EBI (European Molecular Biology Laboratory - European Bioinformatics Institute)	MSA is generally the alignment of multiple biological sequences (more than two) of about similar length. From there we can get the homology and phylogenetic relationships between those sequences.
FastPCR	PrimerDigital	FastPCR is a comprehensive tool for designing PCR primers or oligonucleotide probes, oligonucleotides assembly and analysis, alignment and finding repeats. (We utilised it here to find restriction sites in our sequence)
NEBcutter V2.0	New England Biolabs$_{Inc}$	It is to find the large, non-overlapping ORFs in a DNA sequence using the *E. coli* genetic code and the sites for all Type II and Type III restriction enzymes.

Cas-Designer	CRISPR RGEN Tools	It provides with best gRNAs, targeting the given input sequence. Also, some other relevant information like; potential off-targets with 2nt or 3nt mismatches and out-of-frame scores via Microhomology-predictor.

3.1.2 Chemicals

Chemical	Manufacturer	Quantity
Luria Bertani Broth	HIMEDIA	25g/L (w/v)
Luria Bertani Agar	HIMEDIA	40g/L (w/v)
Ampicillin stock	HIMEDIA	100mg/mL - 50mg/mL (w/v)
Chloramphenicol stock	HIMEDIA	35mg/mL (w/v)
Agarose (1.2%)	HIMEDIA	1.2g/100mL (w/v)
Ethidium bromide	HIMEDIA	0.034%
Tris buffer (1M)	SRL	121.14g/L (w/v)
Tris-Cl (1M)	Fisher Scientific	157.6g/L (w/v)
Glacial acetic acid (10%)	Merck	100mL/L (v/v)
EDTA (0.5M)	Sigma-Aldrich	186.1g/L (w/v)
6X DNA loading dye	Thermo Scientific	1X (diluted) (v/v)

IPTG (IsoPropyl-β-D-1-ThioGalactopyranoside) (1M - stock)	HIMEDIA / GENEI	0.238mg/mL (w/v)
Lysozyme stock	Sigma-Aldrich	10mg/mL (w/v)
RNase A stock	Sigma-Aldrich	10mg/mL (w/v)
Ni-NTA Agarose	Qiagen	5-10mg/mL (Protein binding capacity)
Acrylamide (29.2%)	BIO-RAD	29.2g in 100mL (w/v)
Bis Acrylamide (0.8%)	BIO-RAD	0.8g in 100mL (w/v)
SDS (10%)	HIMEDIA	10g in 100mL (w/v)
Ammonium PerSulfate (APS) (10%)	BIO-RAD	10g in 100mL (w/v)
TetraMethylEthyleneDiamine (TEMED)	Sigma-Aldrich	5-10 µL / reaction (v/v)
Methanol (40%)	Fisher Scientific	400mL/L (w/v)
Coomassie brilliant blue G250	BIO-RAD	0.5%
Glycerol (50%)	Sigma-Aldrich	50mL in 100mL (v/v)
β- Mercaptoethanol	Sigma-Aldrich	5%-12.5%
1kbp DNA ladder	GeneRuler	2-5 µL
SDS PAGE - Protein Ladder (Low and Broad range)	BIO-RAD	4µL / SDS PAGE run

3.1.3 Instrumentation & Equipment

Instruments	Manufacturer
LAF	Microsil India
Incubate Shaker	N- Biotek
Weighing Balance	Denver Instrument
Magnetic Stirrer	RELITECH
pH Meter	EUTECH
Centrifuge 5810 R	eppendorf
Microcentrifuge	HITACHI
Benchtop Centrifuge	Benchmark Scientific
Autoclave	SANYO
Nanodrop	Thermo Scientific (Nanodrop 2000c)
Heat Water Bath	GFL
Agarose Gel electrophoresis setup	BIO-RAD
Gel Doc. System	BIO-RAD
Gel Rocker	GENEI
GS-800 Calibrated Densitometer	BIO-RAD
Freezer (4℃ /-20℃ / - 80℃)	Celfrost / Vestfrost solutions / New Brunswick Scientific, resp.
Sonicator	SONICS
SDS PAGE setup	BIO-RAD

Equipments	Manufacturer
Micropipettes	eppendorf / Thermo scientific / AXYPET
Microtips	Tarson
15 mL glass vial	JSW (Glasswares)
Microcentrifuge tubes (1.5mL & 2mL)	eppendorf

Falcon tubes (15mL & 50mL)	Tarson / Genaxy
Petri Plates	Genaxy
Flasks (250mL / 500mL / 1L)	JSGW
Reagent bottles (100mL / 250mL / 500mL)	JSGW
Column (or Burette - for Protein purification)	JSGW

3.1.4 Biological material

Biological Material	Provider
pRSET-A expression vector	Invitrogen
BL21 (DE3) pLysS cells	Invitrogen

3.2 Methodology

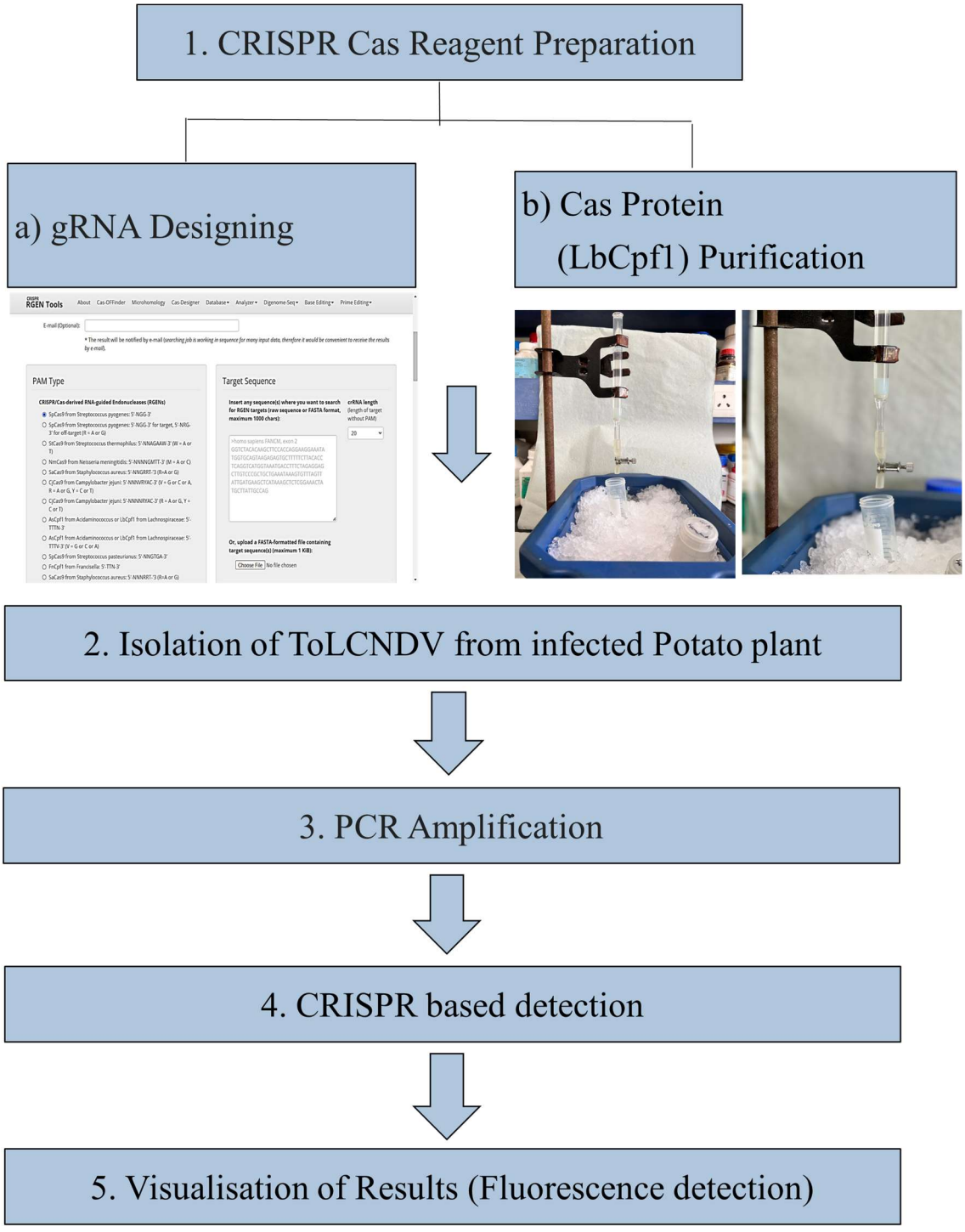


Figure 3.1: Flow chart representation of Methodology for CRISPR based Nucleic acid detection.

3.2.1 *In silico* Primer & gRNA designing

Before starting the experiment in the wet lab, we're required to go through some essential preparations from 'Dry lab'. This Dry lab work is divided mainly in two parts i.e.; gene specific Primer and CRISPR gRNA designing. But before designing these two kinds of oligonucleotides *in silico*, we first need to pick the target regions or genes complementary to which we're going to design these oligonucleotides (primers & gRNA).

3.2.1.1 Selection of ToLCNDV isolate and their targeted genes *in silico*

Go to NCBI -> Taxonomy Browser -> Search for 'Tomato leaf curl New Delhi Virus' -> From the list selected 'Tomato leaf curl New Delhi Virus - potato' (Taxonomy ID: 1296572). There clicked on the 'View & Analyse sequences in NCBI viruses. A list of all the ToLCNDV from potato isolates reported till now came across. From the list selected 'KX951455' & 'KX951456'; KAN-6 isolates of ToLCNDV-Potato (GenBank ID of their DNA-A and DNA-B respectively) **(Figure 3.2)**.

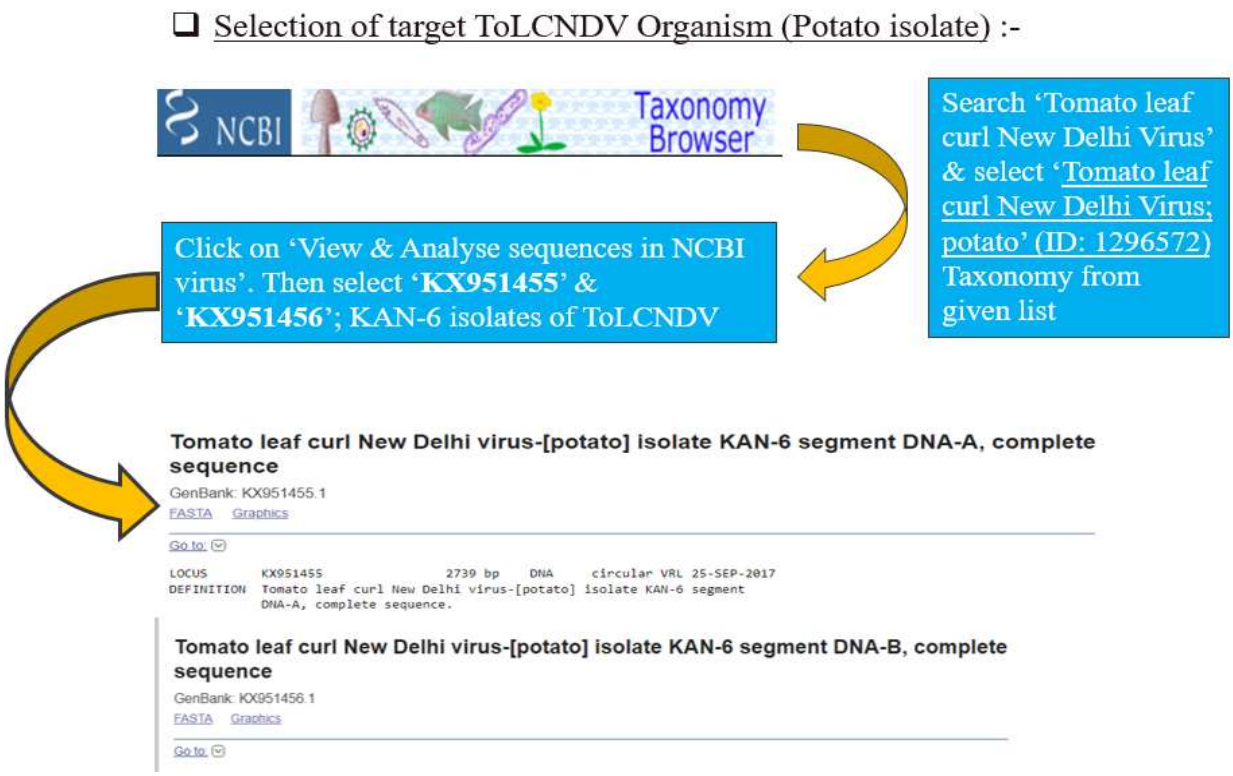


Figure 3.2: Selection of ToLCNDV-Potato variant and their genes.

First through NCBI > Taxonomy browser, then in NCBI > Nucleotide (from their GenBank ID).

As of now I've selected the ToLCNDV-Potato KAN-6 isolate and known with their GenBank IDs, went to their sequence in Genbank format (NCBI > Nucleotide) and selected the most conserved regions or essential genes for viral pathogenicity through BLAST followed by MSA **(Table 4. 1)**.

3.2.1.2 Primer designing for selected targeted sequences

This step has the utmost importance and is very common when coming across the PCR reaction. As for my case, I'm going to isolate ToLCNDV genes (selected) from the large potato genome through PCR directly so it becomes even more important. There are various software's and tools available on the internet for Primer designing such as; Primer-BLAST, Primer3, Primer Bank, Gene fisher Primer designer, Primer3Plus, FAST-PCR, etc. Among these I've done my work on 'Primer-BLAST' > NCBI.

Go to NCBI → Nucleotide, there search for "KX951455 or KX951456" (GenBank ID) for DNA-A or DNA-B segments resp. Clicked on 'Pick Primer' at the right side of the GenBank window for KX951455 & KX951456. Primer BLAST window got opened, made few changes in Parameters, like;

I. In the Primer template box added a sequence of targeted genes (i.e.; AV-1, BV-1, AC-1 and AC-2).
II. Changed 'Database' to custom (; KX951455 or KX951456)
III. At 'Organism' section added '223347' Taxon ID for ToLCNDV (potato)

Leave the remaining parameters on default and click on 'Get Primers'. Shortlisted primer pairs with best of the properties from result. **(Figure 4.2.1 & 4.2.2)**

3.2.1.3 gRNA designing for target regions

Go to RGEN tools (http://www.rgenome.net/) → Cas-Designer, inserted job title as ToLCNDV AV-1/BV-1/AC-1/AC-2 gene (depending on the gene we're targeting at the time).

Added Email for notification of results (optional). Now the parameters are selected as;

I. PAM type - 'AsCpf1 from *Acidaminococcus species* or LbCpf1 from *Lachnospiraceae bacterium*: 5'-TTTN-3'' as LbCpf1 is the Cas protein (Cas12) we're going to utilise.
II. Next, in the Target genome: select Organism type - Plants; and Genome - Solanum tuberosum (PGSC v4.03) – Potato, as they're most related.
III. Added Target sequence against whom we're interested to design gRNA
IV. Selected gRNA sequences with best possible properties, in complementary to the Primer-BLAST results **(Figure 4.2.1 & 4.2.2)**.

3.2.2 LbCpf1 (Cas12): Transformation, Expression, Extraction & Purification

3.2.2.1 Transformation of pRSET-A vector with LbCpf1 gene to BL21 (DE3) host cells & confirmation

First synthesised pRSET-A vector containing LbCpf1 gene sequence (3681bp) downstream of 6x–His tag at BstBI site on MCS (Multiple Cloning site) of pRSET-A expression vector (invitrogen) commercially. Then, transformed this expression vector (along with LbCpf1 gene) into 100µL of BL21 (DE3) competent cells via Heat shock method of transformation.
First, competent cells and vector were incubated on ice for 20 min. then heat shocked at 42 ℃, for 1 min. And again, incubated on ice for 2-3 min. Added 900µL of LB broth and grown the culture for 1 hr. at 37 ℃. Centrifuged the grown culture at 4000 rpm for 4 min and discarded 900µL of supernatant. Resuspended the pellet in remaining 100µL and spread plate over three LB agar plates (triplicates) prepared with antibiotics Ampicillin (50µg/mL) and Chloramphenicol (35µg/mL) in LAF. Incubated these plates at 37℃ and check growth on overnight grown plates **(Figure 4.3)**.

3.2.2.2 Confirm LbCpf1 gene in transformed pRSET-A vector

Picked a single colony from overnight grown culture of Ampicillin and Chloramphenicol resistant plates (above) and inoculated in 5mL LB media having antibiotics Ampicillin (100µg/mL) and Chloramphenicol (35µg/mL) overnight at 37 ℃. Plasmid isolation (via QIAprep Spin Miniprep Kit) from overnight grown culture and double digestion with XhoI & PvuI (pair) and HindIII & BamHI (pair) confirmed the presence of LbCpf1 gene within transformed pRSET-A vector through visualisation of digested fragments (bands) in Gel electrophoresis **(Figure 4.4)**.

3.2.2.3 Expression of LbCpf1 (LbCas12 Protein)

Put a starter culture of 15mL of LB broth with antibiotics Ampicillin (100µg/mL) and Chloramphenicol (35µg/mL) by adding 100µL of glycerol stock and grow overnight at 37 ℃, 150rpm. Then, subculture 5mL each to three 100mL prepared autoclaved LB broth media and grow at similar conditions. On next day first collect the 1mL aliquot of uninduced control (Uninduced-Control) of each then induce two of these overnight grown culture (of 100mL) with 0.5mM IPTG for 4hr. (Sample-1) and 16hr. (Sample-2) and third culture with 1mM IPTG overnight (Sample-3) at 37 ℃ and 150 rpm shaker and collect 1mL aliquot of induced culture

(Induced-Control) from each. Centrifuged all, the main cultures (of 100mL) at 7000 rpm for 15 min. and 1mL aliquots at 8000 rpm for 4 min. Discard supernatant, collect cell pellets & store at 4 ℃ till the next step (recommended for a few days only and for long term storage keep at -20 ℃).

3.2.2.4 Crude Protein Extraction

3.2.2.4.1 Soluble Proteins extraction

Weighed cell pellets of each culture were 2g (Sample-1), 0.8g (Sample-2) & 0.9g (Sample-3) & resuspended it on 10mL, 4mL and 5mL of Resuspension buffer (5mL/g wet weight) respectively with lysozyme (1 mg/mL) and incubate on ice for 30 min. Then sonicate at 200W, 40m amplitude with pulse of 10 sec. burst and 10 sec. of pause on ice for 2 min. Added RNase A (10µg/mL) and incubated on ice for 10 min (should also include DNase I - 5µg/mL, if have). Then centrifugation at 8000rpm for 45 min. at 4 ℃. Collected supernatants of each sample in fresh labelled tubes and stored at 4 ℃ for purification step. Carried out pellets to the future proceedings (i.e.; insoluble protein extraction).

All the steps related to soluble proteins should be done on ice or at 4 ℃ to maintain protein structure stability.

3.2.2.4.2 Insoluble Proteins extraction

Weighed cell lysates of each sample (after soluble protein extraction) were 1.2g (Sample-1), 0.44g (Sample-2) & 0.48g (Sample-3). Resuspend it on 6mL, 3mL and 3mL of Lysis buffer respectively. Mix it thoroughly and incubate at room temperature for 60 min till it becomes translucent. Then centrifuge at 8000 rpm for 45 min at room temperature. Discard pellets and save supernatant for further purification step.

3.2.2.5 Recombinant protein (LbCpf1) Purification

Washed the column properly first with Aqua regia and then rinsed it with ethanol and sterile water 2-3 times. Let it dry for a while and it's ready to use. Add 1mL of Ni-NTA (Nickel-Nitriloacetic acid) Agarose matrix (Qiagen) to each supernatant sample, except for Sample-1 is mixed with 2mL of Ni-NTA Agarose. Then stir it slowly on a shaker at 10 rpm, 4℃ for 1hr. After proper mixing and binding of LbCpf1 fused 6X His tagged (recombinant protein) to Ni-NTA Agarose, add this mixture to the clean and dried column. Keep the bottom valve closed, let agarose beads settle down for a few minutes. When all beads looked settled, open the valve slowly and collected flow through in a 15mL falcon tube. Washed twice with 4mL of Wash

buffer (20mM Imidazole) and collected both the washed samples in 15mL of falcons labelled Wash-1 and Wash-2 (in series its collected). Then added 2-3 mL of Elution buffer (with 250mM Imidazole) and collected ~0.5mL of aliquots in microcentrifuge tubes (Eppendorf) labelled E1-E6. Stored all the collected samples at 4 ℃ till SDS-PAGE analysis

3.2.2.6 SDS-PAGE analysis

Washed all the gel electrophoresis apparatus (gel plates, beakers, running chamber) and assembled it. Set gel plates on a casting tray and poured it with Resolving gel (10%) followed by an overlay of water, let it polymerise for a few minutes (15-20 min.). Removed overlaid water and mounted the resolving gel with Stacking gel (5%) and fixed 10 wells comb immediately to it. Stood by for a few minutes to polymerise the gel, meanwhile prepared samples by mixing it with 5X SDS PAGE sample buffer (in 4:1 ratio), 40µL in total (32µL sample + 8µL 5X SDS PAGE loading buffer). Heated the samples in a water bath at 94 ℃ for 10 minutes. Removed comb from polymerised gel and fixed the gel plates on PAGE running chamber (Bio-Rad). Poured the chamber with 1X SDS running buffer and started loading the samples to wells. Connected wires on cathode (black) and anode (red) in the running chamber to the power supply and run SDS-PAGE at 50V. After running the gel for a few hours when the loading dye seems to have leaked out of the bottom of the gel plate, the gel run is complete. Power supply was turned off and transferred the gel to a staining solution (Coomassie G-250) and put it on the Gel rocker at 5 rpm for a few hours to overnight. Then removed staining solution and added destaining solution, give similar conditions. Changed destaining solution for a few intervals until bands looked clear. Then checked out the gel on GS 800 Calibrated Densitometer and saved results **(Figure 4.6)**.

3.2.2.7 Pilot expression of IPTG induced LbCpf1 culture

Prepared LB broth media of 15mL having Ampicillin (50µg/mL) and Chloramphenicol (35µg/mL), inoculated it with 100µL of glycerol stock of BL21 DE3 (with LbCpf1gene). Incubated the culture at 37 ℃, 150 rpm till OD_{600} reaches 0.6. Then transferred 1mL from this grown culture to 9 sets of microcentrifuge tubes. Induced each 1mL of these aliquots with different IPTG stocks at different concentrations and for different time intervals **(Tabel 3.1)**. After incubation at 37℃ for their respective time centrifuge each induced cultures (of 1mL) at 8000 rpm for 7 minutes. Decant supernatant and suspended cell pellets on 2X SDS PAGE sample buffer. Heat incubated the samples in a water bath (GFL) at 95 ℃ for 10 minutes and loaded 40µL of samples to gel for SDS PAGE analysis.

Tabel 3.1: Induced cultures for pilot expression with different IPTG stocks at varying concentration and time interval.

IPTG Stocks	Concentration	Incubation Time (in hours)
UI	0mM	5hr.
A_1 (RPI)	1mM	4hr.
A_2 (HIMEDIA)	1mM	4hr.
B_3 (HIMEDIA)	1mM	3hr.
B_4 (HIMEDIA)	1mM	4hr.
B_5 (HIMEDIA)	1mM	5hr.
$C_{0.5}$ (GENEI)	0.5mM	4hr.
C_1 (GENEI)	1mM	4hr.
$C_{1.5}$ (GENEI)	1.5mM	4hr.

3.2.3 Sample (ToLCNDV) DNA extraction

Started with genomic DNA extraction of ToLCNDV infected Potato plant leaves using DNeasy Plant mini kit (Qiagen) in quadruplets and quantified on nanodrop. Performed PCR on extracted genomic DNA to amplify or isolate ssDNA regions (Coat protein & Replicase genes) of ToLCNDV from infected potato leaves **(Table 3.2) Figure 3.3)**. The amplified fragments of Coat protein (CP) & Replicase (Rep) with 140bp & 120bp length respectively are visualised and analysed through 1.6% agarose gel electrophoresis **(Figure 4.8)**.

Table 3.2: PCR reaction setup

Components	Quantity
Emerald master mix	10µL
Nuclease free water	7µL
Fwd. Primer (for CP & Rep)	1µL
Rev. Primer (for CP & Rep)	1µL
Template DNA (Extracted genomic DNA)	1µL
Total reaction volume	**20µL**

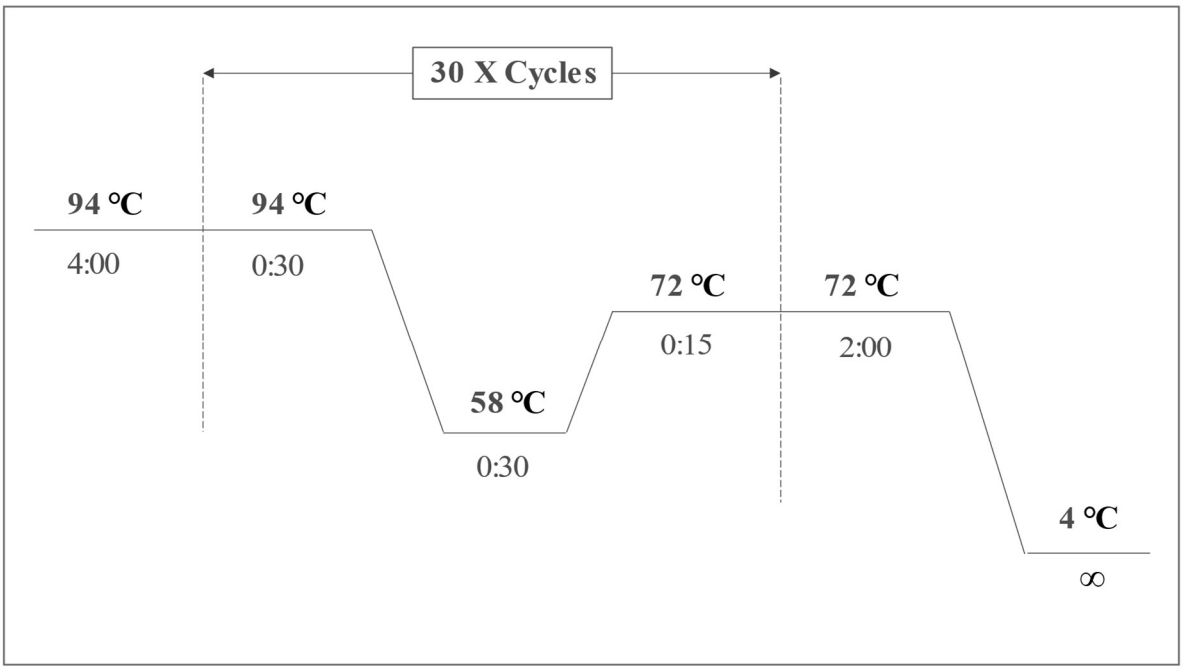


Figure 3.3: PCR reaction protocol in thermal cycler.

CHAPTER 4 – RESULTS & DISCUSSION

4.1 Narrow down selection to ToLCNDV-Potato and their targeted genes

In order to design precise or specific primers and gRNAs for any sequence of an organism (for its nucleic acid detection) we first need to limit down the 'organism type' among large taxonomy of ToLCNDV variants infecting different hosts at varied geographical regions through NCBI Taxonomy browser or NCBI virus database **(Figure 4.1)**.

Figure 4.1: Selection of ToLCNDV-Potato variant.

From there also a number of ToLCNDV-Potato genome sequences have been reported, isolated from varied geographical locations inside or outside of India. So, I selected the "ToLCNDV-Potato KAN–6 isolates; KX951455 & KX951456 (GenBank IDs of DNA-A & DNA-B segments of bipartite ToLCNDV)" isolated from Kanpur, UP, India **(Figure 4.2)**. As they're the most recent sequences reported (on 2017-09-25) and submitted to the Division of Plant

Protection, CPRI, Bemloe, Shimla, Himachal Pradesh 171001 (where my work is also in collaboration with).

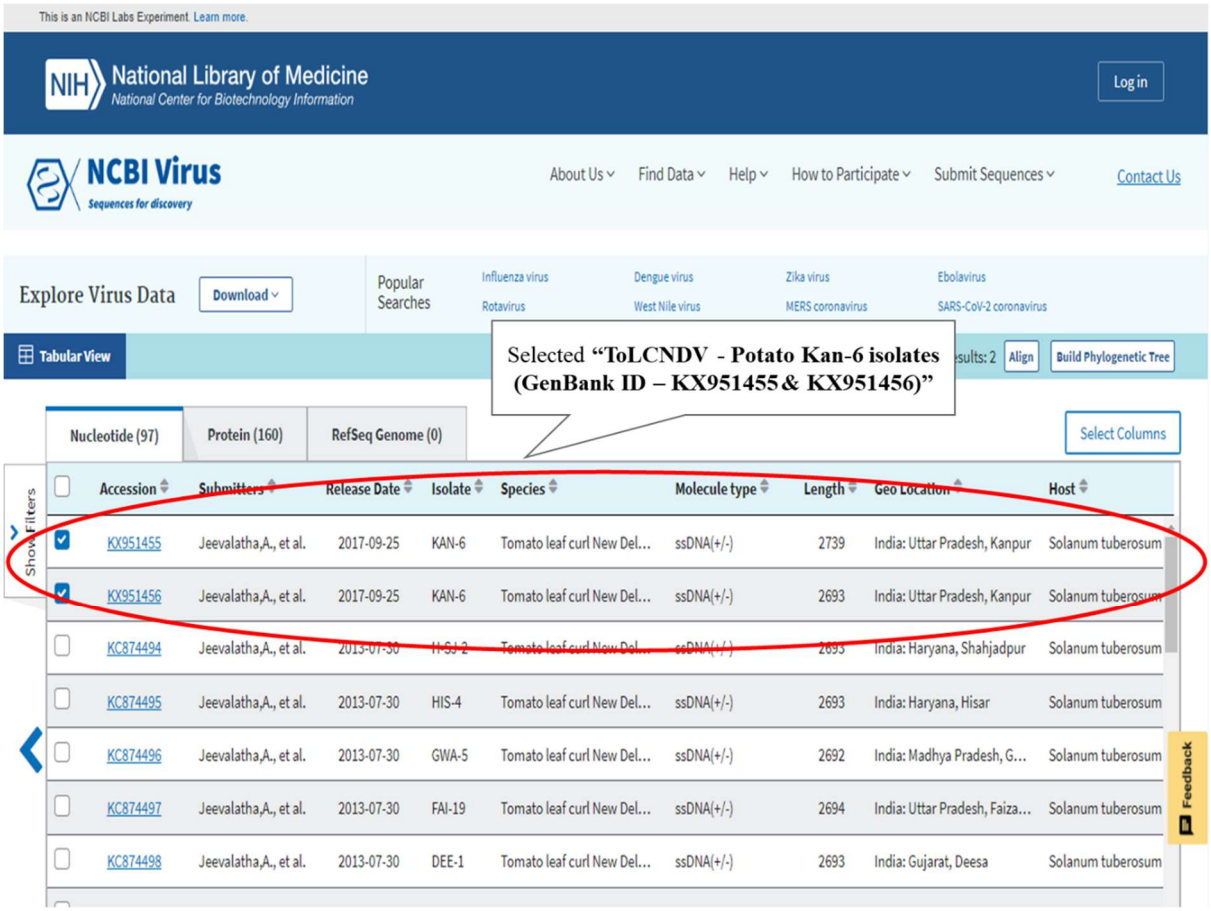


Figure 4.2: Selected ToLCNDV - Potato KAN-6 isolates (Accession/GenBank ID - KX951455 & KX951456).

Now as we have already selected the organism and have access to their GenBank ID's from here we got their nucleotide sequence and other genomic information like about genes, genomic structure (circular ssDNA), genome size (2739 & 2693 of DNA-A & DNA-B segments respectively), etc. And selected the genes which are essentially important for viral pathogenicity or having the most conserved regions, confirmed through BLAST (Basic Local Alignment Search Tool) followed by MSA (Multiple Sequence Alignment) (Hussain et al., 2005) **(Table 4. 1)**

Table 4. 1: Different genes in bipartite genome of ToLCNDV in potato isolate KAN-6. Light blue boxes representing selected target genes for primer & gRNA *in silico* esigning.

DNA-A (KX951455)		DNA-B (KX951456)	
Gene	**Function**	**Gene**	**Function**
AV-1 (280-1050)	Coat Protein	BV-1 (442-1248)	Nuclear shuttle protein
AV-2	Pre-Coat Protein + role in movement	BC-1	Movement protein
AC-1 (1499-2584)	Replication initiator		
AC-2 (1177-1596)	Transcriptional activator protein		
AC-3	Replication enhancer protein		
AC-4	Not Known		
AC-5 (310-795)	Not Known		

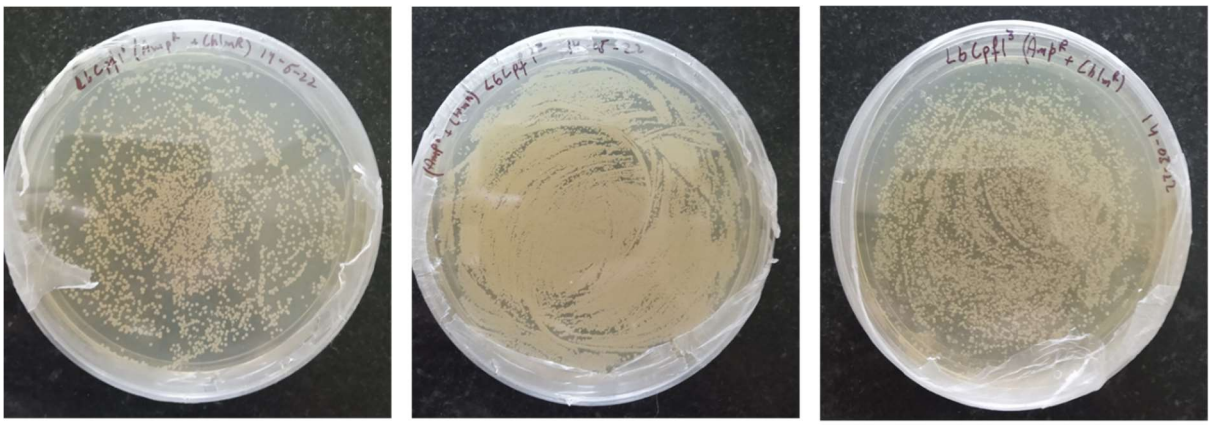
4.2 Designed Primers & gRNAs for selected genes (or targeted regions)

In silico designing of primers (from Primer-BLAST; NCBI) and gRNAs (from Cas designer; RGEN tools) targeting specific regions of selected genes and having best optimised characteristics **(Figure 4.2.1 & Figure 4.2.2)** like for Primers; almost similar Tm for forward & reverse primer, GC% in between 40% to 60% range, length of the primers should be around 18-30bp, low self-complementarity & self 3` complementarity to avoid secondary str. & primer dimers formation resp. And for gRNA designing; GC% should be high (~50 or more) for efficient gRNA and target region binding, Out-of-frame score must be >66 at least to avoid frame shifting & Mismatch score of 0 0 1 is recommended for 0 1 2 mismatch(es) format.

4.3 LbCpf1 (Cas12): Transformation, Expression, Extraction & Purification

4.3.1 Confirmed Transformation of pRSET-A vector to BL21 (DE3)

After transformation of pRSET-A expression vector (along with LbCpf1 gene) to BL21 (DE3) cells three Ampicillin and Chloramphenicol resistant LB agar plates had been prepared (triplicates) and spread plated it with transformed cultures and grown overnight to see the growth. The growth in these plates is only possible if BL21 (DE3) host cells having Chloramphenicol resistant genes are transformed with pRSET-A expression vector having Ampicillin resistant genes. Growth in all the three (triplicates) Ampicillin and Chloramphenicol resistant plates proved that transformation was successful **(Figure 4.3)**.



(a) (b) (c)

Figure 4.3: Growth of Transformed cells on Ampicillin (100µg/mL) & Chloramphenicol (35µg/mL) resistant LB Agar Plates. (a) Plate 1 (b) Plate 2 (c) Plate 3.

4.3.2 Confirmed LbCpf1 gene in transformed pRSET-A vector

LbCpf1 gene sequence was added downstream of 6X His-tag sequence of pRSET-A vector at BstB1 site of MCS. Isolated plasmid from transformed cells and quantified on nanodrop having 307.6 ng/µL of DNA. According to concentration of plasmid, restriction digestion reaction was set up whose results are visualised on 1.2% of agarose gel electrophoresis **(Figure 4.4)**. Isolated and quantified plasmids (307.6 ng/ µL) were double digested in two sets, first with HindIII & BamHI restriction enzymes as MCS of pRSET-A vector flanked by these two restriction sites. Secondly with XhoI & PvuI restriction enzymes flanking the LbCpf1 gene sequence (confirmed through FastPCR tool & NEB cutter). On digestion full-fledged pRSET-A vector backbone was obtained of 2.9 kbp of length in both the cases **(Figure 4.4)** along with that many small fragments were there which when added upto 3.6 kbp of length that's the original size of

LbCpf1 gene. Hence, it confirmed the presence of LbCpf1 gene in transformed pRSET-A vector.

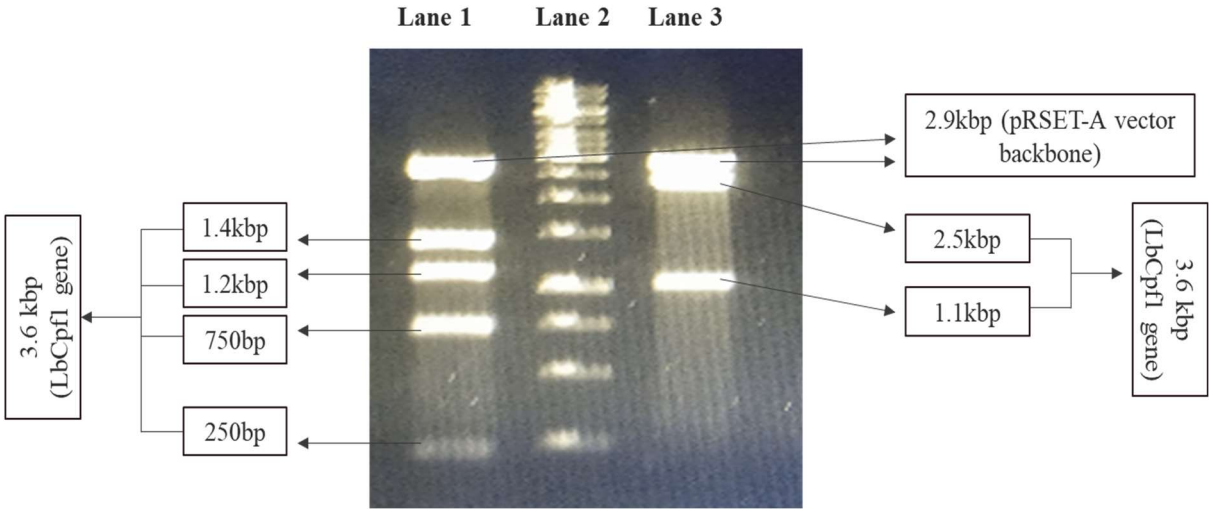
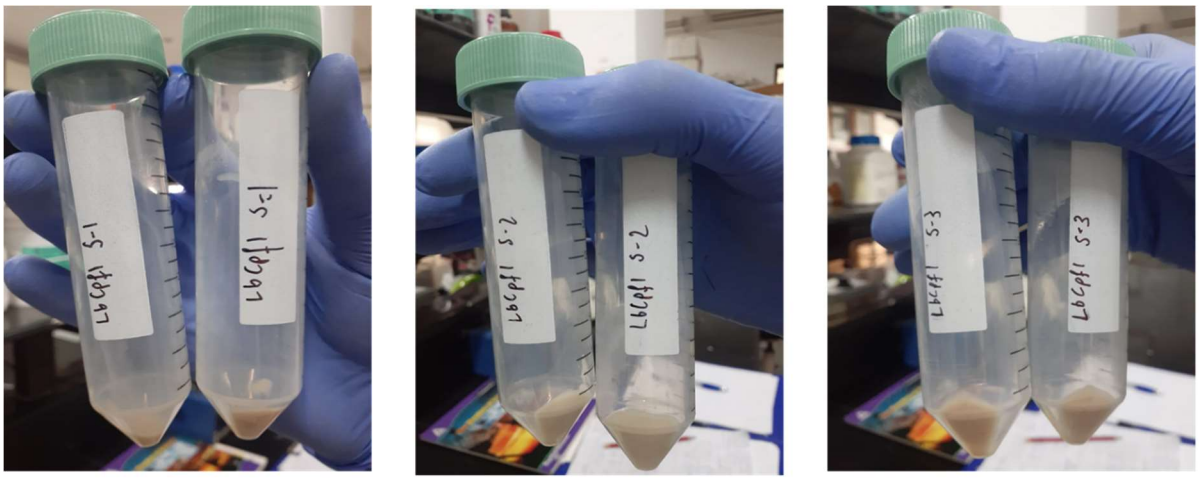


Figure 4.4: Visualisation of pRSET-A vector backbone and fragments of LbCpf1 gene on double digestion of Isolated plasmid in 1.2% agarose gel. (Left to Right) Lane 1 - HindIII & BamHI restricted fragments. Lane 2 - 1 kbp Ladder (generuler). Lane 3 - XhoI & PvuI restricted fragments.

4.3.3 Expression & Extraction of LbCpf1 Protein

Results for expression and extraction can't be visualised until the SDS PAGE analysis. Protein bands under induced control (Lane 2) in SDS PAGE results after purification **(Figure 4.6)** could be considered as expressed or crude protein extract content. As they're the 1mL aliquots obtained from immediate expressed or IPTG induced cultures. But for now, we can just observe the pellets of Expressed or IPTG induced cells **(Figure 4.5)** and supernatant in case of protein extract, protein content of which can be quantified by using Bradford assay. But it wasn't necessarily required in our case as we're focussing more on the purified LbCpf1 protein than crude protein extract. So, we'll quantify the concentration of purified LbCpf1 content (protein) thereupon the successful IMAC (Immobilized Metal Affinity Chromatography).

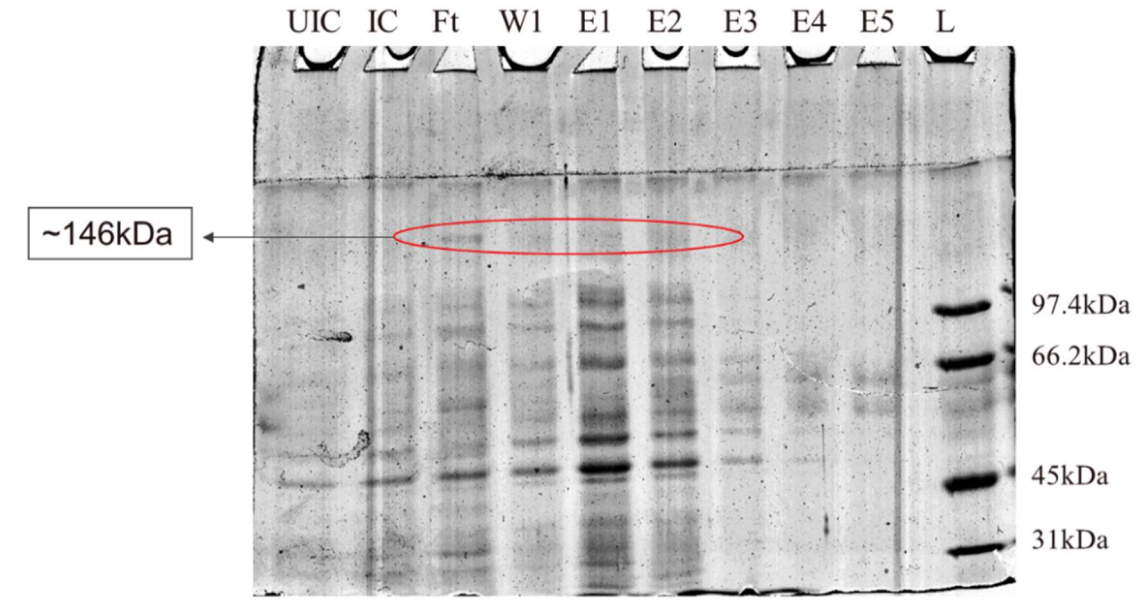


(a) (b) (c)

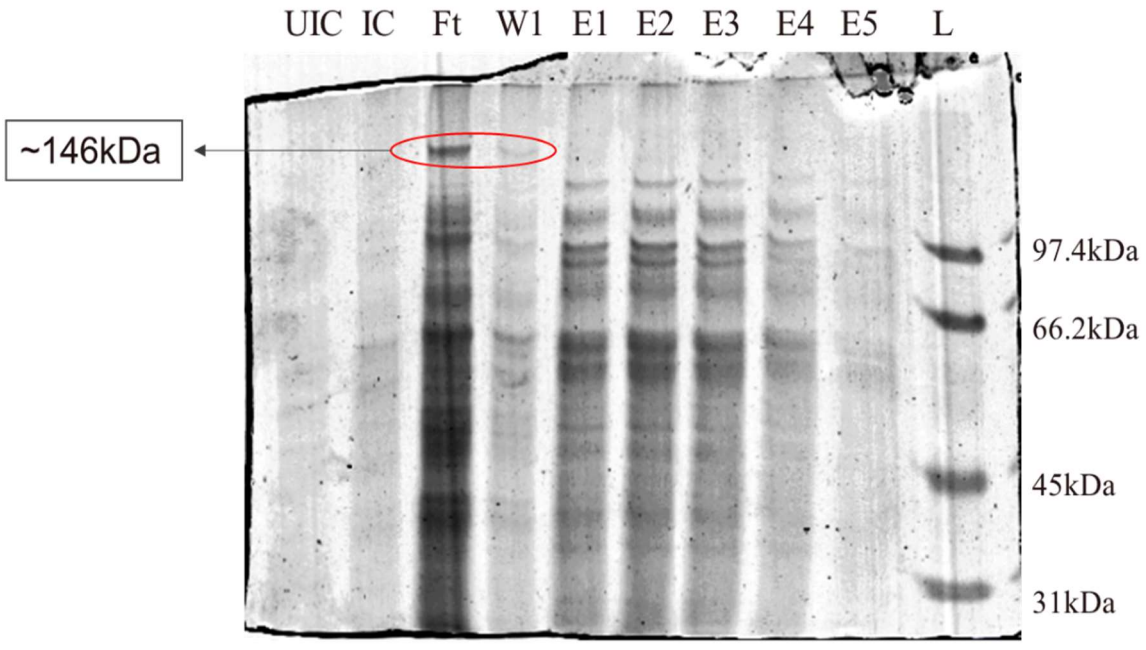
Figure 4.5: Expressed or IPTG induced cell pellets of (a) Sample-1 (b) Sample-2 (c) Sample-3

4.3.4 SDS PAGE Analysis of Purified 6X His-tagged LbCpf1 protein

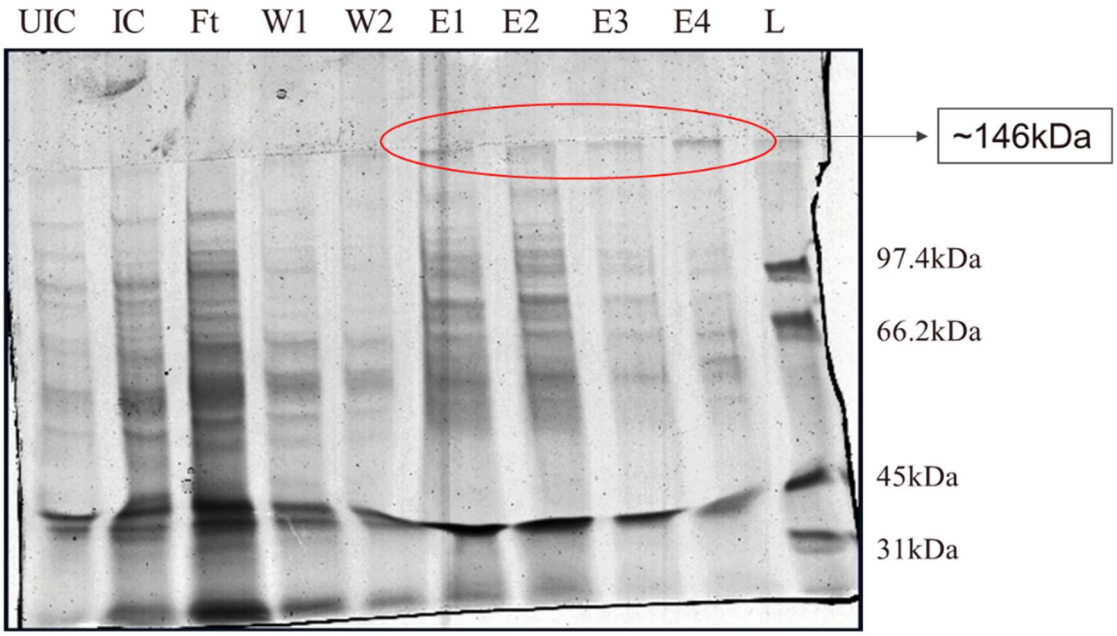
LbCpf1 is a monomer protein, so SDS PAGE of small aliquots of the samples could be performed effectively. On SDS PAGE analysis of purified recombinant protein (i.e.; LbCpf1) in 10% of resolving gel, it's been observed that 6X-His tagged LbCpf1 is getting expressed but purification is not up to the mark. The desired protein (LbCpf1) either getting drawn out of column with flow through or wash buffer in Sample-1 & Sample-2 **(Figure 4.6; a & b)** or if coming into elution then along with other undesirable protein contamination **(Figure 4.6; c)**. This may be due to the turbulent or unmanageable flow of samples through column (Burette; JSGW) or more standardisation need to be done in buffer composition or other steps in purification protocol like; more binding time for 6X - His tag protein to Ni-NTA matrix.


UIC IC Ft W1 E1 E2 E3 E4 E5 L
~146kDa
97.4kDa
66.2kDa
45kDa
31kDa

(a)


UIC IC Ft W1 E1 E2 E3 E4 E5 L
~146kDa
97.4kDa
66.2kDa
45kDa
31kDa

(b)



(c)

Figure 4.6: SDS PAGE analysis of Recombinant protein (6X His-Tagged LbCpf1) Expression and Purification using Ni-NTA agarose matrix; IMAC.
(a) For Sample-1 (b) For Sample-2 (c) Sample-3. UIC – Un-Induced culture, IC – IPTG Induced culture, Ft – Flow through, W1 & W2 – Collected washed samples, E1 to E5 – Elution samples, L – SDS PAGE protein Ladder of Low range (BIO-RAD).

4.3.5 SDS PAGE Analysis of Pilot expression LbCpf1 cultures

Pilot expression of LbCpf1 cultures (BL21-DE3) is done to standardise the induction or expression step. Different IPTG stocks are been used at different concentration over varying time intervals. Finally, it's been observed in 10% SDS PAGE that there is no much difference in expression level of LbCp1 gene in pRSET-A vector (BL21-DE3 pLysS cells) at IPTG concentration from 0.5mM to 1.5mM over incubation time of 3hr. to 5hr **(Figure 4.7)** thus more standardisation needs to be done.

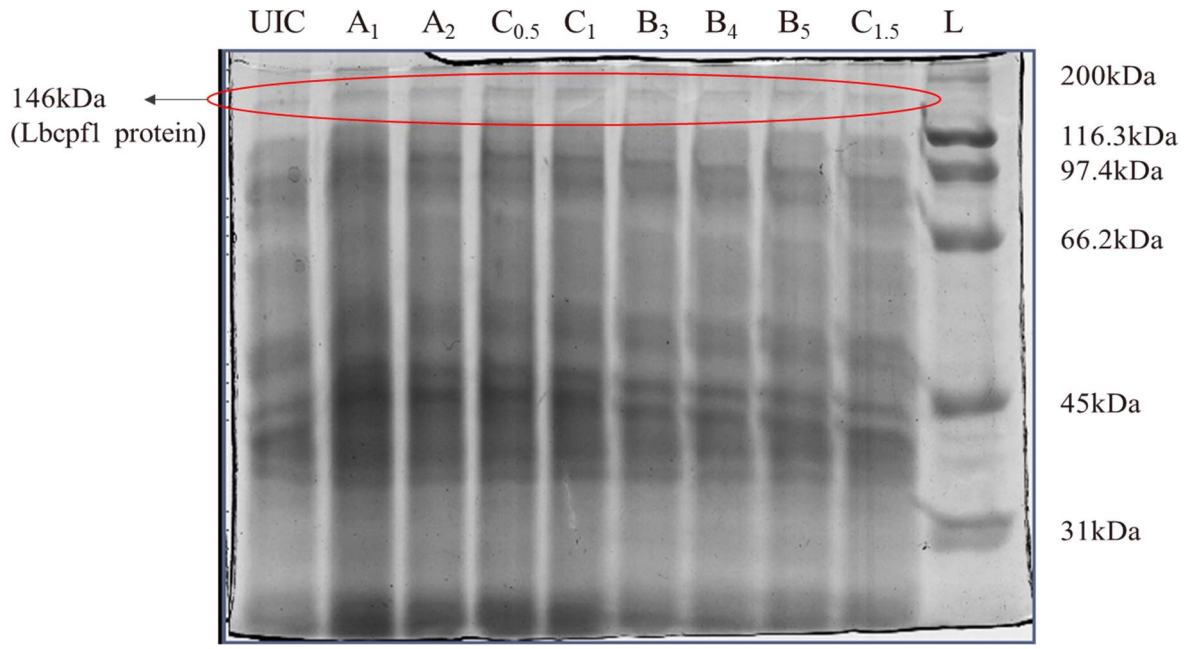


Figure 4.7: SDS PAGE evaluation of Pilot expression induced cultures at varying IPTG concentrations and time intervals. **:** Lane 1 - UIC (Un-Induced Control), Lane 2 to Lane 9 IPTG Induced cultures; A_1 - Induced with 1mM IPTG stock ($A_{1;\ company}$) for 4hr., A_2 - Induced with 1mM IPTG stock ($A_{2;\ company}$) for 4hr., $C_{0.5}$ - Induced with 0.5mM IPTG (GENEI) for 4hr., C_1 - Induced with 1mM IPTG (GENEI) for 4hr., B_3 - Induced with 1mM IPTG (HIMEDIA) for 3hr., B_4 - Induced with 1mM IPTG (HIMEDIA) for 4hr., B_5 - Induced with 1mM IPTG (HIMEDIA) for 5hr., $C_{1.5}$ - Induced with 1.5mM IPTG (GENEI) for 4hr., L - SDS-PAGE Protein Ladder of Broad range (BIO-RAD)

4.4 Sample (ToLCNDV) DNA Extraction & Isolation

The targeted regions of ToLCNDV are extracted, isolated from infected potato leaves and quantified as 49.6 ng/µL, 38.3 ng/µL, 69.9 ng/µL, 77.2 ng/µL (in quadruplets) followed by primer specific amplification of 'coat protein' & 'replicase' gene regions of isolated ToLCNDV genome. The amplified or isolated targeted gene regions of ToLCNDV are analysed and validated from 1.6% agarose gel electrophoresis **(Figure 4.8)**. These amplicons can later be utilised for CRISPR-Cas based nucleic acid detection of ToLCNDV in Potato leaves.

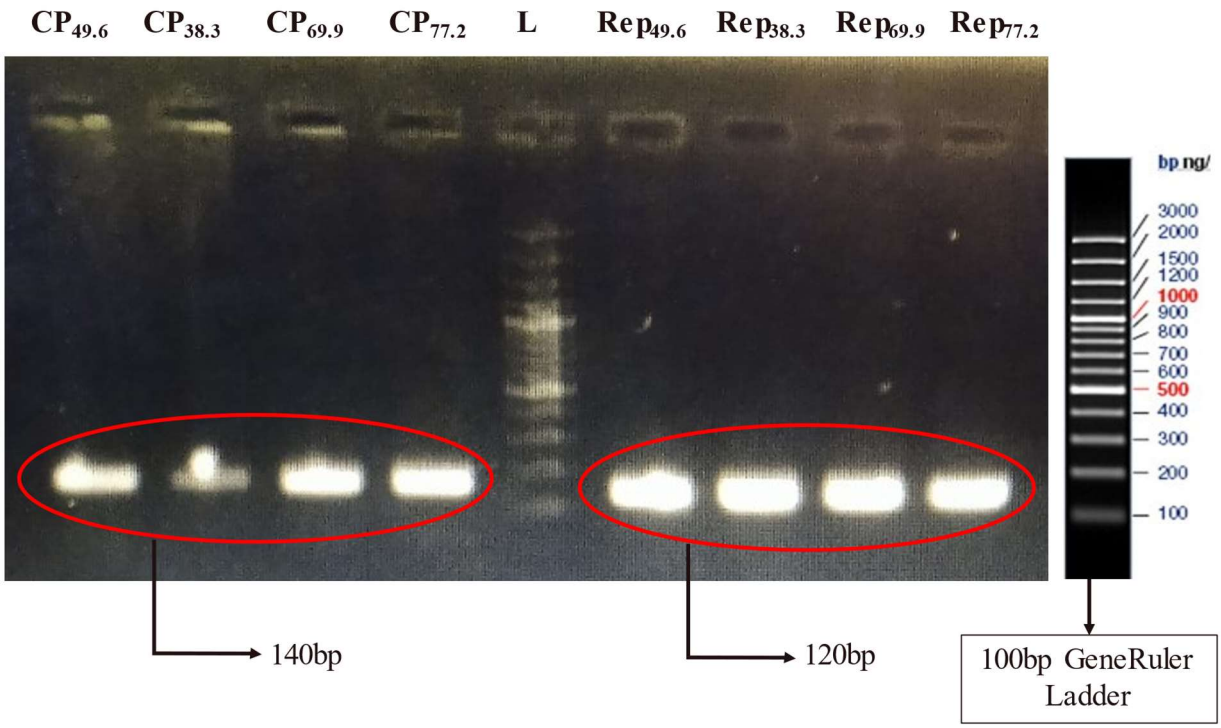


Figure 4.8: PCR amplified or Isolated Coat protein (140bp) & Replicase (120bp) gene regions of ToLCNDV in 1.6% agarose gel with 100bp Ladder (GeneRuler) on right side.

CONCLUSION

The BL21 (DE3) pLysS host cells are successfully transformed with pRSET-A expression vector along with LbCpf1 gene, confirmed through culturing of transformed cells on LB plates having ampicillin and chloramphenicol antibiotics as transformed cells are resistant to both these antibiotics. Also, the presence of LbCpf1 gene was confirmed by restriction double digestion following to plasmid isolation of transformed culture and visualisation of LbCpf1 gene (3.6kbp) on agarose gel electrophoresis.

Then induced transformed cells using IPTG at different concentration over time to optimise the best expression conditions for LbCpf1 gene on the available expression system. After that extraction and purification of expressed LbCpf1 protein tagged with 6X-Histidine is done using Ni^{2+}-NTA Column (Qiagen) via IMAC, that analysed on SDS PAGE. Alongside with recombinant Cas protein, primers & gRNAs were designed for specific targeted gene regions of ToLCNDV-Potato variant with best optimised properties *in silico*.

Both of these CRISPR-Cas components i.e; designed gRNA and Purified LbCpf1 (Cas12a) could be employed for CRISPR based diagnosis of ToLCNDV from infected Potato sample.

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APPENDIX

I. Reagents & Buffers for Recombinant Protein Extraction & Purification:

Reagents or Buffers	Composition
For Soluble Proteins	
Resuspension buffer	50mM $NaH_2PO_4.H_2O$ 300mM NaCl 10mM Imidazole (Maintain pH 8.0)
Wash Buffer	50mM $NaH_2PO_4.H_2O$ 300mM NaCl 20mM Imidazole (Maintain pH 8.0)
Elution Buffer	50mM $NaH_2PO_4.H_2O$ 300mM NaCl 250mM Imidazole (Maintain pH 8.0)
For Insoluble Proteins	
Lysis Buffer	100mM $NaH_2PO_4.H_2O$ 10mM Tris-Cl 8M Urea (Maintain pH 8.0)
Wash Buffer	100mM $NaH_2PO_4.H_2O$ 10mM Tris-Cl 8M Urea (Maintain pH 6.3)
Elution Buffer 1	100mM $NaH_2PO_4.H_2O$ 10mM Tris-Cl 8M Urea (Maintain pH 5.9)
Elution Buffer 2	100mM $NaH_2PO_4.H_2O$ 10mM Tris-Cl 8M Urea (Maintain pH 4.5)

II. SDS-PAGE Buffers:

Reagents	Composition
Gel Casting buffer	
30% Acrylamide/Bis-Acrylamide solution	29.2g Acrylamide + 0.8g Bis-acrylamide in 100mL dH_2O
Tris buffer	For Resolving gel - 1.5M Tris (pH 8.8) Or For Stacking gel - 1M Tris (pH 6.8)
SDS (Sodium Dodecyl Sulfate)	10% SDS
APS (Ammonium Per Sulfate)	10% APS
TEMED (TetraMethylethyleneDiamine)	----
Gel Running buffer (1X)	
Tris base	25mM
Glycine	200mM
SDS	0.1%
(Maintain pH 8.3)	
SDS-PAGE loading buffer (5X)	
Tris-Cl (pH 6.8)	250mM
Glycerol	50%
SDS	10%
Bromophenol blue	0.05%
DTT (Dithiothreitol)	500mM
Staining & De-staining solutions	
Coomassie G-250	0.5%
Methanol	50%
Acetic acid	10%
dH_20 or MiliQ water	40%