Name

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Subject

Bt 511T

Complete handouts final team [Word to word]

Prepare by Muhammad Abdullah

Lesson # 73

Cloning genomic DNA

PCR as an alternative to genomic DNA cloing

- PCR with specific primers could be used be used to isolate genes directly from genomic DNA, obviating the need for the production of genomic libraries.

Lecture # 74:-

Properties of cDNA:-

- cDNA is prepared by reverse-transcibing cellular RNA.
- cDNA lack introns and other non-coding sequence present in the corresponding genomic DADNA.
- Introns are rare in bacteria but occur in genes of higher eukaryotes.

Lecture # 75:-

CDNA libraries cloning:-

- cDNA library is a combination of cloned cDNA fragments inseted into a fragments inseted into a collection of host cells.
- cDNA library is representative of the RNA population from which it was derived.

Lecture # 76:-

Preparation of cDNA for libary construction.

- The cDNA synthesis reaction.
- Development of cDNA cloning strategies.

Lecture # 77:-

Improved method for cDNA cloning.

- A serious disadvantage of the hairpain method is that cleavage with S_1 nuclease.

result in the loss of sequence at the 5' end of the clone.

- This strategy has therefore been superseded with other methods.

Lecture # 78:-

PCR as an alternative of cDNA cloning:-

- Reverse transcription followed by the PCR (RT-PCR) leads to the amplification of RNA sequence in cDNA form.
- RT-PCR is a sensitive means for detecting, quantifying and cloning specific cDNA molecules.

Lecture # 79:-

Screening strategies:-

- Major screening strategies involved.

1). Genetic methods.
2). Sequence-dependent screening
3). Screening expression libraries.

Lecture # 80:-

Screening by hybridization

- Nucleic acid hybridization is the most commonly used method of library screening
- Grunstein and hogness [1975] develop a screening procedure to detect DNA sequence in transformed colonies by in situ ~~by~~ hybridization.

Lecture #75:- [Remaining Part]

cDNA libraries:-

- Phage - λ vectors for cDNA cloning and expression
- λgt10 and λgt11 vectors.

Lecture # 77:- [Remaining Part]

Improved method for cDNA cloning:-

- For cDNA expression libraries, it is advantage if the cDNA can be inserted into the vector in the correct orientation.
- This can be achieved by self-priming method by adding linker molecule to double stranded cDNA.

Lecture # 79:- [Remaining part]

Genetic method:-
Selection for the presence of vector:-

- All useful vector molecules carry a selectable genetic marker or property.
- Plasmid and cosmid vector carry drug resistance or nutritional marker
- In phage vectors, plaque formation it itself the selected property.

Lecture # 80:-

Screening by hybridization:-

- The result of the hybridization can be monitored by autoradiography.

Lecture # 81:-

Sequence-dependent screening Benton and davis plaque lift procedures.

- Benton and davis [1977] devised a method called plaque lift, in which nitrocellulose filter is applied to the upper surface and agar plates, making direct contact b/w plaques and filter.
- Number of alternatives labeling methods are avaible that avoid the use of radioactivity. It incuded digonigenin or bi biotin.

Lecture # 82:-

Probe design:-

- A great advantage of hybridization for library screning is that it is extremly versatile.
- Conditions can be used in which hybridization is very stringent, so that only sequence indentical to the probe are identified.

Lecture # 83

Chromosome walking

- Walking along the chromosome is a term used to describe an approach which allow the isolation of gene sequence whose function is quite unknown but whose genetic location is known.
- A clone genomic fragment must be found as starting point for the walk.
- A cloned genomic fragment must be found as starting point for the walk.
- In human genome, the starting point may be restriction fragment length polymorphism (RFLP) sequence that is closely linked to disease locus.
- One drawback to this method is the requirement that each DNA segment used is not required repeated elsewhere in the genome.

Lecture No 84

Chromosome jumping

- In chromosome jumping the DNA of

interest is identified, cut into fragment which restriction enzyme and circularized.

- It bring together DNA sequence that were originally located a considerable distance apart in the genome.
- These cloned DNAs from the ~~culture~~ closure site make up a jumping library.
- One of the application of chromosome walking and jumping is in the cloning of human cystic fibrosis gene.

Lecture # 85

Screening by PCR

- The PCR is widely used to isolate specific DNA sequences from unclored genomic DNA or cDNA but it also a useful technique for library screening.
- To isolate specific clone, PCR is carried out with gene-specific primer that flank a unique sequence in target.

- There are several application where the use of degenerate primer is favorable.
- A degenerate primer is a mixture of primer, all of similar sequence but with variation at one or more position.

Screening expression libraries:

Lecture # 86

Expression cloning

- If DNA library is established using expression vectors, each individuals clone can be expressed to yield a polypeptide.
- Expression libraries are useful because they allow a range of alternative technique to be employed.

Lecture # 87

Immunochemical Screening

- Immunochemical screening involves the use of antibodies that specifically recognize antigenic determinants on the polypeptides synthesized by a target clone.
- The molecular target for recognition is generally an epitope.
- The method of Broome and Gilbert [1978] exploited the fact that antibodies absorb very strongly to certain types of plastic such as polyvinyl and that IgG antibodies can be readily labelled with ^{125}I by iodination in vitro.

Lecture # 88;

Immunochemical screening of lambda gt11;

Immunochemical screening of λgt11;

- It is must convenient to use bacteriophage -λ insertion vectors, because these have a higher capacity and the efficiency of in vitro packaging allows large numbers of recombinent to be prepared and screened

- The original detection method using iodinated antibodies has been superseded by more convenient methods using non-isotopic labels, which are also more sensitive and have a lower background of non-specific signal.

The original detection method using digoxigenated antibodies has been superseded by more convenient method using non-isotopic labels, which are also more sensitive and have a lower background of non-specific signal.

Lecture # 89

South-western and north-western blotting

- It involve the screening and isolation of clones expressing sequence specific DNA or RNA-binding proteins.
- Screening is carried out without using an antibody by incubating the membrane with radiolabelled double standed DNA probe.

Lecture #90

Screening by functional complementation

- Functional complementation is the process by which a particular DNA sequence compensates for a missing function in a mutant cell and thus restores the wild type phenotype.
- Functional complementation is also possible in transgenic animals and plants.
- It has been used for complementation in transgenic mice to isolate the shaker-2 gene.

Functional complementation

Lecture # 91

Cloned DNA in E. coli

Expression in E. coli of Requirement for expression

- Synthesis of functional protien depend upon transcription of appropriate gene, efficient translation of mRNA and in many cases, Post-Translational Processing and compartm compartmentalization of nascent polypeptide.

Lecture # 92

Secretion of Protien

- Gram-negative bacteria such as E. coli have a complex wall-membrane structure comprising an inner, cytoplasmic membrane separate from an outer membr... by a cell wall and the Periplasmic space.

Lecture #93

Protien Trafficking

- The bacterial inner membrane, periplasmic space and outer membrane all contain protien not found in the cytoplasm.
- All of these protien are first synthesized in the cytoplasm but somehow find their way out.
- There are actually several general mechanisms of protien export in bacteria
- Each system transport a particular type of protien.

Stability of foreign Protiens in E.coli;

- Various strategies have been developed to cope with the instability of foreign protien in E.coli
- In this case of somatostatin, degradation was prevented by producing a fused

Protein consisting of somatostatin and the β-galactosidase.

Maximizing the expression of cloned gene;

Constructing the optimal promoter; and [Lecture # 95] as well

- Large number of promoter for E. coli have been analysed;
- Many promoters has led to the formulation of a consensus sequence which consists of the -35 region [5' TTGACA] and -10 region or Pribnow [5' TATAAT-3].
- Expression from a strong promoter can be the represent 20-40% of cloned gene product of total cell protien.

Optimizing translation initation; and [Lecture # 96] as well.

- Complementarity of shine-Dalgarno [S-D] sequence with 16S rRNA can affect the rate of translation.

- Composition of tiplet immediately preceding the AUG start codon also affect the efficiency of translation.

Stability of mRNA and codon choice;- and [Lecture # 97]

- The rate of synthize of particular protien will depend on the steady-state of mRNA in the cell.
- Degradation of mRNA usually proceeds by a combination of endonuclease and 3'- exonuclease attack.

The effect of plasmid copy number;- and [Lecture # 98]

- The number of ribasomes in a cell far exceeds any one class of mRNA
- One way of increasing expression of a cloned gene in to increase the number of the cossponding transcipt.

[Lecture #99]

Plasmid Stability :-

- Having maximized the expression of a particular gene it is important to consider what effect this will have on the bacterium harbouring the recombinant plasmid.
- Loss of plasmid due to defective partitioning is called segregative instability.

Stuctural instability of plasmid may arise by deletion or reaarangments of DNA.

Host cell Physiology can affect the level of expression;

- Factors which will be important include the choice of nutrients and environmental parameters.

Lecture # 102

Analysing DNA Sequence

DNA sequencing:

Benefits and applications:

- DNA sequence information is a prerequisite for planning any substantial manipulation of DNA
- Information is used to various fields i-e molecular and evolutionary biology. Metagenomics, medicine, forensis etc.
- Technique for DNA sequences become available in the late [1970s]

Lecture # 103:

Maxam-Gibert method

This method for DNA sequencing makes use of chemical reagents to being about base-specific cleavage of the DNA.

Lecture # 104;

Chain termination or dideoxy Prodn Procedure ;-

- This method was developed by sanger et al. [1977]
- It capitalizes on the ability of DNA Polymerase to incorporate dideoxynucleoside triphosphate as substate.
- DNA synthesis is carried out in the presence of four deoxynucleoside triphosphates, one or more of which is labelled with ^{32}P and in four seperate incubation mixes.

Lecture # 105

Modification of chain terminator sequence.

- Improvements to original sanger's method have been made by replacing the

- Klenow fragment of E.coli DNA Polymerase I
- The combination of chain terminator and M13 vectors to produce single standed DNA is very powerful.

Lecture # 106

Automated DNA Sequence:

- To automate the process, it is desirable to acquire sequence data in real time by detecting the DNA bands within gel during the electrophoretic saperation.
- Automated DNA sequencing offering number of Advantage.

Lesson # 107:

Sequencing accuracy

- Sequences that were read beyond 400bp contained an average of 3.2% error. while

these are less than 400 bp had 2-8% error.

Lesson No 108;

DNA sequence Databases

- Since the development of current DNA sequencing technology large amount of sequence data has accumulated that is maintained in 3 data bases.
- National centre for Biotechnology Information.
- DNA Databank of Japan
- European Bioinformatic institute - UK.

Lesson # 109 / Changing gene: site-directed Mutagenesis

- Mutagenesis is a process to change the genetic information of an organisms.
- It can be occure naturally, or as a result of exposure to mutagens or induced the experimentally in laboratory.

- Three different methods of site-directed Mutagenesis has been devised
  - i- Cassette mutagenesis
  - ii- Primer extension
  - iii- Procedures based on PCR

Lesson # 110

Cassette mutagensis

- A synthetic DNA fragment containing the desired mutant sequence is used to replace the corresponding sequence in the wild-Type gene.

Lecture # 111

Primer extension: the single primer method

- It involves priming in vitro DNA synthesis with a chemically synthesized oligonucleotide [7-20 bp] that carries a base mismatch complementary sequence.

- After E.coli transformation, heteroduplex that are formed are either that of orginal wild type DNA or that containing the mutated base.

Lecture # 112;

PCR method for site-directed mutagenesis

- Single bases mismatched between the amplification primer and the template become incorported into the template sequence as a result of the amplification.
- In megaprimer method, the Product of the first PCR is used for second PCR.
- The advantage of a PCR based mutagenic Protocal is that the desired mutation is obtained with 100% efficiency.

Lecutie #113

Polymerase chain reaction

Basic PCR reaction;

- PCR. is a technology in molecular biology is used to amplify a single or a few copies of target DNA across several orders of magnitude, generating millions of copies of particular DNA sequence.
- PCR can be also used to generate DNA fragment for gene cloning by using primer with specific restriction sites.

Lecture #114

PCR Principal and Procedure

- PCR amplifies a specific target region of DNA that may range from a few to several Kilo

base pairs.

- Basic reaction requires several components.
- PCR cycles may consists of several steps of denaturation, annealing and extension.

Lecture # 115

DNA Polymerases

- DNA Polymerase are enzyme that synthesize DNA molecule by adding nucleotide, the building blocks of DNA.

Taq DNA Polymerase

- Taq Polymerase is a thermostable DNA Polymerase isolated from thermophilic bacterium *Thermus aquaticus* and is used in PCR to amply DNA fragment.

Lecture # 116

Mapping and sequence genomes

Genome Mapping

- Assigning of a specific gene to particular region of a chromosome and determining the location of and relative distance between gene on the chromosome

Types

1- Linkage maps
2- Physical maps.

Lecture # 117

Markers for genome mapping

- Any identifiable feature of the genome can serve as a marker in mapping
- The landmark on genome map might include short DNA sequence, regulatory sites and genes themselves.

Lecture # 118

Method for genome mapping

Genetic linkage mapping;

Genetic linkage maps illustrate the order of genes or genetic markers on a chromosome and the relative distance between those genes.

Lecture # 119

Physical Mapping

Physical maps gives the DNA base pair distance from one landmark to the another.

Lecture # 120

Physical Versus linkage maps;

- Chromosomal maps of bacteria has been obtained by combining the mapping technique of interrupted mating, recombination mapping, transformation and transduction

Lecture # 121

The use of RFLPs in Physical maps

- RFLP-Restriction fragment length of Polymorphism exploits variations in the homologous DNA sequence.
- Botstein et al. [1980] were the first to recognize that DNA probes that target RFLPs can be used for mapping.

Lecture #122

STS in Physical maps

Sequence-tagged sites [STS] can more convenient markers than RFLPs because they do not use southern blotting.

Lecture #123

SNPs as Physical markers

Single nucleotide Polymorphisms [SNPs] are single base-Pair Position in genomic DNA at which different sequence alternatives [alleles] exist in a Population

SNPs Prop Probably are the most important sequence markers for Physical mapping of genomes.

Different method to detect SNPs have been developed that based on enzymatic, electrophoretic, solid phase or the chromatographic analysis.

Lecture # 124

Polymorphic DNA detection in the absence of sequence information

- Polymorphic DNA can be detected by amplification in the absence of the target DNA sequence information used to generate STSs.

Lecture # 125

Fluorescence in situ hybridization [FISH]

- FISH is a cytogenetic method that used to fluoresent probe bind to only those parts of chromosome with a high degree of sequence complementarity.

Lecture #127

Happy Mapping

- RH mapping have a few problem that arise from the in vivo step. Happy mapping overcomes these limitations, being an entirely in vitro techniques.

Lecture #128

Map integration

- It is essential that the different mapping methods are integrated as each has its own strengths and weaknesses.

Lecture #129

Sequencing genome

- The process of determining the exact order of nucleotides within a DNA or RNA molecules is called sequencing.

In 1970s DNA sequencing was a very tedious process which involved determining only a few hundred nucleotide at a time.

Lecture # 130

High-Throughput Sequencing

Recent advances in DNA sequencing have make it possible to sequence data very rapidly and at a substantially lower cost. It mainly describe a number of different modern sequencing technologies to sequence genome.

Lecture # 131

Shotgun Sequencing

Shotgun sequencing is used for sequencing long DNA stands.
In this strategy, DNA shredded into smaller fragments that can be sequenced individually and then reassembled into original order.

Lecture # 132

Clone-by-clone sequencing

In clone-by-clone sequencing, a map of each chromosome of the genome is made before the DNA is split up into fragments for sequencing.

Lecture # 133

Comparative genomics

- **Orthologs and Paralogs**

Orthologs are homologous gene in different organisms that encode protiens with the same function and which have evolved by direct vertical descent.

Lecture # 134

Functional genomics & Proteomics;

- **DNA Microarrays;**

Microarrays is used to study the expression of many genes at once. It involves the immobilization of thousand of gene sequences on glass slide

that are then detect with complementary base pairing between the sample and the gene sequences on the chip.
Two major types of DNA array are used in expression analysis;

i- Spotted DNA arrays
ii- Printed oligonucleotide chips.

Lecture # 135

Spotted DNA Arrays

Spotted DNA arrays are produced by printing DNA samples on treated microscope slides. It is made by transferring or spotting DNA clones or PCR product individually onto a solid support where they are immobilized.

Lecture # 136

Oligonucleotide chips

- Oligonucleotide chips are manufactured by in situ oligonucleotide synthesis.

Lesson # 137

Application of microarrays

Microbial gene expression analysis

- The predominate application of DNA microarray has been to measure gene expression levels.

Lecture # 138

Profiling in human disease

- Arrays have been used to investigate the transcriptional profiles associated with human disease and to identify novel disease markers and potential new drugs targets
- **Genotyping;** Microarrays have been widely used as single nucleotide polymorphism [SNP] genotyping platforms.

Lecture #139

Phage display

- Phage display is the technology that allows expression of exogenous polypeptide on the surface of phage particals.

Lecture # 140

Screening Phage display libraries

- The most common screening method is based on enriching the phage clones with binding affinity for the target by a process called Biopanning.

Lecture # 141

Application of Phage display.

- Application of phage display include determination of protien to protien ~~interction~~ interactions, to determine enzyme specificity and to generate target specific antibodies.

Lecture # 142

Knock outs

- A gene Knock out is a genetic technique in which one of an organisms's gene is made inoperative that is simply called as Knocked out
- In molecular cloning, a Knock-in refers to gene manipulation method that involve the insertion of a Protien coding cDNA sequence at a particular locus in a organisms's chromosomes.

Lecture # 143

SiRNA technology

- Small interfering RNA [siRNA] is most commonly used RNA interference [RNAi] tool for inducing short-term silencing of Protien coding gene, siRNA is a synthetic RNA duplex designed to specifically target

a particular mRNA for degradation.

Lecture # 144

Application of siRNA

- siRNA] is the method of choice to target specific gene for silencing and has provided immense potential as therapeutic tools.

Topics which are not in each lecture slide but include in handouts.

Cloning DNA Libraries;

Cloning genomic DNA;

- Producing representative genomic libraries in λ cloning vector
  Cloning of large DNA fragments of 20Kb in λ replacement vector, fewer clones are required for ready complete library.
  How to appropriately size random fragments be products?
- Average fragment size can be controlling by mechanical shearing
  More commonly used procedure is restriction endonucleases.

λEMBL vectors for genomic library construction

- High molecular weight genomic DNA is digested with Sau3AI and subsequently ligated in λEMBL vector digested with BamH1

Sequence-dependent screening
Lecture #80

Screening by hybridization

- Nucleic acid hybridization is most commonly used method of library screening Grunstein and Hogness [1975] developed a screening ~~Prod~~ procedure to ~~dete~~ detect DNA sequence in transformed colonies by in situ hybridization.

Expression in E.coli of cloned DNA

Stability of foreign Protien in E.coli;

- Various strategies have been developed to cope with the instability of foreign protien in E.coli
- In the case of somatostatin, degradation was prevented by producing a fused protien consisting of somatostatin and β-galactosidase.

PCR

- **Primers;**

  Primer is a short stand of oligonucleotide that serves as a starting point for DNA synthesis

- **Degenerate Primer**

  It is actually a mixture of primer, all of similar

sequence but with variation at one or more positions.

Types of PCR:

Recently, varient have been developed from basic PCR method to improve performance and specificity and to amplify other molecule such as RNA.

- **RT-PCR:-**

Reverse Transcriptase was designed to amplify RNA sequences [mainly mRNA] through synthesis of cDNA by reverse transcriptase.

- Various strategies can be adopted for first stand cDNA synthesis
- **Competitor RT-PCR:**

One use of RT-PCR is in determining amount of mRNA in a sample. [Competitor R-T PCR]

Real time Quantitative PCR [qPCR]

- qPCR used to measure quantity of a target sequence
- It is used to measure starting amount of DNA, CDNA, RNA

Nested PCR

Two sets of primer is used in two successive PCRs.
First set of primers allows first amplification
Product of first PCR is subjected to second PCR using second set of primers.

Inverted PCR;-

Used to identify the flanking sequences around genomic inserts.

Multiplex PCR;-

Used to amplify several different DNA sequence simultaneously.

- **RAPD**

Random amplification of Polymorphic DNA is a type of PCR reaction but the segment of DNA that are amplified are random

- **RFLP:**

Restriction fragment length Polymorphism exploits variation in the homologous DNA sequence.

- In RFLP, DNA sample is digested by restriction enzyme and saperated by gel electrophoresis.

- **AFLP-PCR:-**

Amplified fragment length Polymorphisms are different in restriction fragment length caused by SNPs or INDELS

- AFLP is a PCR based tool used in genomic engineering.

Application of PCR

PCR has widespread application in various field of science include genetic engineering, medical, forensic, agriculture, environment etc.

- **PCR - Gene cloning and expression**

PCR is used in gene cloning and screening of genomic libraries.

- **PCR - Medicine;-**

PCR has major impact on medicine especially in the field of clinical microbiology or diagnosis

- Molecular tools have also allowed to perform
- Prenatal genetic diagnosis.

- **PCR - Forensic Science;**

Application of scientific tool to solve criminal and legal matters

- Molecular method are used to established the filiation of a person or to obtain evidence

from minimal sample of saliva, semen or other tissue.

- **PCR-DNA Profiling:-**

DNA Profiling or DNA fingerprinting is a forensic technique used to identify individuals by characteristics of their DNA

- **PCR-Agriculture science and environment:-**

PCR has also facilitated research in detection of pathogens in plants, animals and environment.

- **Molecular Palentology-PCR:,**

It is refer to the recovery and analysis of DNA and protien from ancient human, animal and plant remains.

Method for genome mapping

- **Radiation hybrid [RH] Mapping**

RH mapping used X-ray breakage of the chromosome to determine the distance between DNA markers as well as their order on the chromosome.

- **Comparative genomics of Bacteria**

It is may be variable in size. For example, it may vary from **0.49Mb** [Nanoarchaeum equitans] to **9.1Mb** [Bradyrhizobium japonicum] or Streptomyces.

- **Comparative genomics of eukaryotes;**

The minimal eukaryotic genome is smaller than the many bacterial genomes

Comparative genomics used to identify gene and regulatory elements.

It gives insight into evolution of key proteins.

- **Comparative genomics of organelles;**

- Mitochondrial genomes exhibit an amazing structural diversity
- Gene transfer has occured between mtDNA and nuclear DNA.

Selection for the Presence of vector:-

- All useful vector molecule carry a selectable genetic marker or property.
- Plasmid and cosmid vector carry drugs resistance or nutritional marker.
- In phage vectors, plaque formation is itself the selected property.

AFLPs detection in the absence of sequence information;-

- Amplified fragment length Polymorphism [AFLP] is a diagnostic fingerprinting technique that detect genomic restriction fragments and in that respect resembles the RFLP technique.

• Radiation hybrid [RH] mapping;-

RH mapping used x-rays breakage of chromosome to determine the distance between DNA markers as well as their order on the chromosome.