IIT Guwahati Lecture 25

Course BT 631

Protein Structure, Function and Crystallography

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Combinatorial strategies for X-ray Crystallography

Combinatorial strategies at DNA Level

- The targeted design at the DNA level demands that at least some insight about where and which nucleotide to mutate, which is always not Known.
- In such cases Combinatorial Design is used to produce a library of random DNA constructs which need to be screened for expression, solubility, crystallisation and diffraction.
- This process requires extensive screening of colonies and may require use of robots.

Combinatorial libraries can be produced by

i) Random truncation,

ii) Fragmentation of DNA and

iii) Directed evolution methods

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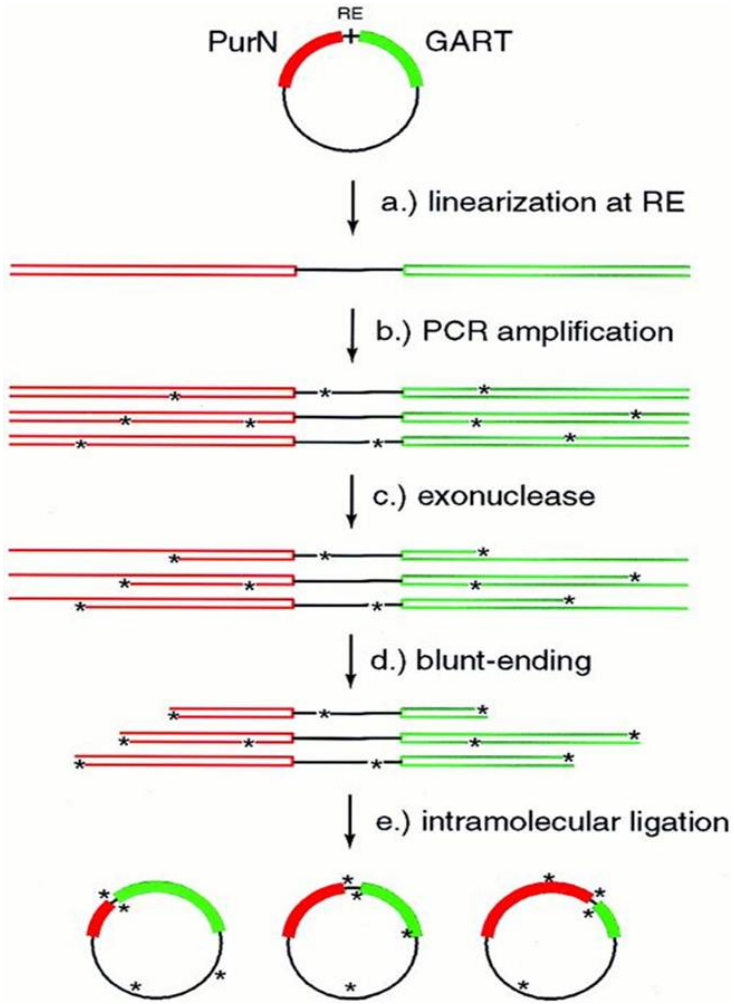
i) Random Truncation

- Generation of such random unidirectional truncation libraries involves enzymatic digestion of PCR amplified DNA by exonuclease III followed by treatment with 5' nuclease (such as mung bean nuclease) to remove any overhangs.
- The truncated fragments are then ligated to a vector. A screening method is also required to select colonies.
- The positive clones are then sequenced to determine the clones containing sequences which are in reading frame.
- The library can now be transformed into expression host. Alternatively clones can be directly screened by checking for expression followed by sequencing.



Construction of random truncation libraries

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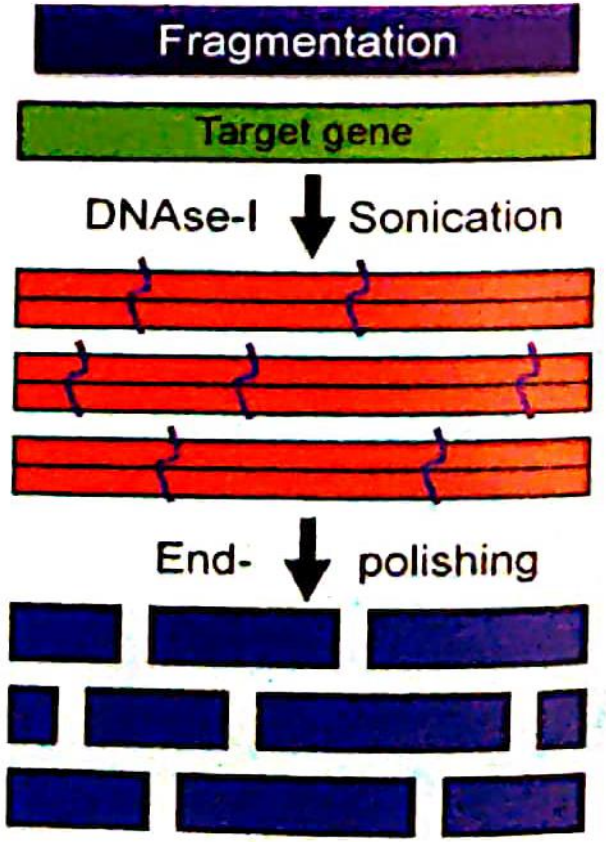
ii) DNA fragmentation libraries

- When expanse of domains can not be established by sequence analysis or enzymatic proteolysis, random fragmentation can be used to produce stable and folded proteins.
- DNA is fragmented by sonication and/or enzymatic treatment with DNaseI followed by treatment with a polymerase with proofreading activity.
- The colonies are then screened for soluble protein expression.



- Target DNA is randomly fragmented by DNaseI or sonication.
- Fragments are then made blunt ended and transformed.
- Only few will contain expressing and folding domains.
- So extensive screening is required.

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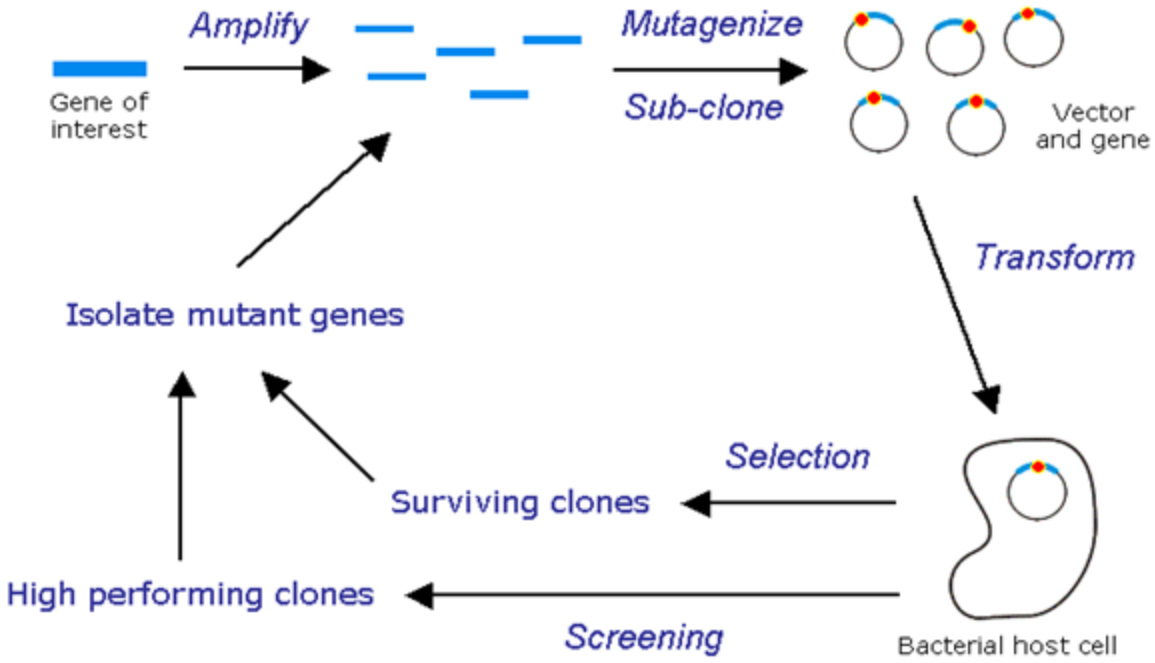
iii) Directed Evolution

- A library of random point mutations is constructed by using error prone PCR by recruiting special mutazyme polymerases in lieu of high fidelity proofreading enzymes.
- Point mutation may be introduced by using mutator strains deficient in DNA repair genes.
- The library is screened for a phenotype marker, for instance, expression level or solubility.
- The selected mutants produced by error prone PCR are fragmented using DNAasel and reassembled by PCR.



Directed evolution by Random mutations

- The mutant construct produced by directed evolution has been used to produce improved Green Flourescent Protein (GFP) variants.

Combinatorial strategies for X-ray Crystallography

Modifications at the protein level

A) At the protein level modifications are usually enzyme mediated e.g.

i) Limited proteolysis

ii) Deglycosylation

B) Mild chemical treatments like surface lysine methylation are also used.

The modifications at this level are mostly applied only when it is established that the purified protein does not crystallize.

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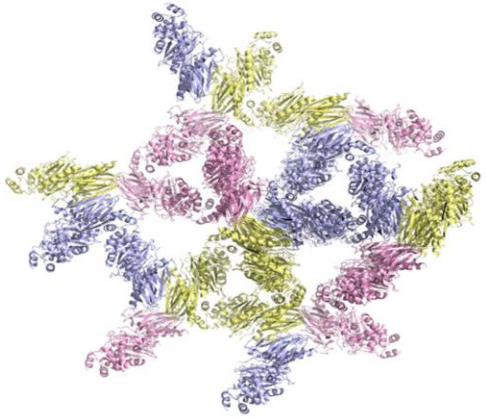
i) Limited Proteolysis

- Proteases require that target peptides adapt and bind according to the specific stereochemistry of the protease, thus ensuring that only flexible regions of disordered stretches or exposed loops are cleaved.
- It has been also used for domain analysis by cleaving flexible linkers and connecting multiple domains of a protein molecule.
- Human lipoprotein E crystallised and diffracted well, when C-terminal domain and N-terminal disordered linkers were removed. However, large disordered regions are not always a problem as there are proteins that have been studied without removing the terminal Histidine tag residues forming a disordered region.
- This enzymatic treatment has been used for removal of flexible stretches of residues to generate more compact and homogeneous molecules. e.g.

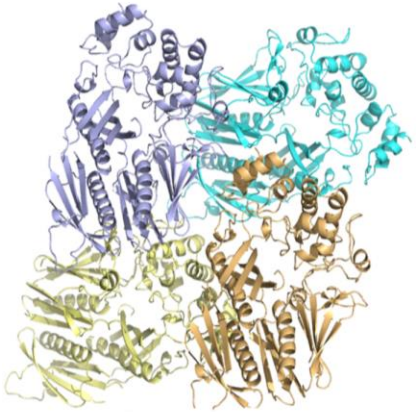
Crystal structure of minor pseudopilin (secretary protein) ternary complex of XcpVWX from *Pseudomonas aeruginosa*



Hexagonal structure (Less compact)

In situ proteolysis by Chymotrypsin

Removal of flexible regions



Orthorhombic structure (More compact)

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Deglycosylation

- Proteins glycosylation is fairly common in eukaryotes, which causes problem during crystallisation due to the presence of flexible surface containing oligosaccharides.
- Glycosylations are mostly heterogeneous and vary depending on host. Glycosylations may be N-linked occurring at Asn residues of tripeptide consensus Asn-X-Ser (X-non proline residue), while O-linked glycosylation occurs at Ser and Thr.
- Mild enzymatic deglycosylation is used to obtain conformationally homogeneous glycoprotein. e.g. Endoglycosidases (release oligosaccharides) are frequently used for truncation of N-linked glycosylations.
- Site-directed mutagenesis at the DNA level to convert Asn residue from Asn-X-Ser sequence to Asp removes N-linked glycosylations completely.
- Expression in glycosylation deficient host cell lines and use of glycosylation inhibitors in growth medium have also been used.

Chemical treatment by Lysine methylation

- The technique of chemical modification of side-chain has been used to improve crystallisation as well as to induce proteins for crystallisation in cases of non-crystallisable proteins.
- Lysine methylation also occurs naturally during post-translational modification in eukaryotic cells.