CHAPTER-3

Materials and Methods
3.1 CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

3.1.1 Materials

3.1.1.1 Bacterial strains

Cloning host used is *E. coli* DH5α ultra competent cells, expression host included *E. coli* strain BL21 (DE3) and Rosetta (DE3) (Novagen).

3.1.1.2 Bacterial cell culture medium

- **2 % Luria Broth (LB) medium**

  20 g Luria Broth powder (Himedia) was dissolved in deionized water and the final volume made to 1000 ml. The medium was sterilized by autoclaving at 15 lbs for 20 min.

- **2 % Luria Broth agar medium**

  2.0 % Luria Broth medium, 1.5 % Agar (Himedia RM 301): 15 g of agar was added to the LB medium and the mixture was autoclaved at 15 lbs for 20 min.

- **Antibiotics**

  *Ampicillin sodium salt (Sigma A-9518):*

  Stock: A solution of 100 mg/ml was made in deionized water and filtered through a 0.22 μm filter. LB media containing 100 μg/ml ampicillin was used for growing cultures with plasmid harboring ampicillin resistance gene.

  *Kanamycin sulfate (Sigma K4378):*

  Stock: A solution of 50 mg/ml was made in deionized water and filtered through a 0.22 μm filter. 34 μg/ml kanamycin was used in LB media.

  *Chloramphenicol (MP Biomedicals):*

  A solution of 34 mg/ml was made in deionized water and filtered through a 0.22 μm filter. LB media containing 34 μg/ml chloramphenicol was used for growing Rosetta 2(DE3).
3.1.1.3 Reagents for preparation of E. coli DH5-α ultra competent cells

- **SOB medium**

  2 % bacto-tryptone (Himedia RM 014), 0.5 % yeast extract (Himedia RM 027), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, pH 6.7 to 7.0

  To make SOB medium, 6 g bacto-tryptone, 1.5 g yeast extract, 175.32 mg NaCl, 55.91 mg KCl were dissolved in 297 ml deionized water and autoclaved. After autoclaving, 3 ml of 2 M Mg solution was added prior to use.

- **2 M Mg solution**

  1 M MgSO₄·7H₂O (6.15 g in 25 ml deionized water),

  1 M MgCl₂·6H₂O (5.075 g in 25 ml deionized water)

  25 ml of 1 M MgSO₄·7H₂O and 25 ml MgCl₂·6H₂O were mixed to make 2 M Mg solution, and it was filtered through 0.22 μm filter.

- **TB buffer**

  10 mM PIPES, 15 mM CaCl₂, 250 mM KCl, 55 mM MnCl₂

  393 mg PIPES, 286.65 mg CaCl₂, and 2.42 g KCl were dissolved in 120 ml deionized water and pH was adjusted to 6.7 with 5 N NaOH. After adjusting pH, 1.42 g MnCl₂ was added to the solution and volume was made up to 130 ml with deionized water. The solution was filtered through 0.22 μm filter.

- **Dimethyl Sulfoxide (DMSO) (Sigma D-8779)**

3.1.1.4 Plasmids

CloneJET PCR cloning kit (Thermo Scientific), pMALc5-E (NEB), pGEX2T (GE Healthcare), pEGFPn1 and pEGFPc1 (Clontech), pmCherry (a kind gift from Dr. Bhattacharya lab, ACTREC), pcDNA3-HA (Life Technologies)
Polymerase Chain Reaction (PCR)

High fidelity DNA polymerase-Pfu Turbo (Chem-agilent), 10X Pfu Turbo buffer, 25 mM MgCl₂, dNTPs mix (2 mM) from Stratagene

3.1.1.5 Primers

PCR primers were commercially synthesized from Sigma Genosys. The primers were designed using DNA star software.

For the complete list of primers please find Table 3.1. The lyophilized primers were reconstituted in autoclaved deionized water to obtain final concentration of 1000 ng/μl. A working dilution of 125ng/μl was prepared for further experiments.

Table 3.1 List of oligos for site-directed mutagenesis and sub-cloning of different gene of interest.

<table>
<thead>
<tr>
<th>HPV18 E2 mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Q35A, Q38A</strong></td>
</tr>
<tr>
<td>CAG CCA AAT AGC GTA TTG GGC ACT AAT ACG T</td>
</tr>
<tr>
<td><strong>R41A</strong></td>
</tr>
<tr>
<td>GCA ACT AAT AGC TTG GGA AAA TG</td>
</tr>
<tr>
<td><strong>W42A</strong></td>
</tr>
<tr>
<td>CAA CTA ATA CGT GCG GAA AAT GCA</td>
</tr>
<tr>
<td><strong>R41A, W42A</strong></td>
</tr>
<tr>
<td>CAACATAAGCTGCAGAAATGC</td>
</tr>
<tr>
<td><strong>F48A</strong></td>
</tr>
<tr>
<td>GCA ATA TTC GCT GCA GCA AGG</td>
</tr>
<tr>
<td><strong>E51A, H53A</strong></td>
</tr>
<tr>
<td>GCA GCA AGG GCA GCT GGC ATA CAG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Procaspase-8 mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L42A</strong></td>
</tr>
<tr>
<td>CAA GGA TGC CGC GAT GTT ATT CC</td>
</tr>
<tr>
<td><strong>F45A</strong></td>
</tr>
<tr>
<td>CCT TGA TGT TAG CCC AGA GAC TC</td>
</tr>
<tr>
<td><strong>L42A, L45A</strong></td>
</tr>
<tr>
<td>CAA GGA TGC CGC GAT GTT AGC CCA GAG ACT C</td>
</tr>
<tr>
<td><strong>F122A</strong></td>
</tr>
<tr>
<td>GTC TTT TAA GTT TGC TTT GCA AGA GG</td>
</tr>
<tr>
<td><strong>F122Y</strong></td>
</tr>
<tr>
<td>GAGGCTTTTTAAGTATCTTTGCAAGAG</td>
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<tr>
<td><strong>L123A</strong></td>
</tr>
<tr>
<td>GTC TTT TAA GTT TGC TTT GCA AGA GG</td>
</tr>
<tr>
<td><strong>L123A, Q125A</strong></td>
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<tr>
<td>CTT TTA AGT TTG CTT TGG CAG AGG AAA TCT C</td>
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<tr>
<td><strong>F122A, L123A</strong></td>
</tr>
<tr>
<td>GTC TTT TAA GGC TGC TTT GCA AGA GG</td>
</tr>
<tr>
<td><strong>Y8G</strong></td>
</tr>
<tr>
<td>CAG AAA TCT TGG TGA TAT TGG GGA AC</td>
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<tr>
<td><strong>R5A, Y8A</strong></td>
</tr>
<tr>
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<tr>
<td><strong>S119A, E126A</strong></td>
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<tr>
<td>CAG AAT TGA GGG CTT TTA AGT TTC TTT TGC AAG CGG AAA TCT C</td>
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</table>
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<table>
<thead>
<tr>
<th>D158A</th>
<th>GGA AAG TTG GCC ATC CTG AAA</th>
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<tbody>
<tr>
<td>R162A</td>
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<tr>
<td>Q166A</td>
<td>GTCTGTGCGCAATCAACAAAGAG</td>
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<tr>
<td>K161A, R162A</td>
<td>TGG ACA TCC TGG CAG CAG TCT GTG CC</td>
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<tr>
<td>Q166A, N168A</td>
<td>GTC TGT GCC GCA ATC GCC AAG AGC CTG CTG</td>
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<tr>
<td>C360A</td>
<td>TTA TTC AGG CTG CTC AGG GGG ATA ACT</td>
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#### FADD mutants

<table>
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<tr>
<th>V6A</th>
<th>CGT TCC TGG CGC TGC TGC AC</th>
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<tbody>
<tr>
<td>H9A</td>
<td>GTG CTG CTG GCC TCG GTG TCG</td>
</tr>
<tr>
<td>H9G</td>
<td>CTG GTG CTG CTG GGC TCG GTG TCG TC</td>
</tr>
<tr>
<td>T21A</td>
<td>GCG AGC TGG CCG AGC TCA AG</td>
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<tr>
<td>F25Y</td>
<td>GAG CTC AAG TAC CTA TGC TCT AGG</td>
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<td>L28A</td>
<td>GCT CAA GTT CCT ATG CGG GCG GCG CTG GC</td>
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<td>K33R</td>
<td>GGC GCG TGG GCG AGC GCA AGC TGG AG</td>
</tr>
<tr>
<td>R34A</td>
<td>GCA AGC GCA GCC TGG AGC GCG TG</td>
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<tr>
<td>K35R</td>
<td>GCA AGC TGG CGC GCG TGC AG</td>
</tr>
<tr>
<td>E37A</td>
<td>GTG GCC AAG GCC AAG CTG G</td>
</tr>
<tr>
<td>R38A</td>
<td>CGC AAG CTG GAG GCC TGT CAG AGC GGC C</td>
</tr>
<tr>
<td>L43A</td>
<td>CAG AGC GGC GAT GAC CTC TTC</td>
</tr>
<tr>
<td>L43D</td>
<td>CAG AGC GGC GCA GAC CTC TTC</td>
</tr>
<tr>
<td>D44A</td>
<td>CAG AGC GGC CTA GCC CTC TTC TCC ATG C</td>
</tr>
<tr>
<td>E51A</td>
<td>CCA TGC TGC TGG CGC AGA ACG ACC TGG AGC</td>
</tr>
<tr>
<td>L62A</td>
<td>GCA CAC CGA GGC CCT GCG TGA GCT G</td>
</tr>
<tr>
<td>L63A</td>
<td>CAC ACC GAG CTC GCG CTG GAG CTG CTC</td>
</tr>
<tr>
<td>A68F</td>
<td>CGT GAG CTG CTC TCC CTG CGG CGC</td>
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</table>

#### HtrA2 mutants

<table>
<thead>
<tr>
<th>G230A</th>
<th>GTC CCA GCG GCG CTA CAT TGC GGT GAT GAT G</th>
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<tbody>
<tr>
<td>AVPS</td>
<td>GAG ATA TAC ATA TGG TCG CTA GCC CGC CG</td>
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#### XIAP mutants

<table>
<thead>
<tr>
<th>E219R, H223V</th>
<th>GTG CCT GGT CAC GAC ACA GGC GAG TCT TCC CTA ATT G</th>
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</thead>
<tbody>
<tr>
<td>Q319R, W323V</td>
<td>CTT GGG AAC GAC ATG CTA AAG TGT ATC CAG GGT GC</td>
</tr>
</tbody>
</table>

#### Procasparse-8 DED-B deletion constructs

<table>
<thead>
<tr>
<th>Helix-1</th>
<th>GGGAGAATCTTTTATTTTCAGGGCGGGATTCATGGAAAGTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGCAGATCAGAATTTGAG</td>
</tr>
<tr>
<td>Helix-2</td>
<td>GAGAATCTTTTATTTTCAGGGCGGGATCCATGGAAATCTC</td>
</tr>
<tr>
<td></td>
<td>CAAATGCAAACACTGATG</td>
</tr>
<tr>
<td>Helix-3</td>
<td>GAGAATCTTTTATTTTCAGGGCGGGATCCATGGATGACAT</td>
</tr>
<tr>
<td></td>
<td>GAACCTGCTTGATATTTTCA</td>
</tr>
<tr>
<td>Helix-4</td>
<td>CAAATGCAAAACTGATGACATGACGGAATTCGAA</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Helix-5</th>
<th>GCTTGGCACTGGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix-6</td>
<td>GAGCTGTGCCCCAACATCAAACAGTGGAATTCGAAGCTGGCCTGG</td>
</tr>
</tbody>
</table>

For all these mutants, the reverse primer is the complementary sequence.

### Mammalian procaspase-8 constructs

| DED-A (F)     | CGGGAATTCATGGACTTCAGCAGAAATC |
| DED-A (R)     | CGGTGGATCCCGCCTGTAGGGAATTTTGAC |
| DED-B (F)     | CTCAAGCTTCCAGATTCTGATGATGCTCTATCATGTCATTAC |
| DED-B (R)     | CGGTGGATCCCGGCTATTACACACACAC |
| FADD (F)      | GAATCCATGGACCGCTTCTGATGCTGTG |
| FADD (R)      | CAGAAATTTGAGC |
| Caspase-8 full length (R) | GGATCCCTCAATCAGAGGAAGACACAGGTTTT |

### Bacterial procaspase-8 constructs

| DED-AB (F)    | CGCGGATCCATGGACTTCAGCAGAAATC |
| DED-AB (R)    | CGCCTATCTCAGTCTGCTTCTGATGCTCTATCATGTCATT |
| DED-A (R)     | CGCAATTCATGGACTTCAGCAGAAATC |
| DED-B (F)     | GGATCCATGGAGACACCGAAGGAAAAAC |
| DED-B (R)     | CTGGAATTCCTGCTCTGTGACTGATGCTG |

### Bacterial HPV18 E2 constructs

| HPV18 E2 (1-201) (F) | CGCGGATCCATGGAGACACCGAAGGAAAAAC |
| HPV18 E2 (1-201) (R) | CGCAATTCATGGACTTCAGCAGAAATC |
| HPV18 E2 full length (F) | GGATCCATGGAGACACCGAAGGAAAAAC |
| HPV18 E2 full length (R) | CAGAAATTTGAGC |

### XIAP

| BIR2 (124-240) (F) | GAATTCATATGAGAGATCATATTTTGCCTTA |
| BIR2 (124-240) (R) | GGAATCCATATGAGAGATCATATTTTGCCTTA |
| BIR3 (241-356) (F) | GAATTCATATGAGAGATCATATTTTGCCTTA |
| BIR3 (241-356) (R) | GGAATCCATATGAGAGATCATATTTTGCCTTA |

### Sequencing oligos

| CMV promoter | CGCAAAATGGGGCGGTAGGCGGT |
| T7 promoter  | TAATACGACTCTATAGGG |
| T7 terminal  | GTAGTTATTGCTGACCCG |
| MBP reverse  | GGGCCAGTCTTTCGACTGAG |
| MBP forward  | GATGAAAGCCTGAAAGACGCAG |

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3.1.1.6 Restriction Digestion and Ligation

Fast Digest DpnI, BamHI, EcoRI, NcoI, NdeI, XhoI and 10X Fast digest buffer supplied along with the restriction enzymes (Fermentas)

T4 DNA ligase and ligase buffer from NEB

3.1.1.7 Agarose Gel Electrophoresis

- 10 X TBE buffer
  0.89 M Tris base, 0.89 M Boric acid, 0.02 M EDTA:
  108 gm Tris base, 55 g boric acid and 7.44 g EDTA were dissolved in deionized water to a final volume of 1 litre.

- Ethidium bromide (Sigma E-8751) – Stock of 10 mg/ml in deionized water

- Agarose (Sigma A-6013) – 1% Agarose in 1X TBE buffer

- 1kb DNA ladder – 0.5 μg/μl (Fermentas SM0311)

- Loading dye (6X) – 0.25 % Bromophenol blue, 0.25 % Xylene cyanol, 30 % Glycerol

3.1.1.8 Reagents for expression and purification of recombinant GST fusion proteins

- 1M Isopropyl-β-D-thiogalactopyranoside (Sigma I-5502)
  1.19 g Isopropyl-β-D-thiogalactopyranoside (IPTG) was dissolved in 5 ml deionized water, filter sterilized and stored at -20 °C.

- Lysis buffer for bacterial cells

- Protease inhibitor cocktail (Sigma P8340)

Resins – Ni-IDA (BIOTEX), GST-sepharose (Novagen), Amylose (NEB), Pre-packed gel filtration column: Superdex 200 and 75 – 16/600 and 10/300 from GE Healthcare
3.1.2 Methods

3.1.2.1 Preparation of E.coli DH5-α ultra competent cells

The procedure followed was adopted from Inoue et al with some modifications [123]. E.coli DH-5α cells were streaked from the glycerol stock on LB agar plate and were incubated overnight at 37 °C. A single colony of cells were picked up and grown in 250 ml of SOB medium at 18 °C until OD$_{600}$ reached to 0.6. The cells were then immediately kept on ice for 10 min and harvested by centrifugation at 6,000 rpm for 10 min at 4 °C using a SS-34 rotor in a Sorvall RC5C centrifuge. After centrifugation, cells were re-suspended in 80 ml of pre-chilled TB buffer and kept on ice for 10 min. The cell suspension was then centrifuged at 5000 rpm for 10 min at 4 °C. Post centrifugation, cells were re-suspended gently in 20 ml ice-cold TB buffer and 1.4 ml DMSO was added to attain final concentration of 7% (v/v). Cells were kept on ice for 10 min and aliquots of 100 μl each were made and snap frozen in liquid nitrogen. The ultra competent cells were then stored at -80 °C.

3.1.2.2 Strategy for cloning of genes

There are different kinds of gene cloning methods such as homology based cloning, ligation-independent cloning and restriction enzyme based cloning. In the present study, we employed restriction enzyme based cloning which is described in detail in the following section:

Restriction enzyme based cloning

Here, both expression vector and target genes are subjected to digestion with the same set of sticky ends generating restriction enzymes that enables insertion of target gene into a plasmid backbone.

a) Preparation of expression vector: The target genes were cloned into different bacterial and mammalian expression vectors to perform various in vitro and ex vivo experiments,
respectively. The plasmid DNA was extracted and purified as per the manufacturer’s protocol (GeneElute Plasmid Miniprep Kit from Sigma). The isolated plasmid vector was digested with the respective restriction endonucleases and the digestion was carried out at 37 °C for 1 h. The digested plasmid was resolved on 1% agarose gel followed by purification using Gel Extraction Kit (Sigma) according to manufacturer’s protocol.

b) Preparation of target gene: The gene of interest was PCR amplified using gene specific forward and reverse primers. The primers were designed using Primer-X software online tool. The parameters taken under consideration for designing primers included GC content: 40-60%, Tm: 50-65 °C, presence of 3’-GC clamp. Detailed list of oligos synthesized for amplifying specific gene of interest is given in Table 3.1.

Table 3.2 A typical PCR reaction composition.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Water</td>
<td>15.5</td>
</tr>
<tr>
<td>10X Pfu Turbo buffer</td>
<td>2</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>0.5</td>
</tr>
<tr>
<td>Template DNA (80ng)</td>
<td>0.5</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>0.5</td>
</tr>
<tr>
<td>(125 ng/μl) Forward primer</td>
<td>0.5</td>
</tr>
<tr>
<td>(125 ng/μl) Reverse primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Pfu Turbo</td>
<td>0.5</td>
</tr>
<tr>
<td>Total volume</td>
<td>20</td>
</tr>
</tbody>
</table>

The typical program set for PCR was as follows:

1. Denaturation – 95 °C for 5 min
2. Denaturation – 95 °C for 30 sec
3. Annealing – 53 °C for 50 sec
4. Extension – 72 °C for min (depending upon rate of amplification and no. of bases)
5. Repeat steps from 2 to 4 for 25 cycles
6. Final Extension – 72 °C for 10 min
7. Hold at 4°C forever

The amplified PCR product was purified using PCR clean-up kit from Sigma by following the manufacturer’s protocol. The purified PCR product was subjected to restriction digestion with the respective endonucleases used for preparing the vector backbone. The digested PCR product was further purified by gel extraction technique.

c) **Ligation:** The digested vector and PCR product was mixed in a molar ratio of 1:3 or 1:6, and then ligated using T4 DNA ligase according to the manufacturer’s protocol. The ligation reaction was carried out at 22 °C for 2 h. The ligation mixture was then transformed into ultra-competent DH5α cells.

The plasmid DNA can be incorporated into the competent cells in the process of bacterial transformation. The ultra competent *E.coli* DH5α cells were taken out from -80 °C and thawed on ice. 5 μl of ligation mixture was added to the cells and the suspension was incubated for 30 min. Cells were given heat shock at 42 °C for 90 sec and immediately kept on ice for 2-5 min. After heat shock, 750 μl of sterile LB media was added and the mixture was incubated at 37 °C for 30 min on shaker. Post incubation, cells were pelleted down by centrifugation at 5,000 rpm for 3 min at RT. The supernatant was discarded and fresh 100 μl of LB was added in the tube. Cells were re-suspended and spreaded on LB-agar containing appropriate antibiotic for selection. The plate was incubated at 37 °C for 16-18 h.

d) **Screening of potential clones:** The colonies were screened by isolating the plasmid DNA and subjecting it for restriction digestion using the respective enzymes. The digested product was resolved on 1% agarose gel to check the release of target gene from the vector backbone.
The positive clones are further confirmed by DNA sequencing using specific sequencing oligos listed in Table 3.1.

### 3.1.2.3 Site-Directed Mutagenesis (SDM)

The method relies on amplification of entire plasmid using mutagenic primers and DNA polymerase lacking primer displacement activity. SDM involves point mutation at a particular position in the gene of interest and comprises following three major steps.

a) **Overlapping PCR:** It requires two site-specific primers with the incorporated mutation and should be self complementary. The desired mutation should preferably be at the centre of the primer sequence of about 25-45 bases in length. The PCR reaction can be setup according to the reaction conditions described in Table 3.2.

b) **Dpn I digestion:** DpnI is an endonuclease which specifically targets the methylated DNA strands. Most of the plasmid DNA synthesized in *E. coli* is dam methylated and is therefore susceptible to DpnI digestion. One Unit of DpnI is added to 30 μl of reaction mixture and incubated for about 1 h at 37 °C.

c) **Transformation of SDM PCR product and sequencing:** The Dpn I digested product is transformed into competent *E.coli* DH5α cells and selected on LB agar plate containing appropriate antibiotic for selection. Single colony is inoculated in 10 ml of LB broth supplemented with the respective antibiotic and grown overnight in a shaker incubator at 37 °C. The plasmid DNA isolated from these colonies was sequenced to confirm the presence of mutation.
3.1.2.4 Bacterial Protein Expression

a) Selection of expression host: Different bacterial strains having specific or unique properties can be utilized for protein expression. Primarily, the choice of bacterial host strain depends on level of gene expression. For routine purposes, *E. coli* BL21 (DE3) strain, a lysogen of lambda-prophage (DE3), is used because it lacks the Lon and OmpT proteases. It carries a chromosomal copy of the T7 RNA polymerase under the control of lacUV5 promoter (inducible by IPTG) and therefore can conveniently express genes driven by the T7 promoter. *E.coli* Rosetta 2(DE3) is a derivative of BL21 (DE3) which can be used for the expression of eukaryotic proteins having rare codons. The stain is equipped with additional tRNAs that can recognize codons such as AUA, AGG, AGA, CUA, CCC, and GGA encoded on a compatible chloramphenicol-resistant plasmid call pRARE. Also, a modified version of BL21 (DE3) called BL21 pLysS can be used for expression of toxic genes. The strain contains a chloramphenicol resistant pLysS plasmid which encodes for T7 lysozyme to prevent basal or leaky expression.

b) Preparation of seed culture: A single transformed colony of the expression host is inoculated in 20 ml of sterile LB broth containing suitable antibiotic. The culture is allowed to grow overnight at 37 °C with continuous agitation at 200 rpm.

c) Scale up and induction: The scaling up of protein expression is carried out by inoculating the starter culture in 1000 ml LB broth in a ratio of 1:100. The culture is grown at 37 °C till the OD$_{600}$ reaches between 0.6-0.8. Depending on the expression vector, the protein is induced by adding IPTG (at a concentration of 0.4 mM) or arabinose (2 mg/ml). The culture flasks are incubated at 18 °C for 16-18 h under constant shaking of 200 rpm. Cells are then
harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. The bacterial pellet is stored at -
80 °C until further use.

3.1.2.5 Recombinant Protein Purification

Protein purification procedure utilizes differences in physical properties of the bio-molecule
such as protein size, charge, ligand specificity and biological activity. Commonly,
purification steps contain one or more chromatographic separation techniques. These
techniques that exploit different physiochemical properties are listed below:

- Affinity chromatography – Based on ligand specificity or bio recognition
- Ion exchange chromatography – Dependent upon charge of the protein
- Gel filtration chromatography – Separation on the basis of molecular size/mass
- Hydrophobic interaction chromatography – Relies on overall hydrophobicity

In the present study, we followed two step purification using affinity chromatography
followed by gel filtration.

a) Affinity chromatography: Affinity chromatography separates proteins on the basis of a
reversible interaction between a protein and a specific ligand coupled to a chromatography
matrix. It provides highest specificity and selectivity for purification of bio-molecules. The
interactions between ligand and target protein can be a result of electrostatic or hydrophobic
interactions, van der Waals’ forces and/or hydrogen bonding. To elute the target molecule
from the affinity medium the interaction is reversed, either specifically using a competitive
ligand, or non-specifically, by changing the pH, ionic strength or polarity. In a single step,
affinity purification offers immense time-saving over the less selective multi-step procedures.
The affinity matrix, used in our experiments is listed below:

- Glutathione Sepharose – glutathione-S-transferase for GST fusion proteins
- Metal ions (Ni-IDA) – Poly (His) fusion proteins, native proteins with histidine
- Amylose resin – Proteins fused to maltose-binding protein (MBP)

b) Gel filtration chromatography: Gel filtration also called size-exclusion chromatography is the separation technique based on the molecular size and volume of the components. The molecule with larger molecular hydrodynamic volume migrates faster compared to the smaller one. Separation is achieved by the differential exclusion of the sample molecules from the pores of the packing material. The principle feature of this technique is its gentle non-adsorptive interaction with the sample, enabling high retention of bimolecular activity and achieving homogeneous purified proteins. It can be used for varied applications including protein purification, buffer exchange and desalting.

3.1.2.6 General protocol for protein purification

The cell pellet is re-suspended with pre-chilled lysis buffer (10 ml for 1 litre of pellet). The cells are lysed by ultra-sonication under ice-cold condition at 50 pulse rate and 50 power for 10 cycles of 1 min each. The lysed homogenous suspension is centrifuged at 18,000 rpm for 20 min. For soluble proteins, collect the supernatant and discard the cell debris. All the proteins analyzed or characterized in the present study was purified using two stages of purification. The pre-cleared lysate is carefully added on the equilibrated affinity resins and incubated at room temperature (RT) for an hour. After binding, the resin is extensively washed with the lysis buffer to remove non-specific or bacterial proteins. Depending upon the tag, the protein is eluted using a gradient of imidazole (25-500 mM) for His-tag fusion protein or maltose (2-20 mM) for MBP-fused proteins. The purity of proteins is checked on 12-15% SDS-PAGE based on the molecular weight of the proteins being analyzed. Further, the desired fractions are concentrated by ultra-filtration method using Amicon Ultra
centrifugal filter devices of 3-30 kDa molecular weight cut-off. The second step of purification involves gel filtration chromatography using Superdex 200 or 75 matrixes. 1 ml of concentrated protein is injected in the pre-equilibrated column (instrument-AKTA purification system). Elution of the protein is monitored by recording the absorbance at wavelength 280 nm. The purity of the eluted fractions is checked on 12-15% SDS-PAGE depending on the molecular weight of the proteins being analyzed. The detailed purification protocols for different proteins have been mentioned in the respective chapters.

3.2 ANIMAL CELL CULTURE

3.2.1 Routine maintenance of cell lines

3.2.1.1 Materials

- **Medium**
  Powdered Dulbecco’s modified Eagle’s medium (DMEM) from GIBCO was dissolved in 1 litre of deionized water (3.7 g of sodium bicarbonate per litre was added and the pH of was adjusted to 7.4). The medium was filtered using millipore assembly – 0.22 μm membrane filter (Whatman). One ml of the filtered medium was added to the sterility test medium and incubated at room temperature for 6 days under observation. Sterility test medium (14.9 g of Fluid-thioglycolate was dissolved in ~250 ml of water. The volume was made up to 500 ml in measuring flask and boiled. After aliquoting 6 ml of the medium in glass tubes a pinch of CaCO₃ was added to each tube and autoclaved.)

- **Phosphate Buffered Saline** (PBS) - 150 mM NaCl, 2 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄

- **0.4% Trypan Blue solution** - Prepared in 0.81% NaCl and 0.06% KHPO₄, dibasic

- **Trypsin-EDTA** (0.025% Trypsin, 0.2 mM EDTA)

- **Complete medium**
DMEM with 10% Fetal bovine serum and 1% antibiotic solution (Amphotericin B 20 μg/ml, Penicillin 2500 Units/ml, Streptomycin 800 μg/ml)

- **Freezing mixture** (90% Fetal bovine serum, 10% DMSO)
- **Cell lines** – Human Embryonic Kidney (HEK) 293, HeLa

### 3.2.1.2 Protocols

**a) Revival of cells:** Revival of frozen cell cultures was carried out by thawing the freezing vials with the cells into a glass beaker containing water at 37 ºC. Immediately the thawed cell suspension was added drop wise into a sterile centrifuge containing 5 ml of complete medium. The cell suspension was centrifuged for 10 min at 1,000 rpm. The supernatant was discarded and the cell pellet was washed with 1X PBS twice for complete removal of freezing mixture. Finally, the cell pellet was re-suspended in 3 ml of complete medium. The cell suspension was mixed gently with a Pasteur pipette to remove any clumps and finally transferred in a culture plate or flask. The cells were incubated in a humidified CO₂ (5%) incubator at 37 ºC and their growth was observed each day under inverted microscope.

**b) Subculture/trypsinization and transfer of cells:** Cells with 70-80% confluency were washed with 1X PBS twice and 1 ml of trypsin-EDTA was added to the culture plate. Excess trypsin-EDTA was discarded and the plate was incubated till the cells partially detached. Complete medium was added into the plate and the resulting cell suspension was mixed with pipette to make a single cell suspension. Total cell count and the percent viability were calculated by dye exclusion method using Trypan Blue dye on a haemocytometer using an inverted microscope. Appropriate amount of cells depending upon cell type were seeded in culture plates. The plates were further incubated in humidified CO₂ incubator at 37 ºC.
c) **Freezing and cryopreservation of cells:** For freezing the cells, 70-80% confluent culture flask or dish was subjected to trypsinization. After noting the total cell count, the cell suspension was spun at 1,000 rpm for 10 min. The supernatant was discarded and the pellet was dislodged by tapping the tube gently. One ml of freezing mixture was added drop wise and mixed gently with pipette. The cells suspension was then transferred to freezing vials. The freezing vials were labelled and suspended in a cylinder with liquid nitrogen vapour and cooled gradually before plunging it into liquid nitrogen for long term storage.

### 3.2.2 Transient transfection

**Reagents:** Plasmid DNA, 0.5 M CaCl₂, 2X BBS (50 mM BES, 1.5 mM Na₂HPO₄ and 250 mM NaCl), autoclaved water, Lipofectamine 2000 (Invitrogen)

Note: Working aliquot of calcium chloride and water were stored at 4 °C for not more than a month. BBS was stored at -20 °C. About half an hour prior to use, calcium chloride and sterile water were warmed to 37 °C and BBS was thawed at RT.

**Protocol:** Cells at a confluency of 40-60% were transfected by calcium phosphate precipitation protocol as described [124] or with Lipofectamine 2000 as per the manufacturer’s protocol. Following is the composition of calcium phosphate transfection mixture in different culture dishes-

<table>
<thead>
<tr>
<th>Diameter of culture dish</th>
<th>DNA</th>
<th>Water</th>
<th>CaCl₂</th>
<th>2X BBS</th>
<th>Total volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 mm</td>
<td>5 μg</td>
<td>45 μl</td>
<td>50 μl</td>
<td>100 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>60 mm</td>
<td>10 μg</td>
<td>90 μl</td>
<td>100 μl</td>
<td>200 μl</td>
<td>400 μl</td>
</tr>
<tr>
<td>100 mm</td>
<td>25 μg</td>
<td>225 μl</td>
<td>250 μl</td>
<td>500 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>
3.2.3 Live cell confocal imaging

**Material:** Optimum Minimum Essential Media (Opti-MEM), buffered with HEPES and sodium bicarbonate, 35 mm glass-bottomed dishes (Cell E&G, USA)

**Protocol:** Cells were grown in the glass-bottomed dishes and were transfected at the confluency of 40-50% with the desired plasmids. Post 24-30 h of transfection, the complete medium was replaced with minimally fluorescent Opti-MEM. To monitor colocalization, confocal imaging was performed with Zeiss LSM 510 META equipped with 100× or 63× 1.4 NA (numerical aperture) objectives. Single- or dual-colour images were obtained using separate excitation in 12-bit format and with line averaging of two. GFP fluorescence was excited with a 488 nm argon laser and collected between 495 and 550 nm. Similarly, mCherry was excited with a 543 nm helium-neon laser and emission was captured between 650 and 760 nm. Transmitted light images were captured in the blue channel. Z-stacks were collected at intervals of 2–4 seconds and laser illumination was minimized to limit photodamage. Images acquired were further processed using LSM 510 image examiner software.

3.2.4 Preparation of whole cell lysate post transfection

**Reagents:** SDS lysis buffer (5 mM EGTA, 5 mM EDTA, 0.4% SDS and protease inhibitor cocktail in 25 mM Tris-HCl pH 7.2) and non-denaturing lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA)

**Protocol:** Post 30 h of transfection, the cells were spun at 1,000 rpm for 10 min at 4 °C. Cells were suspended in SDS or denaturing lysis buffer and incubated on ice for 40 min. The cell suspension was then subjected to ultra-sonication for 1 min, followed by centrifugation at 13,000 rpm for 15 min to separate cell debris. The supernatant and pellet were loaded on SDS-PAGE followed by Western blot analysis using respective antibodies.
3.2.5 **Protein estimation using Peterson method**

**Reagents:** Working stock of BSA (1mg/ml) prepared in sterile distilled water, Copper Tartarate Carbonate (CTC) reagent: CTC reagent was prepared by adding 20% Na$_2$CO$_3$, 0.2% CuSO$_4$, and 0.4% Sodium potassium tartarate in the ratio of 1:1:2.

Solution A: Prepared by mixing CTC, 10% SDS, 0.8 N NaOH, and deionised water in the ratio of 1:1:1:1 and Solution B was prepared by diluting Folin Ciocalteau’s Phenol Reagent with distilled water in the ratio of 1:6.

**Protocol:** One ml of 5 – 25 μg /ml of BSA standard tubes were prepared in duplicates along with the blank. For estimating the concentration of unknown proteins, 5 μl of the lysate was added to 995 μl of distilled water. 1 ml of solution A was added and the tubes were vortexed followed by incubation at 37 ºC for 10 min in dark. To each tube add 500 μl Solution B followed by incubation at room temperature (RT) for 30 min in dark. The absorbance was read at 750 nm, and the concentration of the unknown lysate was calculated by extrapolating the standard curve.

3.2.6 **SDS-PAGE**

**Reagents:** 30% Acrylamide (29.2% acrylamide (w/v) and 0.8% bis acrylamide(w/v)), 1.5 M Tris-HCl pH 8.8, 1 M Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED and 6X SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 25% Glycerol, 2% SDS, 0.5% bromophenol blue), Electrode buffer (5X): 15.1 g Tris base, 94 g glycine and 5 g of SDS were dissolved in deionized water to a final volume of 1 litre.

- **Molecular weight markers** – Fermentas page ruler prestained protein ladder-SM 0671
- **Coomassie staining solution** – 0.25% coomassie brilliant blue R 250, 45% methanol and 10% acetic acid in distilled water
- **Destainer** – 45% methanol (v/v) and 10% acetic acid (v/v) in distilled water
**Protocol:** The samples were separated on 6-15% SDS-PAGE depending on the molecular weight of the proteins being analyzed with 5% stacking gel.

### 3.2.7 Western Blotting

**Reagents:** 1X Transfer Buffer (190 mM glycine, 20% methanol, 0.05% SDS, 25 mM Tris base), Tris-buffered saline (TBS) – 150 mM NaCl, 10 mM Tris-HCl pH 8.0, Tris-buffered saline with 0.1% TWEEN-20 (TBST), Ponceau staining solution (0.2% ponceau stain in 5% acetic acid), Blocking buffer (3% BSA or 5% Milk in TBS), Antibodies dilutions were made in 1% BSA in TBS, ECLprime Kit (GE Healthcare), X-ray film (Kodak), Antibodies- anti-His antibody (Abcam, USA), anti-HPV18 E2 (sc-26939), anti-caspase-8 (1C12, Cell signaling), anti-FADD (sc-56093), APO 1–3 anti-Fas (clone CH11, 05-201, Millipore), anti-GFP (sc-9996), anti-β actin (AC-74, Sigma), secondary anti-HRPO mouse, rabbit, goat from Millipore and Sigma

**Protocol:** Proteins resolved on SDS-PAGE were transferred to the activated polyvinyl difloride (PVDF) membrane placed in form of the sandwich and wet electro-blotting was performed at constant voltage of 100V for 2 h. The transfer of proteins was visualized using reversible Ponceau-S staining. The membrane was incubated in blocking solution for 1 h at RT on a rocker. The blots were rinsed in TBST to remove residual BSA. After blocking, the membrane was incubated with diluted primary antibody for 1 h at RT on the rocker. The blot was then washed at least thrice with TBST for 5 mins each. It was then incubated with horseradish peroxidase (HRPO) conjugated secondary antibody (anti mouse/ anti rabbit/ anti goat) for 1 h at RT. The unbound secondary antibody was removed and the blot was washed thrice with TBST for 10 mins each. The blots were developed using ECL prime chemiluminescence reagent according to the manufacturer’s protocol and the signal captured onto X-ray films.
3.2.8 Cell death assays

a) Cell viability analyses by flow cytometry: The estimation of the viability of a cell population by flow cytometry is based on a simple yet powerful principle, that the dead cells are leaky. All the different methods for evaluating viability are based on either direct leak detection or measurement of a direct consequence of this leakage. Dyes are used that either do not leak into or out of viable cells. These methods can clearly identify whether the cells are damaged or dying but not completely dead.

Propidium iodide (PI) is a membrane impermeant dye that is generally excluded from viable cells. It binds to double stranded DNA by intercalating between base pairs. PI is excited at 488 nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. Because of these spectral characteristics, PI can be used in combination with other fluorochromes with same excitation wavelength such as green fluorophores including fluorescein isothiocyanate (FITC) and phycoerythrin (PE).

Reagents: 1X PBS, PI staining solution - 1mg/ml PI (Sigma) in PBS.

Protocol: Post transfection, cells were trypsinized, and washed twice with PBS. Approx. one million cells were re-suspended in 500 μl PBS containing 4 μg/ml PI, and analyzed by FACS Calibr (BD Bioscience) for GFP fluorescence and PI content. Percent of dead cells are the % of GFP and PI-positive cells /GFP-positive cells.

b) Caspase 8 activity assay: Caspase-8 is activated early in apoptosis and is involved in the proteolysis and activation of downstream executioner procaspase-3. To identify and quantify caspase-8 activity in apoptotic cell lysates, an upstream sequence of the site recognized by active caspase-8, IETD (Ile-Glu-Thr-Asp), is utilized as a basis for the highly specific caspase-8 substrate. The caspase-8 fluorimetric assay is based on the hydrolysis of the...
peptide substrate N-Acetyl-Ile-Glu-Thr-Asp-7-amino-4-(trifluoromethyl) coumarin (Ac-IETD-AFC) by caspase-8 resulting in the release of a 7-amino-4-(trifluoromethyl) coumarin (AFC) moiety. AFC release can be monitored in a spectrofluorometer at an excitation wavelength of 400 nm and an emission wavelength range of 480-520 nm.

**Reagents:** Caspase assay buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 0.5 % NP-40), Ac-IETD-AFC (Enzo Life Sciences)

**Protocol:** Cells were extracted in caspase assay buffer containing 0.5% NP-40. Caspase activity was measured in cell lysate containing 20 - 30 μg proteins with 0.002 μM of Ac-IETD-AFC in 100 μl of caspase assay buffer for 2 h at 37 °C. Ac-IETD hydrolysis was measured by monitoring emission at 510 nm on excitation at 405 nm.

c) **DNA ladder assay:** Apoptosis is characterized by the activation of endonucleases with subsequent cleavage of chromatin DNA into inter-nucleosomal fragments of roughly 180 base pairs (bp) and multiples thereof (360, 540 etc.). Separation of DNA fragments by agarose gel electrophoresis and subsequent visualisation by ethidium bromide staining results in a characteristic "ladder" pattern. While most of the morphological features of apoptotic cells are short-lived, DNA laddering can be used as final state read-out method and has therefore become a reliable method to distinguish apoptosis from necrosis.

**General Protocol**

1. Collect culture media; add 1 ml of trypsin to cell monolayer on 100-mm dishes, scrape the cells, harvest cells (culture media and cell monolayer) by centrifugation (2,500 rpm, 5 min), and wash cell pellets with 1X PBS

2. Add 100 μl of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl pH 7.5)
3. Centrifuge at 3,000 rpm for 5 min and obtain the supernatant

4. Add 10 µl of 10% SDS solution to pooled supernatant (final: 1% SDS), treat with 10 µl of 50 mg/ml RNase A (final 5 µg/µl) and incubate for 2 h at 56 °C

5. Add 10 µl of 25 mg/ml Proteinase K (final 2.5 µg/µl) and incubate for 2 h at 37 °C

6. Add 1/2 vol. (65 µl) of 10 M ammonium acetate and 2.5 vol. (500 µl) of ice-cold ethanol and mix thoroughly. Stand for 1 h in -80 °C (“ethanol precipitation”)

7. Centrifuge for 20 min at 12,000 rpm, wash the white pellet with 200 µl of 80% ice cold ethanol followed by air-drying for 10 min at room temperature

10. Dissolve the pellet in 50 µl of TE buffer

11. Determine the DNA concentration (Abs 260 nm), and resolve about 4 µg of DNA on 2% agarose gel.

### 3.3 Protein-protein interaction analyses

There are several techniques by which protein-protein interaction can be studied; however, the requirements of each of these methods are unique and dependent on many properties of the ligand and analyte. Usually a combination of techniques is necessary to validate, characterize and confirm protein interactions. Following were the techniques used for measurement of protein-protein interaction in our study:

#### 3.3.1 Pull-down assay

Pull-down assays are commonly used for qualitative measurement of interactions between two or more proteins. A "bait" protein is tagged and captured on an immobilized ligand (support beads) through an affinity tag such as GST, MBP or 6X His. This forms a complex, a "secondary affinity support", which is then incubated with cell lysate or [³⁵S] methionine
labeled or unlabeled proteins. Protein complexes are either eluted or directly loaded onto SDS-PAGE and are analyzed by staining, western blotting or autoradiography.

### 3.3.2 Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) is similar in methodology to pull-down assays because of the use of beaded support to purify interacting proteins. The difference between these two approaches, though, is that while pull-down assays use a bait protein to purify any proteins, co-IP uses antibodies to capture protein complexes in a lysate. In a typical experiment, cells are lysed and a whole cell extract is prepared under non-denaturing conditions. It is essential to use non-denaturing conditions in order to maintain any interactions that occur. An antibody specific to the bait is then added to the extract, forming a primary complex. This complex is then immobilized on protein A or protein G sepharose beads. Proteins that do not bind are removed by a series of washes. The protein complex is then eluted from the beads and dissociated by SDS sample buffer. Samples are then evaluated by SDS-PAGE followed by western blotting with specific antibodies against the binding partners.

### 3.3.3 Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) is the most quantitative probe available for measuring the thermodynamic properties of a protein-protein interaction. It measures the binding equilibrium directly by determining the heat evolved (exothermic) or absorbed (endothermic) on association of a ligand with its binding partner. In a single experiment, the values of the binding constant ($K_a$), stoichiometry (n), and the enthalpy of binding ($\Delta H$) are determined. The free energy ($\Delta G$) and entropy ($\Delta S$) of binding is then calculated using the equation:

$$\Delta G = -RT\ln K_a = \Delta H - T\Delta S$$  \hspace{1cm} (Eq.1)

(where R is the gas constant and T is the absolute temperature in Kelvin)
An isothermal titration calorimeter consists of two identical cells (reference cell and sample cell) composed of highly thermal conducting and chemically inert substance (Hastelloy alloy or gold), surrounded by an adiabatic jacket. The temperature difference between the two cells containing the macromolecule is detected and maintained using sensitive thermocouple circuits. A known concentration of ligand (loaded in the syringe) is titrated into the sample cell, in precisely known aliquots, which upon interaction results into heat dissipation or generation. Heat change during the reaction is quantified in terms of time-dependent input of power required to maintain equal temperatures between the sample and reference cells. In an exothermic reaction, feedback power of the sample cell is decreased because of increase in the temperature of sample cell while opposite occurs in case of an endothermic reaction.

We used an iTC200 system from Microcal (GE Healthcare), one of the most sensitive isothermal titration calorimeters available. The sample cell is only 200 µl. It can directly measure sub-millimolar to nanomolar binding constants ($10^{-7}$ to $10^{-9}$ M$^{-1}$).

### 3.3.4 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is one of the fastest methods that allow real-time, label free detection of bimolecular interactions. SPR phenomenon occurs when a polarized light, under conditions of total internal reflection, strikes an electrically conducting surface at the interface between two media. This generates electron charge density waves called plasmons, reducing the intensity of reflected light at a specific angle known as the resonance angle; in proportion to the mass on a sensor surface. The basic principle involves the changes in the reflective index as a result of absorption of molecules onto prefixed ligand.

The molecular interaction studies were performed using Biacore 3000 (GE Healthcare). In Biacore instruments, the sensor surface comprises a micro-fluidic flow cell (~ 20 - 60 nl).
Through this flow cell an aqueous solution (running buffer) is passed under a continuous flow rate (1 - 100 µl/min). To allow the detection of an interaction, desired molecule (termed as the ligand) is immobilized onto the sensor surface. Its binding partner (analyte) is then injected in aqueous solution (ideally with the same components and composition as the running buffer) through the flow cell, under continuous flow. As the analyte interacts with the ligand the accumulation of mass on the sensor surface causes a change in refractive index. This is measured in real time, and the result is plotted as response units (RU) versus time (termed a sensorgram). Importantly, a signal (background response) will also be generated if there is a difference in the refractive indices of the running and sample buffers. This background response must be subtracted from the sensorgram to obtain the actual binding response. The background response is recorded by injecting the analyte through a control or reference flow cell, which has no ligand or an irrelevant ligand immobilized to the sensor surface. The real time measurement of association and dissociation of a binding interaction allows for the calculation of association ($k_a$) and dissociation rate constants ($k_d$) and the corresponding equilibrium dissociation constants ($K_D$) can be determined from the ratio of the dissociation and association rate constants ($k_d/k_a$).

Detailed experimental procedures for each of these techniques are given in the respective chapters.

### 3.4 Biophysical characterization

Various biophysical techniques are used to structurally characterize a given protein in solution. The techniques used in the present work are described below:

#### 3.4.1 Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectroscopy measures differences in the absorption of left-handed versus right-handed polarized light which arise due to structural asymmetry. Once the protein
molecule differentially absorbs the left and right handed light, the emerging light is elliptically polarised. CD spectra in the far UV range (260-180 nm) can be analyzed for the different secondary structural components of the protein such as alpha helix, parallel and antiparallel beta sheet, turn, and others. Absorption minima at λ 208 nm and 222 nm indicate α-helical structure, whereas a minimum at λ 218 nm is a characteristic of β-sheets. The disordered secondary structural elements or random coil protein are characterised by a low ellipticity at 210 nm and negative band near λ 195 nm. Based on the high agreement between secondary structures derived from CD and X-ray crystallography, several algorithms have been developed to provide an estimation of the secondary structure composition of proteins from CD data. Widely used algorithms include SELCON (self-consistent), VARSLC (variable selection), CDSSTR, K2D and CONTIN. An online server DICHROWEB that provide flexibility of analyzing data by various algorithms and databases as well as using several reference sets is used for the data analysis. CD spectra therefore can rapidly determine the overall folding and secondary structural elements of the protein. The method is also very reliable for monitoring changes in the conformation of proteins under different conditions such as denaturation studies, unfolding experiments, mutational analysis, etc.

3.4.2 Fluorescence spectroscopy

Fluorescence spectroscopy, also known as fluorometry, exploits the phenomenon of fluorescence in which a molecule absorbs a lower wavelength photon, undergoes electronic excitation, and then emits longer wavelength. The fluorescence of a folded protein is a combination of the signal from individual aromatic residues with the major contribution from tryptophan residue. It can be an indirect probe of the proteins environment, describing the solvent-solute as well as inter- and intra-molecular interactions. After exciting a sample, a
‘Fluorometer’ allow us to obtain and measure various attributes including intensity, spectral wavelengths and lifetime.

Protein emission scans are acquired from \( \lambda \) 310 to 400 nm, following excitation at \( \lambda \) 280 or 295 nm. The \( \lambda_{280} \) monitors the environmental changes associated with tryptophan and tyrosine side chains due their absorption wavelengths at 280 and 275 nm, respectively. However, emission spectrum corresponding to \( \lambda_{295} \) selectively follows tryptophan emission because of least contribution from tyrosine. The fluorescence intensity and \( \lambda_{\text{max}} \) are monitored to study the protein-folding, ligand-binding and so on. Using these techniques structural alterations, protein unfolding was monitored and the melting temperature was determined for different proteins.

**a) Secondary structural analysis**

**Material:** Phosphate buffer (20 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\) buffer pH 7.8 with 20 \( \mu \)M \( \beta \)-mercaptoethanol), Quartz cuvette (1 mm), CD Polarimeter (Jasco, J815)

**Protocol:** Far-UV CD data of 10 \( \mu \)M protein in phosphate buffer were collected between \( \lambda \) 250 and 190 nm (Settings: Scan speed 20 nm/s, accumulation-3, data pitch 0.1, and temperature 25 ° C).

**Data analysis**

CD data from at least three independent experiments were used for data analysis. The spectra were plotted as ellipticity (0) on Y-axis and wavelength (nm) on X-axis. Data is represented in the form of the Mean Residual Ellipticity (MRE or [0]) given as \text{deg. cm}^2 \text{. mol}^{-1}, since the value is concentration independent and constant for a protein. Ellipticity was converted to mean residue ellipticity using the formula, \([0]_{\text{MRE}} = (\theta \times \text{MRW})/(10 \times c \times d)\)

where, MRW (Mean residue weight) = Molecular weight / (number of amino acids -1)
'c' is concentration of protein (mg/ml), d is the pathlength in cm

The data is then saved in Dichroweb format and subsequently analyzed by Dichroweb server (http://dichroweb.cryst.bbk.ac.uk).

**b) Thermal Denaturation**

Thermal stability of protein is an index of its overall stability. It can be assessed by monitoring the CD spectrum with increasing temperature. Far-UV CD region can be used to evaluate the secondary structural changes in proteins. The co-operative nature of melting curve shows that the protein is well folded.

**Reagents:** Phosphate buffer (20 mM Na₂HPO₄/NaH₂PO₄ buffer pH 7.8 with 20 μM β-mercaptoethanol)

**Protocol:** Thermal denaturation of wild-type and mutant proteins was done simultaneously using multi-cell cuvette holder. A Far-UV CD spectrum (λ 250 to 190 nm) was collected in a temperature range of 10 ºC to 90 ºC with an increment of 1 ºC/min. At each data point, the sample was equilibrated for 5 mins.

**Data analysis**

Ellipticity corresponding to 222 nm at different temperatures was obtained for calculation of melting temperature (Tₘ). Firstly, ellipticity of fully folded (θₕ) and unfolded forms (θₜ) were estimated using nonlinear regression (GraphPad Prism). This was then used to calculate fraction folded at any temperature (α) with the following formula,

\[ α = \frac{[F]}{([F] + [U])} = \frac{θₜ-θₜ}{θₚ-θₜ} \]

where [F] and [U] are concentration of folded and unfolded forms respectively, and θₜ is the observed ellipticity at a given temperature. To calculate the Tₘ, the fraction folded at given temperature was further analyzed using nonlinear regression (Igor Pro).
c) **Equilibrium unfolding studies**

**Reagents:** Phosphate buffer (20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.8, 20 mM β-mercaptoethanol), 10 M urea stock solution prepared in phosphate buffer. The solutions were made freshly for each experiment and were filtered (0.22 μm pore size) prior to use.

**Protocol:** The experiments were performed as described previously [125]. Briefly, a protein stock solution (ten times the final concentration to be used in the experiment) was prepared in phosphate buffer. Phosphate buffer, urea from 10 M stock solution, and protein solutions were mixed in 2 ml siliconized microcentrifuge tubes to obtain final urea concentration ranging between 0 M and 8 M. For renaturation experiments, the protein was incubated in 8 M urea-containing buffer. After incubation for 1 h at 25 °C, the protein was diluted in phosphate buffer such that the final urea concentration was as indicated in the figure legends. The samples were incubated for 20 h at 25 °C prior to data collection. The incubation time was such that it was sufficient to allow all samples to equilibrate.

Fluorescence emission at each urea concentration was measured using JOBIN YVON Horiba Fluorolog 3 spectrofluorometer. The measurements were acquired at excitation wavelengths of 280 nm and 295 nm with fluorescence emission in the range of λ 310 and 400 nm. Raw data corrected for buffer background was normalized between zero (unfolded) and one (native), as shown in Eq. 2, in order to visualize different spectroscopic signals on a single scale.

$$Y_{\text{Normalized}} = \frac{(Y_X - Y_U)}{(Y_N - Y_U)}$$  \hspace{1cm} (Eq. 2)

(where $Y_X$ is the signal being normalized, $Y_U$ is the signal of the unfolded protein, and $Y_N$ is the signal of the native protein).

Circular dichroism at 228 nm was measured with a Jasco J-815 spectropolarimeter using a cuvette of 1 mm pathlength. Both instruments were equipped with thermostated cell holders, and the temperature was held constant at 25 °C using a circulating water bath.
Data analysis

For equilibrium studies using fluorescence spectroscopy, average emission wavelength was determined for emission scans using the following equation,

\[ <\lambda> = \frac{\sum_{i=1}^{N} (I_i \lambda_i)}{\sum_{i=1}^{N} (I_i)} \]  
(Eq. 3)

where \(<\lambda>\) is the average emission wavelength and \(I_i\) is the fluorescence emission at wavelength \(\lambda_i\).

Fluorescence and circular dichroism data set were fitted using the three state (dimeric intermediate) or two state equilibrium model using Igor Pro (6.03A).

i) Three state dimeric intermediate model

\[ N_2 \rightleftharpoons I_2 \rightleftharpoons 2U \]  
(Eq. 4)

In this model, the protein is assumed to be either in the native homodimeric state (\(N_2\)), a non-native dimeric state (\(I_2\)), an unfolded monomeric state (U) and \(K_1\), \(K_2\) are the equilibrium constants for the two steps, respectively. If we consider the total molar concentration of the polypeptide chain as \(P_T\), as shown in equation 5,

\[ P_T = 2[N_2] + 2[I_2] + [U] \]  
(Eq. 5)

the mole fraction of each species can be defined as shown in equations 6-8.

\[ f_{N_2} = \frac{2N_2}{P_T} \]  
(Eq. 6)

\[ f_{I_2} = \frac{2I_2}{P_T} \]  
(Eq. 7)

\[ f_{U} = \frac{U}{P_T} \]  
(Eq. 8)

The sum of all fractions is equal to unity as shown in equation 9.

\[ f_{N_2} + f_{I_2} + f_{U} = 1 \]  
(Eq. 9)

The equilibrium constants \(K_1\) and \(K_2\) are related to the mole fraction of each species and to \(P_T\), as shown in equations 10 and 11.
Equating equations 9, 10, substituting in terms of $f_U$, and rearranging yields the following equation,

$$\frac{2f_U^2p_T}{K_1K_2} + \frac{2f_U^2p_T}{K_2} + f_U - 1 = 0$$  \hspace{1cm} (Eq. 12)

By solving the quadratic equation 11, the fraction of each species is obtained, as shown in equation 13-15

$$f_U = \frac{-K_1K_2 \sqrt{(K_1K_2)^2 + 8p_T(K_1K_2 + K_1^2K_2)}}{4p_T(1+K_1)}$$ \hspace{1cm} (Eq. 13)

$$f_{I_2} = \frac{2f_U^2p_T}{K_2}$$ \hspace{1cm} (Eq. 14)

$$f_{N_2} = \frac{f_U}{K_2}$$ \hspace{1cm} (Eq. 15)

From equations 12-14 and eq. 1, one can calculate the equilibrium constant and the values of $\Delta G$ at each urea concentration. We assume the free energy change for each step in the reaction to be linearly dependent on denaturant concentration as described earlier (Equations 16 and 17).

$$\Delta G_1 = \Delta G_1^{H_2O} - m_1 \text{ [denaturant]}$$ \hspace{1cm} (Eq. 16)

$$\Delta G_2 = \Delta G_2^{H_2O} - m_2 \text{ [denaturant]}$$ \hspace{1cm} (Eq. 17)

where $\Delta G_1^{H_2O}$ and $\Delta G_2^{H_2O}$ are the free energy changes in the absence of denaturant corresponding to $K_1$ and $K_2$ respectively, and $m_1$ and $m_2$ are the cooperativity indices associated with each step. The amplitude of the spectroscopic signal determined at each urea concentration is assumed to be a linear combination of the fractional contribution from each species (equation 18),

$$Y = Y_{N_2f_{N_2}} + Y_{I_2f_{I_2}} + Y_{Uf_U}$$ \hspace{1cm} (Eq. 18)
where $Y_N$, $Y_I$, and $Y_U$ are the amplitudes of the signals for the respective species.

In order to determine the unknown parameters $\Delta G_{1H_2O}$, $\Delta G_{2H_2O}$, $m_1$ and $m_2$, the data sets shown in figures 4.3.4 and 4.3.5 were fit simultaneously using Igor Pro.

ii) Two state monomer model

In this model, the protein is assumed to be either in the native monomeric state (N) or in an unfolded monomeric state (U), and $K$ is the equilibrium constant for the unfolding process.

$$N \leftrightarrow U$$  \hspace{1cm} (Eq. 19)

Therefore the total protein concentration is,

$$P_T = [N] + [U]$$  \hspace{1cm} (Eq. 20)

The equilibrium constant $K$ is related to the mole fraction of both the species and is given as,

$$K_1 = \frac{[U]}{[N]}$$  \hspace{1cm} (Eq. 21)

The mole fraction of each species can be defined as shown in equations 22 and 23,

$$f_N = \frac{1}{1+K}$$  \hspace{1cm} (Eq. 22)

$$f_U = \frac{K}{1+K}$$  \hspace{1cm} (Eq. 23)

Therefore, the fractional contribution from each species is given by the equation,

$$Y = Y_N f_N + Y_U f_U$$  \hspace{1cm} (Eq. 24)

(where $Y_N$ and $Y_U$ are the amplitudes of the signals for the respective species)

To determine the unknown parameters $\Delta G_{H_2O}$ and $m_1$, the data set was fitted simultaneously using Igor Pro.

3.5 Protein oligomerization and size characterization

An oligomer is a macromolecular complex formed mostly due to non-covalent bonding of the molecules, and may or may not have biological significance. Homo-oligomerization involves
assembly of identical molecules together, whereas, hetero-oligomer involves two or more different macromolecules. Protein oligomerization can be detected and characterized using different methodologies. However each technique has its own pros and cons, therefore several approaches should be combined to draw a conclusion. The methodologies used in the present work are described below:

3.5.1 **Size-exclusion chromatography**

Size-exclusion chromatography (SEC) is an analytical technique that separates dissolved macromolecules based on their size and shape (hydrodynamic radius). There are two basic types of size exclusion chromatography. One is gel permeation chromatography, which uses a hydrophobic column packing material and a non-aqueous mobile phase (organic solvent) to measure the molecular weight distribution of synthetic polymers. The other is gel filtration chromatography, which uses a hydrophilic packing material and an aqueous mobile phase to separate, fractionate, or measure the molecular weight distribution of molecules soluble in water, such as polysaccharides and proteins. Separation is carried out by means of a porous separation matrix with different sized cavities. Completely solvated polymer chains diffuse according to their hydrodynamic volume into the cavities of the separation matrix and are sorted in terms of their size.

The molecular weight of an unknown protein is calculated based on the time taken to move through the gel column (Superdex 75 or 200) as compared to the time by the mixture of standard known proteins such as alcohol dehydrogenase, bovine serum albumen, lysozyme, and MBP. The proteins with the higher molecular weights travel faster as they are excluded from the gel compared to low molecular weight proteins. Elution volume ($V_e$)/void volume ($V_0$) vs. log of molecular masses of standards was plotted to generate the calibration curve. The molecular weight of the unknown protein is determined from plot of the log molecular weight.
weight of the standard proteins versus $k_{av}$ ($k_{av} = V_e - V_0 / V_c - V_0$), where $V_e$ is elution volume of the protein, $V_c$ is the volume of the column, and $V_0$ or void volume calculated by determining the elution volume of blue dextran (1 mg/ml).

### 3.5.2 Glutaraldehyde cross-linking

Crosslinking is the process of chemically joining two or more molecules by a covalent bond. Crosslinking reagents such as glutaraldehyde contain two or more reactive ends capable of chemically attaching to specific functional groups (primary amines, sulfhydryls, etc.) on proteins. Two physically interacting proteins can be covalently cross-linked and the formation of bridge between two proteins suggests their existence in a close proximity.

**Reagents:** Freshly prepared 0.23% gluteraldehyde stock, phosphate buffer (pH 7.5), 2 M Tris-HCl (pH 8.0).

**Protocol:** Reaction mixtures with 20 μg of purified protein in 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (pH 7.5) was treated with freshly prepared solution of gluteraldehyde (final concentration 0.1%) for 2 min at 37 °C. This reaction was terminated by adding 5 μl of 2 M Tris-HCl, pH 8.0. Cross-linked product was mixed with equal amount of Laemmli SDS sample buffer and analyzed on 12% SDS-PAGE.

### 3.5.3 Dynamic light scattering

Dynamic Light Scattering (Photon Correlation Spectroscopy) is one of the most popular technique for measuring the size and distribution of molecules and particles typically in the submicron region. The Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein
relationship. Most of the "particle size analyzed" operate at 90° and use red light of wavelength 675 nm. DLS is also capable in measurements of several parameters such as molecular weight, radius of gyration, translational diffusion constant etc.

The molecular size estimation in the present work was done using Wyatt technology DynaPro particle size analyzer.

**Reagents:** Protein solution (1 mg/ml) in phosphate buffer

**Protocol:** Protein and buffer solution was filtered (0.44 µm pore size) and degassed prior to measurement. 1mg/ml protein in 10 mM Na₂HPO₄/NaH₂PO₄ buffer (pH 7.5), 100 mM NaCl was loaded into a 45 µl quartz cuvette. Measurements were performed at temperature 25 °C and at least 30 – 40 measurements each of 12 s duration were collected. The refractive index and viscosity values were taken for the water as provided by the software. The translational diffusion coefficient of the protein was calculated from the autocorrelation of scattered light intensity. Histogram analyses of DLS results were carried out using the software DYNAMICS v.6.0

### 3.6 Serine protease enzymatic assay

The protease activity of wild-type HtrA2 and its variants were determined using a generic substrate for serine proteases, FITC (fluorescein isothiocyanate) labelled β-casein (Sigma). FITC-fluorescence was monitored in a multi-well plate reader (Berthold Technologies) using excitation and emission wavelengths of 485 and 545 nm respectively. Reaction rates ($v_0$) were calculated using linear regression analysis. The steady-state kinetic parameters were calculated from the reaction rates by fitting the data to Hill form of Michaelis-Menten equation, velocity $= \frac{V_{\text{max}}}{1+(K_{0.5}/ \text{[substrate]})^n}$, where ‘$V_{\text{max}}$’ is the maximum velocity and
$K_{0.5}$ is substrate concentration at half maximal velocity using KaleidaGraph (Synergy software). All the experiments were done independently in triplicate.

3.7 Molecular Modeling, Docking and Simulation

Molecular modeling involves representation of three-dimensional structures of bio-molecules and their physico-chemical properties. It can be obtained using in-silico tools which produce, estimate and predict reasonable atomic level structures and related characteristics of molecules. It is based on the principle of molecular mechanics which is combined with various computational tools to obtain energetic and structural information of target bio-molecules using theoretical and experimental data. The entire essence of molecular modeling resides in the relationship between the microscropic and the macroscopic observations that belong to statistical mechanics. Macroscopic observations defined by solvation energy, hydrogen-hydrogen bond distance, affinity between two bio-molecules and molecular conformation show a great harmonization with average of observable over selected microscopic states. Thermodynamically determined macroscopic behavior of a system is equivalent to a quantity called the partition function ($Z$). The partition function is a rather very complex to compute therefore, generally a numerical approximations is considered.

$$Z=\sum_i (e^{-\beta E_i})$$

Numerical approximations involve the following contents:

1) Calculation of the system energy for microstate $i$ - performed using semi-empirical force fields by GROMOS/Ambcr/CHARMM.

2) Sampling of the microstates accessible in a given macroscopic state, i.e. micro-canonical sampling, canonical sampling and isothermic-isobaric sampling for fixed $N, V, E$; $N, V, T$ and $N, P, T$ systems respectively.
3.7.1 *Types of Interactions:* Different kinds of non-bonded interactions that are present in interacting molecules are described below:

1) **Electrostatic interactions**

Coulomb law: The attraction and repulsion between charged bodied is directly proportional to product of their charges and inversely to distance between them which is given as,

\[
V_{\text{Ele}} = \sum_{i>j} \frac{q_i q_j}{4 \pi \varepsilon r_{i,j}}
\]

where, \( \varepsilon \) represents dielectric constant (value 1 for vacuum, 4 - 20 for protein core and 80 for water), \( q_i \) and \( q_j \) are the charge on interacting bodies and \( r \) is the distance between them.

2) **van der Waals interactions**

It involves *attractive part* which exist due to induced-dipole/dipole and *repulsive part* due to Pauli’s Exclusion Principle. It is usually represented by the Lennard-Jones potential which is obtained from the single atom parameter \( \varepsilon \) and \( \sigma \).

3) **Hydrogen bonds**

Interaction of the type is D-H \( \cdots \) A. The origin of this interaction is a dipole-dipole attraction.

Typical ranges for distance and angle: 2.4 - 4.0 Å (D-A) and 90º- 180º (D-H\( \cdots \)A).

4) **Hydrophobic interactions**

This interaction exists due to combined effect of contact between water, polar medium and hydrophobic groups, and is usually present with in a distance of 4.7 Å.

**Docking:** The interaction between bio-molecules such as carbohydrates, lipids, nucleic acids and proteins play an essential role in cellular processes. Furthermore, the binding orientation of the two molecules may affect the type of signal produced such as agonism and antagonism. Docking is a computational simulation method of predicting the preferred orientation of one molecule to a second in a stable complex. The relative preferred orientation may be used to...
determine the strength of association between two molecules. It aims to reach an optimized conformation and relative orientation for both the protein and ligand such that the system attains a lowest energy state.

Docking efficiency can be evaluated from a particular pose by counting the number of favorable intermolecular interactions such as hydrophobic contacts and hydrogen bonds. This method is most widely used for classifying a ligand which interacts favourably to a particular receptor, based on the predicted free-energy of binding. Docking is also very frequently used to predict the association of drug and receptor targets in order to determine the binding affinity and molecular kinetics.

**Docking model:** Earlier assumption about docking was a *lock-and-key* mechanism between receptor and ligand in which only the favorable conformation of both interacts and form a stable complex. However, soon the concept adapted to a more feasible aspect of *induced fit* model in which the ligand and the protein adjust their conformation to achieve an overall "best-fit". Due to the flexible nature of both the ligand and protein, the later model is widely accepted but not necessarily applicable to all proteins and ligand.

**Molecular dynamics (MD):** It is a computer simulation that allows interaction of atoms and molecules for a period of time, providing a view of their physical movements. The trajectories of atomic and molecular motion are determined by considering forces between the particles and potential energy as defined by molecular mechanics force fields. MD simulation use numerical methods to find the properties of molecular systems. In simulation of docking process, the protein and the ligand are physically separated, and then ligand is allowed to find its position into the protein’s active conformation. The moves incorporate internal changes to the ligand’s structure, translations and rotations. The advantage of
docking simulation is that it more accurately models reality, and ligand flexibility is easily incorporated.

### 3.7.2 General steps for molecular modeling, simulation and docking:

1. **Crystal structures retrieval** from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) which can be used as a model template.

2. **Modeling**: Homology modeling or *ab-initio* modeling

3. **Model energy minimization**: Energy level is the most basic property of molecules that can be calculated using three major theoretical methods, (i) molecular mechanics, (ii) semi-empirical (quantum mechanics), (iii) *ab-initio* (quantum mechanics) approach. Energy minimization is the first step carried out for geometry optimization of the molecular structure.

4. **Dynamics simulation and conformation search**: Integration of molecular dynamics (solving Newton’s law of motion for the nuclei) for all atoms in the system generates molecular trajectories. Conformation search of bio-molecules is carried out by repeating the procedure of rotating dihedral angles to achieve lowest energy conformations of molecular systems.

5. **Calculation of molecular properties**: Some physicochemical properties such as thermodynamic quantities, solubility, molar volume, heat capacity, molar refractivity density, dipole moment, magnetic susceptibility, partial atomic charge, ionization potential, electrostatic potential, solvent accessible surface area and van der Waals surface area are computed.

6. **Structure superposition and alignment**: It involves comparison of series of homologous molecules to get the best energy minimized model, which requires superposition or alignment of structures.
7. **Molecular interactions, docking**: In intermolecular interaction studies, usually the receptor (e.g., protein) is kept rigid or partially rigid while the conformation of ligand/protein is dynamic. Various docking software available includes AUTODOCK, HAADOCK, PATCHDOCK, ClusPro, etc.