Brief content of lecture

Discipline *"Methods of molecular biotechnology"*

- **LECTURE 8.** ***DNA Sequencing technques***
- Classical chain termination sequencing uses **dideoxynucleotides** (ddNTPs) to terminate DNA synthesis at particular nucleotides.
- **Primer -** A single stranded nucleic acid molecule with a 3′ –OH used to initiate DNA polymerase replication of a paired template strand.
- 3.7 DNA Sequencing
- Fluorescently tagged ddNTPs and capillary gel electrophoresis allow automated, high-throughput DNA sequencing.
- The next generations of sequencing techniques aim to increase automation and decrease time and cost of sequencing.
- 3.8 PCR and RT-PCR
- **Polymerase chain reaction (PCR)** permits the exponential amplification of a desired sequence, using primers that anneal to the sequence of interest.
- 3.8 PCR and RT-PCR
- **RT-PCR** uses reverse transcriptase to convert RNA to DNA for use in a PCR reaction.
- 3.8 PCR and RT-PCR
- **Real-time**, or **quantitative**, **PCR** detects the products of PCR amplification during their synthesis, and is more sensitive and quantitative than conventional PCR.
- PCR depends on the use of thermostable DNA polymerases that can withstand multiple cycles of template denaturation.
- 3.8 PCR and RT-PCR
- **fluorescence resonant energy transfer** (**FRET**) – A process whereby the emission from an excited fluorophore is captured and reemitted at a longer wavelength by a nearby second fluorophore whose excitation spectrum matches the emission frequency of the first fluorophore.
- 3.9 Blotting Methods
- **Southern blotting** involves the transfer of DNA from a gel to a membrane, followed by detection of specific sequences by hybridization with a labeled probe.
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- Northern blotting is similar to Southern blotting, but involves the transfer of RNA from a gel to a membrane.
- Western blotting entails separation of proteins on a sodium dodecyl sulfate (SDS) gel, transfer to a nitrocellulose membrane, and detection of proteins of interest using antibodies.
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- **epitope tag –** A short peptide sequence that encodes a recognition site ("epitope") for an antibody, typically fused to a protein of interest for detection or purification by the antibody.
- 3.10 DNA Microarrays
- DNA microarrays comprise known DNA sequences spotted or synthesized on a small chip.
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- Genome-wide transcription analysis is performed using labeled cDNA from experimental samples hybridized to a microarray containing sequences from all ORFs of the organism being used.
- SNP arrays permit genome-wide genotyping of **single-nucleotide polymorphisms**.
- Array comparative genome hybridization (array-CGH) allows the detection of copy number changes in any DNA sequence compared between two samples.

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Lecture 9-10. ***DNA Microarrays as new molecular biotechnology tool.***

- A **DNA microarray** (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface.
- Scientists use ***DNA microarrays*** to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* (or *reporters* or *oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called antisense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. The original nucleic acid arrays were macro arrays approximately 9 cm × 12 cm and the first computerized image based analysis was published in 1981.
- ***Figure 1***
- Simplified view of a DNA array. The upper rectangles show two spots of DNA on a solid surface (sequences "A" and "B") prior to and after hybridization. The lower rectangles show highly idealized side views of the same surfaces.
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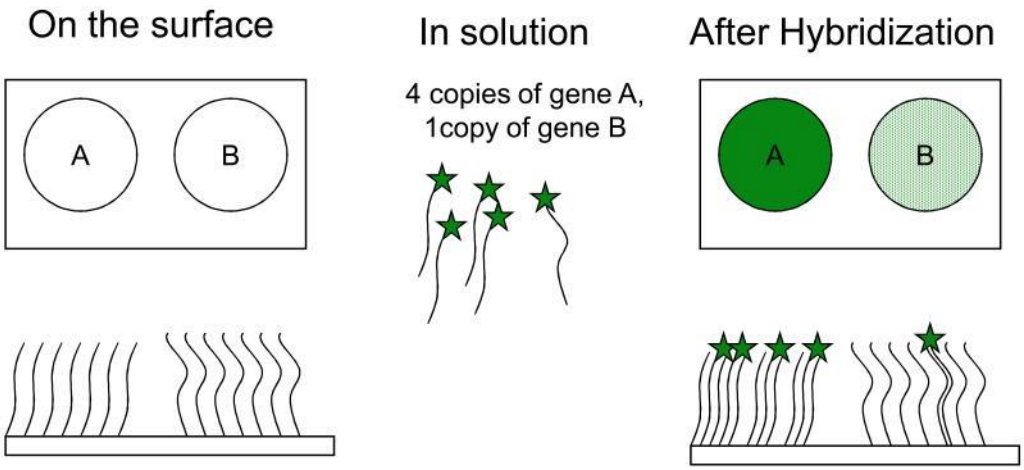

The early history of DNA arrays

One could argue that the original DNA array was created with the colony hybridization method of Grunstein and Hogness, 1975). In this procedure, DNA of interest was randomly cloned into *E. coli* plasmids that were plated onto agar petri plates covered with nitrocellulose filters. Replica plating was used to produce additional agar plates. The colonies on the filters were lysed and their DNA's were denatured and fixed to the filter to produce a random and unordered collection of DNA spots that represented the cloned fragments. Hybridization of a radiolabeled probe of an DNA or RNA of interest was used to rapidly screen 1000's of colonies to identify clones containing DNA that was complimentary to the probe (Unit 6.3).

The birth of the modern DNA array

In the late 90's and 2000's, DNA array technology progressed rapidly as both new methods of production and fluorescent detection were adapted to the task. In addition, increases in our knowledge of the DNA sequences of multiple genomes provided the raw information necessary to assure that arrays could be made which fully represented the genes in a genome, all the sequence in a genome or a large fraction of the sequence variation in a genome. It should also be noted that during this time, there was a gradual transition from spotting relatively long DNA's on arrays to producing arrays using 25-60bp oligos. The transition to oligo arrays was made possible by the increasing amounts of publicly available DNA sequence information. The use of oligos (as opposed to longer sequences) also provided an increase in specificity for the intended binding target as oligos could be designed to target regions of genes or the genome that were most dissimilar from other genes or regions. Three basic types of arrays came into play during this time frame, spotted arrays on glass, *in-situ* synthesized arrays and self assembled arrays ***(Figure 2).***

Spotted arrays

In 1996 Derisi et. al. published a method which allowed very high-density DNA arrays to be made on glass substrates(DeRisi et al., 1996). Poly-lysine coated glass microscope slides provided good binding of DNA and a robotic spotter was designed to spot multiple glass slide arrays from DNA stored in microtiter dishes. By using slotted pins (similar to fountain pens in design) a single dip of a pin in DNA solution could spot multiple slides. Spotting onto glass, allowed one to fluorescently label the sample. Fluorescent detection provided several advantages relative to the radioactive or chemilluminescent labels common to filter based arrays. First, fluorescent detection is quite sensitive and has a fairly large dynamic range. Second, fluorescent labeling is generally less expensive and less complicated than radioactive or chemilluminescent labeling. Third, fluorescent labeling allowed one to label two (or potentially more) samples in different colors and co-hybridize the samples to the same array. As it was very difficult to reproducibly produce spotted arrays, comparisons of individually hybridized samples to ostensibly identical arrays would result in false differences due

to array-to-array variation. However, a two-color approach in which the ratio of signals on the same array are measured is much more reproducible.

In-situ, Synthesized arrays

In 1991 Fodor et.al. published a method for light directed, spatially addressable chemical synthesis which combined photolabile protecting groups with photolithography to perform chemical synthesis on a solid substrate(Fodor et al., 1991). In this initial work, the authors demonstrated the production of arrays of 10-amino acid peptides and, separately, arrays of di-nucleotides. In 1994, Fodor et.al. at the recently formed company of Affymetrix demonstrated the ability to use this technology to generate DNA arrays consisting of 256 different octa-nucleotides (Pease et al., 1994). By 1995-1996, Affymetrix arrays were being used to detect mutations in the reverse transcriptase and protease genes of the highly polymorphic HIV-1 genome(Lipshutz et al., 1995) and to measure variation in the human mitochondrial genome(Chee et al., 1996). Eventually, Affymetrix used this technology to develop a wide catalogue of DNA arrays for use in expression analysis(Lockhart et al., 1996; Wodicka et al., 1997), genotyping (Chee et al., 1996; Hacia et al., 1996) and sequencing (G Wallraff, 1997)(see www.Affymetrix.com for the current catalog of arrays).

A major advantage of the Affymetrix technology is that because the DNA sequences are directly synthesized on the surface, only a small collection of reagents (the 4 modified nucleotides, plus a small handful of reagents necessary for the de-blocking and coupling steps) are needed to construct an arbitrarily complex array. This contrasts with the spotted array technologies in which one needed to construct or obtain all the sequences that one wished to deposit on the array in advance of array construction. However, the initial Affymetrix technology was limited in flexibility as each model of array required the construction of a unique set of photolithographic masks in order to direct the light to the array at each step of the synthesis process. In 2002, authors from Nimblegen Systems Inc., published a method in which the photo-deprotection step of Fodor et. al (Fodor et al., 1991; Lipshutz et al., 1999) is accomplished using micro-mirrors (similar to those in video computer projectors) to direct light at the pixels on the array(Nuwaysir et al., 2002). This allows for custom arrays to be manufactured in small volumes at much lower cost than by photolithographic methods using masks to direct light (which are cheaper for large volume production). One constraint with this method is that the total number of addressable pixels (e.g. unit oligos that can be synthesized) is limited to the number of addressable positions in the micro-mirror device (of order 1M).

In 1996, Blanchard et.al. proposed a method use inkjet printing technology and standard oligo synthesis chemistry to produce oligo arrays(Blanchard et al., 1996). In brief, inkjet printer heads were adapted to deliver to the four different nucleotide phosphoramidites to a glass slide that was pre-patterned to contain regions containing hydrophilic regions (with exposed hydroxyl groups) surrounded by hydrophobic regions. The hydroxylated regions provided a surface to which the phosphoramidites could couple, while the surrounding hydrophobic regions contained the droplet(s) emitted by the inkjets to defined regions. This technology was eventually commercialized by Rosetta Inpharmatics (Hughes et al., 2001) and licensed to Agilent Technologies who produces these arrays at present. The inkjet array approach shares the advantage of the Affymetrix/Nimblegen approach in that one only need to have available a small number of reagents to produce an array. In addition, similar to the Nimblegen approach, the production of a new type of array only requires that a different set of sequence information is delivered to the printer. Hence, the inkjet array technology has been particularly useful for the design of custom arrays that are produced in low volume.

Self assembled arrays

An alternative approach to the construction of arrays was created by the group of David Walt at Tufts University(Ferguson et al., 2000; Michael et al., 1998; Steemers et al., 2000; Walt, 2000) and ultimately licensed to Illumina. Their method involved synthesizing DNA on small polystryrene beads and depositing those beads on the end of a fiber optic array in which the ends of the fibers were etched to provide a well that is slightly larger than one bead. Different types of DNA would be synthesized on different beads and applying a mixture

of beads to the fiber optic cable would result in a randomly assembled array. In early versions of these arrays, the beads were optically encoded with different fluorophore combinations in order to allow one to determine which oligo was in which position on the array (referred to as "decoding the array")(Ferguson et al., 2000; Michael et al., 1998; Steemers et al., 2000; Walt, 2000). Optical decoding by fluorescent labeling limited the total number of unique beads that could be distinguished. Hence, the later and present day methods for decoding the beads involve hybridizing and detecting a number of short, fluorescently labeled oligos in a sequential series of steps(Gunderson et al., 2004). This not only allows for an extremely large number of different types of beads to be used on a single array but also functionally tests the array prior to its use in a biological assay. Later versions of the Illumina arrays used a pitted glass surface to contain the beads instead of a fiber option arrays.

The above is not intended to be a comprehensive history or survey of all DNA microarray technologies. However, it does cover the major advances in the field and the predominate methods of manufacture of arrays.

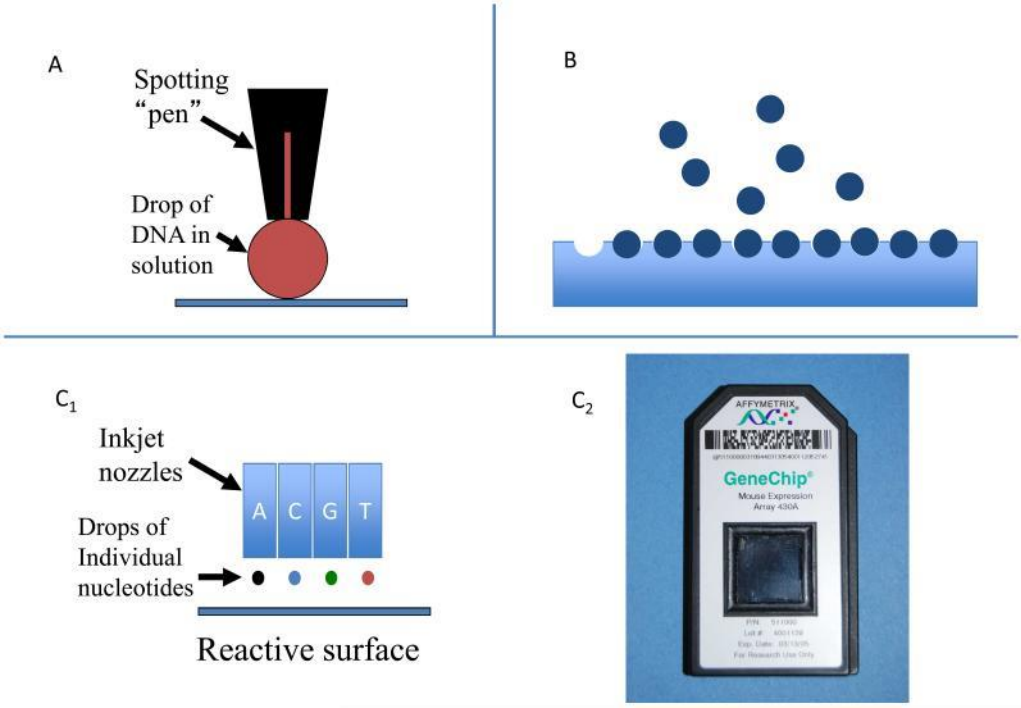

Figure 2

Three basic types of microarrays: (A) Spotted arrays on glass, (B) self assembled *arrays* and (C) *in-situ* synthesized arrays.

A. With spotted arrays, a "pen" (or multiple pens) are dipped into solutions containing the DNA of interest and physically deposited on a 1"x 3" glass microscope slide. Typically the glass slide surface is coated with something to help retain the DNA such as polylysine {DeRisi, 1997 #28191}, a silane {Call, 2001 #28277} or a chemically reactive surface {Rogers, 1999 #28278} (to which chemically reactive oligos or PCR products would be added).

B. Self assembled arrays can be created by applying a collection of beads containing a diverse set of oligos to a surface with pits the size of the beads. After the array is constructed a series of hybridizations determine

which oligo is in what position on each unique array (Ferguson et al., 2000; Michael et al., 1998; Steemers et al., 2000; Walt, 2000) (Gunderson et al., 2004).

C1 and C2. In-situ synthesized arrays can be produced by inkjet oligo synthesis methods (C1) or by photo-lithographic methods such as used by Affymetrix (C2).

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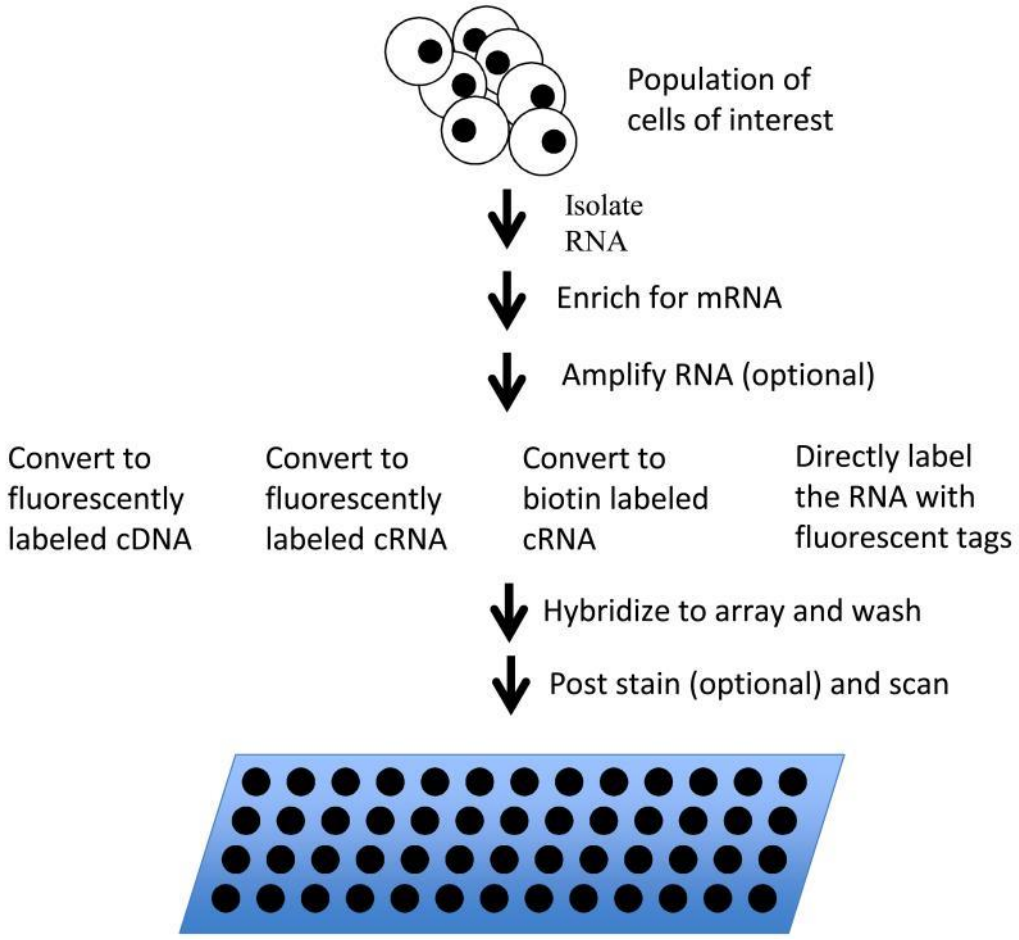
Lecture 11 . Applications of microarrays in gene expression analysis

Gene expression analysis

The predominate application of DNA microarrays has been to measure gene expression levels (Figure 3). In this application, RNA is extracted from the cells of interest and either, labeled directly, converted to a labeled cDNA or converted to a T7 RNA promoter tailed cDNA which is further converted to cRNA through the Eberwine amplification process (Van Gelder et al., 1990). A wide variety of methods have been developed for labeling of the cDNA or cRNA including: incorporation of fluorescently labeled nucleotides during the synthesis, incorporation of biotin labeled nucleotide which is subsequently stained fluorescently labeled streptavidin, incorporation of a modified reactive nucleotide to which a fluorescent tag is added later, and a variety of signal amplification methods (an early review of different labeling methods is provided in (Richter

et al., 2002)). The two most frequently used methods are the incorporation of fluorescently labeled nucleotides in the cRNA or cDNA synthesis step or the incorporation of a biotin labeled nucleotide in the cRNA synthesis step (as is done by Affymetrix).



The labeled cRNA or cDNA are then hybridized to the microarray, the array is washed and the signal is detected by measuring fluorescence at each spot. In the case of biotin labeled samples, the array is stained post-hybridization with fluorescently labeled streptavidin. Laser induced fluorescence is typically measured with a scanning confocal microscope. The intensity of the signal(s) on each spot is taken as a measure of the expression level of the corresponding gene. Gene expression analysis is described in more detail in Units 22.2-22.4.

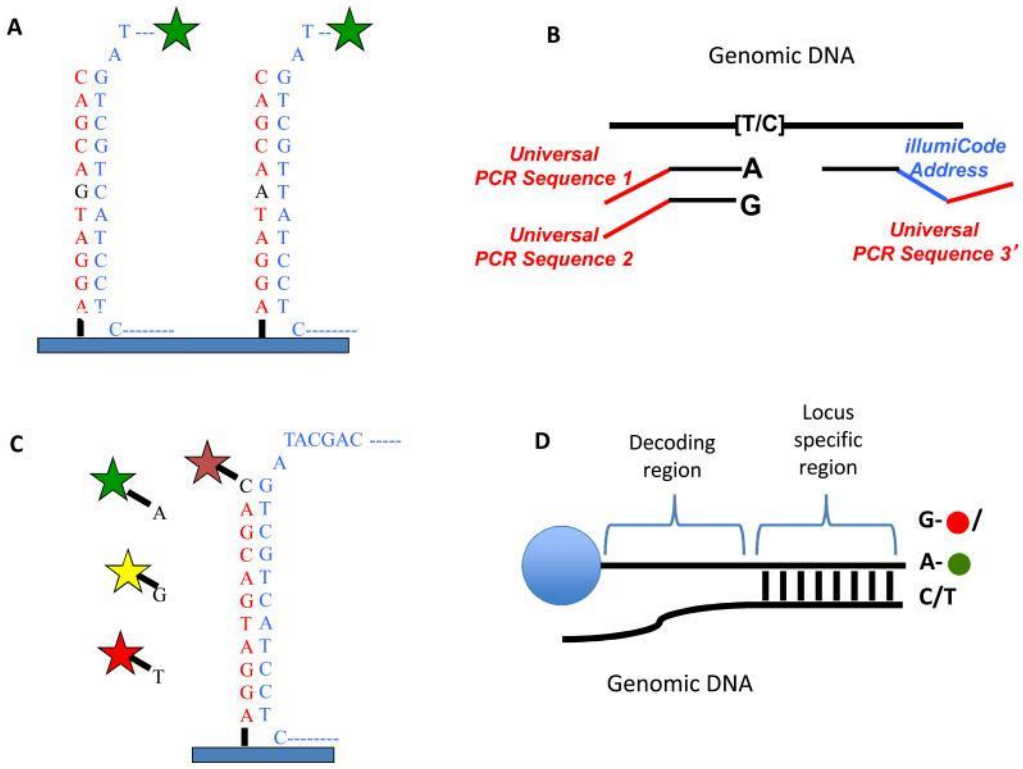
Transcription factor binding analysis

Microarrays have also been used in combination with chromatin immunoprecipitation (Solomon et al., 1988) to determine the binding sites of transcription factors (Horak and Snyder, 2002; Iyer et al., 2001). In brief, transcription factors (TFs) are cross linked to DNA with formaldehyde and the DNA is fragmented. The TF(s) of interest (with the DNA to which they were boud still attached) are affinity purified using either an antibody to the TF or by tagging the transcription factor with peptide that's amenable to affinity chromatography (for example a FLAG-, HIS-, myc or HA-tag). After purification, the DNA is released from the TF, amplified, labeled and hybridized to the array. This technique is commonly referred to as "ChIP-chip" for Chromatin Immuno-Precipitation on a "chip" or microarray.

As TF's often bind quite a distance away from the genes that they regulate, the design of the array and size distribution of the fragment length are interrelated. E.g. the array must contain probes that will interrogate the region of DNA bound to the transcription factor. For bacteria or yeast, the intergenic regions are fairly small and the same arrays used for gene expression work can be applied to ChIP-chip. For mammalian genomes, the intergenic regions are large and the TF often bind many kbp away from the gene of interest. Hence, for mammalian genomes, oligo arrays with oligo's spaced evenly across the entire genome are typically used for ChIP-chip experiments. Buck et. al. provide a good review(Buck and Lieb, 2004) of the considerations for the design and analysis of ChIP-chip experiments and the technique is discussed in detail in Units 21.9 and 21.13.

Genotyping

Microarrays have been widely used as single-nucleotide-polymorphism (SNP) genotyping platforms. Several alternative approaches have been used to detect SNP's but the most commonly used are allele discrimination by hybridization as used by Affymetrix (Wang et al., 1998), allele specific extension and ligation to a "bar-code" oligo which is hybridized to a universal array (the Illumina "Golden Gate Assay"(Fan et al., 2003)) or approaches in which the arrayed DNA is extended across the SNP in a single nucleotide extension reaction (the Arrayed Primer Extension assay of Kurg et.al. (Kurg et al., 2000) or the Infinium Assay of Illumina (Gunderson et al., 2006)). Figure 4 explains the detection approaches in more detail. Allelic discrimination by hybridization suffers background due to non-specific hybridization in complex genomes. In order to reduce this background, Affymetrix developed a PCR based approach to reduce genomic complexity.



igure 4

SNP detections strategies for arrays. A) Allele discrimination by hybridization – Oligos that are complimentary to each allele are placed on the array and labeled genomic DNA is hybridized to the array. The variant position is placed in the center of the oligo (typically 25bp on Affymetrix arrays) as this position has the greatest affect on hybridization. Typically, multiple array positions are used for each allele to improve signal to noise. B) Illumina's "Golden Gate Assay"- two allele specific oligos are each tailed with a different universal primer (1 and 2) and hybridized in solution to genomic DNA. A third oligo that is complementary to the

same locus is tailed with a “barcode” sequence and a third universal primer (3). Polymerase is used to extend the allele specific primers across the genomic sequence and the extended products are ligated to the third oligo. PCR is performed using primers complimentary to universal sequences 1, 2 and 3. The PCR primers complimentary to the universal sequences 1 and 2 are labeled with a unique fluorophore. The barcode sequence on the third oligo allows the PCR product to be uniquely detected on an array containing oligos complimentary to the barcode sequence. The use of multiple barcodes (one for each locus of interest) allows the assay to be multiplexed to sample many loci. C) Arrayed primer extension (APEX) – In this assay, the array contains DNA oriented with the 5′ end attached to the array and the 3′ end stopping one nucleotide short of the SNP. Genomic DNA is fragmented and hybridized to the array and the oligo on the array is extended in single nucleotide dye terminator sequencing reaction. D) Illumina’s Infinium assay – This assay is similar to the APEX assay except that the oligo to be extended is on a bead and the single nucleotide that is added is labeled with a nucleotide specific hapten as opposed to a fluorophore. The haptens are then detected by staining with fluorescently labeled proteins that bind each hapten.

Collection and analysis

The collection of data is done by using a microarray scanner. This scanner consists of a laser, a computer, and a camera. The laser excites fluorescence of the cDNA, generating signals.

When the laser scans the array, the camera records the images produced. Then the computer stores the data and provides the results immediately. The data thus produced are then analyzed. The difference in the intensity of the colors for each spot determines the character of the gene in that particular spot.

1. **Creation of labeled cDNA -** to create cDNA (complementary DNA strand), reverse transcription of the mRNA is done. Both the samples are then incorporated with different fluorescent dyes for producing fluorescent cDNA strands. This helps in distinguishing the sample category of the cDNAs.
2. **Hybridization -** the labeled cDNAs from both the samples are placed in the DNA microarray so that each cDNA gets hybridized to its complementary strand; they are also thoroughly washed to remove unbounded sequences

Applications

To study transcriptomes and proteomes

To diagnose pathogenic as well as genetic diseases in man

To identify microbes in the environment with the help of species-specific probes

To genotype genomes through single nucleotide polymorphism (SNP) analysis

To detect gene expression of mRNAs of a particular cell at different times

To measure changes in the level of gene expression

To observe DNA mutations

To study genomic gains and losses

Types of DNA microarrays

DNA microarrays are of four types:

a. cDNA microarrays: uses complementary DNA strands formed by transcription of mRNA
b. Oligo DNA microarrays: uses chemically synthesized oligo DNA as probes
c. BAC microarrays: uses template amplified by polymerase chain reaction as the probe
d. SNP microarrays: used to detect polymorphisms within a population

Data standards and data exchange

With the exception of DNA sequencing, microarrays were perhaps the earliest technology that allowed biologists to vast amounts of complex digital data. As the technology came into use, it rapidly became apparent that in order for others to be able to reproduce a given microarray experiment a detailed description of the array, the sample, the protocols and the data analysis methods needed to be available. Moreover, it also became apparent that access to the raw and processed data would allow others to perform analyses and meta analyses (on combinations of data) that the original data producers had not conceived. To address these issues of reproducible science and data exchange, members of the Microarray Gene Expression Data Society (now the Function Genomics Data Society – www.FGED.org) created the MIAME (Minimum Information About a Microarray Experiment) standards for the description of microarray experiments(Ball and Brazma, 2006; Brazma et al., 2001) and for the exchange of microarray data(Rayner et al., 2006; Spellman et al., 2002). These efforts influenced the creation public databases for microarray data (Barrett et al., 2007; Brazma et al., 2006; Brazma et al., 2003) and subsequent standards efforts in other areas (Deutsch et al., 2008; Field et al., 2008; Taylor et al., 2007).

Transcription factor binding analysis

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LECTURE 13. ***Different types of PCR in molecular biotechnology.***

Polymerase chain reaction (PCR) is an efficient and cost-effective molecular tool to copy or amplify small segments of DNA or RNA. PCR combines the principles of complementary nucleic acid hybridization with those of nucleic acid replication that are applied repeatedly through numerous cycles. It results in the exponential production of the specific target DNA/RNA sequences **by a factor of 10^7 within a relatively short period.**

This **in vitro amplification technique** can amplify a single copy of nucleic acid target by using two synthetic oligonucleotide "primers" that bind to the target genomic sequence, which are extended by a Taq polymerase (a thermostable DNA polymerase) . An automated process of repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94°C), annealing of primers to their complementary sequences (50°C), and primer extension (70°C) is employed for the amplification of target sequence.

PCR was originally developed in 1983 by the American biochemist and Nobel Laureate Kary Mullis.

Primer: *A short segment of nucleotides, which is complementary to a section of the DNA or RNA, which is to be amplified in the PCR. Two short DNA sequences designed to bind to the start (forward primer) and end (reverse primer) of the target sequence is used in PCR.*

Taq polymerase: *A thermally stable DNA polymerase originally isolated from the thermophilic bacterium Thermus aquaticus, which resist inactivation during denaturation temperatures and allows primer extension at high temperature.*

Components of Polymerase Chain Reactions (PCR)

- DNA template (the sample DNA that contains the target sequence to amplify)
- Deoxyribonucleoside triphosphates (dNTPs)
- PCR buffer
- Primers (forward and reverse)
- Taq polymerase

Steps of polymerase chain reaction-PCR

To perform PCR, extracted sample (which contains target DNA template) is added to a tube containing primers, free nucleotides (dNTPs), and Taq polymerase. The PCR mixture is placed in a PCR machine. PCR machine increases and decreases the temperature of the PCR mixture in automatic, programmed steps which generates copies of the target sequence exponentially.

Polymerase Chain Reaction (PCR) has **three major steps.**

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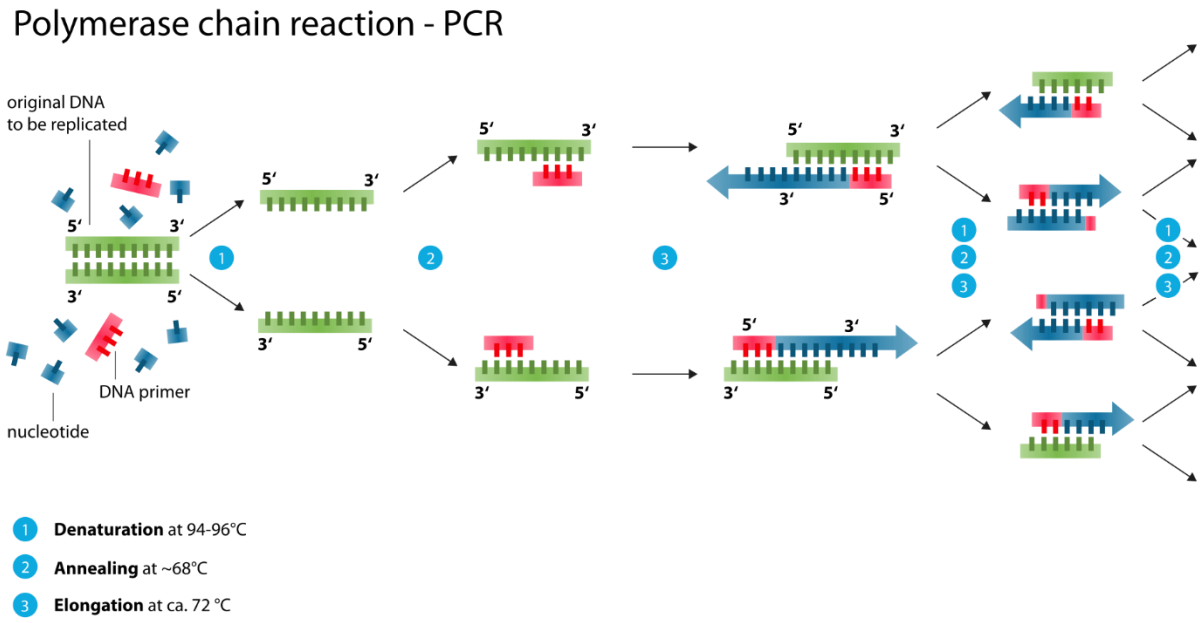

Types of polymerase chain reaction-PCR

Steps of Polymerase Chain Reactions (PCR)

1. **Denaturation (strand separation)** : The separation of the two hydrogen-bonded complementary chains of DNA into a pair of single stranded polynucleotide molecules by a process of heating (94°C to 96°C)
2. **Annealing (primer binding):** The temperature is lowered (45-60 °C) so the primers can attach themselves to the single stranded DNA strands.
3. **Extension (synthesis of new DNA):** It starts at the annealed primer and works its way along the DNA strand (72°C).

Once the first round is completed, the process is repeated by cycling back to the first reaction temperature and next round of denaturation, annealing and extension is started*(an automatic process in thermocycler)*. This 3 steps temperature cycle is repeated approximately 30 times which results exponential amplification of target gene sequence.

Several modification of PCR methods have been developed to enhance the utility of this method in diagnostic settings based on their applications. Some of the common types of PCR are;

1. Real-Time PCR
2. Nested PCR
3. Multiplex PCR
4. Quantitative PCR
5. Arbitrary Primed PCR

Detection of PCR products

Labeled probe that is specific for the target gene sequence is used to detect PCR amplified gene product (also known as amplicon). Based on the nature of the reporter molecule used, probe generates radioactive, colorimetric, fluorometric, or chemiluminescent signals. Probe based detection of amplicons serves two purposes

1. It allows visualization of the PCR product
2. It provides specificity by ensuring that the amplicon is the target sequence of interest and not the result of non-specific amplification.

Apart from DNA based hybridization method, sometimes simple gel electrophoresis method is sufficient to confirm the presence of specific amplicons.

Applications of PCR

- Identification and characterization of infectious agents
    - Direct detection of microorganisms in patient specimens
    - Identification of microorganisms grown in culture
    - Detection of antimicrobial resistance
    - Investigation of strain relatedness of pathogen of interest
- Genetic fingerprinting (forensic application/paternity testing)
- Detection of mutation (investigation of genetic diseases)
- Cloning genes
- PCR sequencing

LECTURE 14 . *Molecular markers types and applications*

1. 1. Utilization of Molecular Markers for PGRFA Characterization and Pre-Breeding for Climate Changes Aug. 31st- Sept. 4th, 2014
2. Based on Hybridization Markers Morphological Biochemical Molecular Based on PCR RFLP Mini-satallite Microsatallite Isozyme Protein Banding Pattern RAPD SSRISSR AFLPSTS SCARSCoT ESTAP-PCR
3. Molecular Cytogenetic Techniques Types of Molecular Markers Molecular Markers Techniques FISH Extended DNA fiber–FISH RAPD SSR AFLP SCoT RFLP EST
4. Morphological markers
5. • Assessment Genetic diversity in a crop species is fundamental to its improvements. • Genetic variability is considered the reservoir that plant breeders fall upon in their continuous strive to develop improved varieties and hybrids. • Knowledge of germplasm diversity and of relationship among elite breeding materials has a significant impact improvement of crop plants. • This information is useful in planning crosses for hybrid and line development, in assigning lines to heterotic groups. Genetic Diversity
6. •Limited genomic coverage
7. Molecular Markers They may be due to: • Base pair changes. • Rearrangements (translocation or inversion). • Insertions or deletions. • Variation in the number of tandem repeats. Reflect heritable differences in homologous DNA sequences among individuals.
8. ⎪ Ubiquitous. ⎪ Stably inherited. ⎪ Multiple alleles for each marker. ⎪ Devoid of pleiotropic effects. ⎪ Detectable in all tissues, at all ages. ⎪ Long shelf life of the DNA samples. Advantages of Molecular Markers

9. Hybridization based (non-PCR) Technique RFLPs Restriction Fragment Length Polymorphism analysis Botstein et al. (1980)
10. Genetic markers resulting from the variation or change in the length of defined DNA fragments produced by digestion of the DNA sample with restriction endonucleases RFLPs :
11. RFLPs (restriction fragment length polymorphisms) Electrophoretic comparison of the size of defined restriction fragments derived from genomic DNA 1. Isolate high quality DNA 2. Digest with a combination of restriction enzymes 3. Fractionate digested samples by electrophoresis 4. Transfer fragments to membrane 5. Hybridize with radioactively labeled DNA probe(s); detect by autoradiography. Can also use non-radioactive labeling systems
12. RFLP analysis Polymorphism revealed by different probe/enzyme combinations among 13 different accessions.
13. Considerations for use of RFLPs - Relatively slow process -Use of radioisotopes has limited RFLP use to certified laboratories (but non-radioactive labeling systems are now in wide use) - Co-dominant markers; often species-specific - Need high quality DNA - Need to develop polymorphic probes - expensive
14. PCR based techniques (RAPD, ISSR, SSR, AFLP, EST ,SCoT)
15. Random Amplified Polymorphic DNA (RAPD) Randomly Amplified Polymorphic DNA (RAPDs) are genetic markers resulting from PCR amplification of genomic DNA sequences recognized by ten-mer random primers of arbitrary nucleotide sequence (Williams et al., 1990). RAPDs are dominant markers that require no prior knowledge of the DNA sequence, which makes them very suitable for investigation of species that are not well known (Williams et al. 1993).
16. RAPD profiles for the 14 Date Palm accessions as detected with primers OPB-06 (A), OPB-08 (B), OPB-11 (C), and OPO-07 (D). Lanes 1 to 14 represent: SAK-AK, SAK-AB, BRT-AK, BRT-AB, MLK-AK, MLK-AB, GND-AK, GND-AB, SIW-KH, SIW-DK, SIW-HB, SIW-TZ, FRA-HB and FRA-TZ. M: 1 Kb ladder DNA marker.
17. Inter-Simple Sequence Repeats (ISSR) The generation of ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite repeated sequence and in some cases primer also contains 1- 4 base anchor at either 3′ or 5′ or at both ends, which target a subset of 'simple sequence repeats' (SSRs) and amplify the region between two closely spaced and oppositely oriented SSRs (Fang et al., 1997; Fang and Roose, 1997; Moreno et al., 1998). ISSR technique permits the detection of polymorphisms in microsatellites and inter-microsatellites loci without previous knowledge of the DNA sequence (Moreno et al., 1998).
18. ISSR profiles of the 14 Date palm accessions using the primers: IS3 (A), IS4 (B), IS7 (C), IS9 (D). M: 1 Kb ladder DNA marker. Lanes 1 to 14 represent: SAK-AK, SAK-AB, BRT-AK, BRT-AB, MLK-AK, MLK-AB, GND-AK, GND-AB, SIW- KH, SIW-DK, SIW-HB, SIW-TZ, FRA-HB and FRA-TZ.
19. Are DNA sequences with repeat lengths of a few base pairs. Variation in the number of repeats can be detected with PCR by developing primers for the conserved DNA sequence flanking the SSR. As molecular markers, SSR combine many desirable marker properties including high levels of polymorphism and information content, unambiguous designation of alleles, even dispersal, selective neutrality, high reproducibility, co-dominance, and rapid and simple genotyping assays. Microsatellites have become the molecular markers of choice for a wide range of applications in genetic mapping and genome, genotype identification and variety protection, seed purity evaluation and germplasm conservation, diversity studies, paternity determination and pedigree analysis, gene and quantitative trait locus analysis, and marker-assisted breeding. Simple Sequence Repeats (SSR)
20. A B C D FE M 1 20 M 1 20 200 bp 100 bp Photograph of EtBr stained polyacrylamide gels of polymorphic SSR products from 20 maize inbred lines as detected by SSR primers (A: M28, B: M27, C: M25, D: M20, E: M18 and F: M22). M (100bp DNA ladder)
21. AFLP (Amplified Fragment Length Polymorphisms) ¬ A combination of PCR and RFLP ¬ Informative fingerprints of amplified fragments
22. Amplified Fragment Length Polymorphism (AFLP) AFLP process 1. Digest genomic DNA with restriction enzymes 2. Ligate commercial adaptors (defined sequences) to both ends of the fragments 3. Carry out PCR on the adaptor-ligated mixture, using primers that target the adaptor, but that vary in

the base(s) at the 3’ end of the primer. AFLP technology is a DNA fingerprinting technique that combines RFLP and PCR. It is based on the selective amplification of a subset of genomic restriction fragments using PCR.

23. AFLP profiles of the 14 Date Palm accessions as revealed by the primer combination Eact X Mcta. (A) and the primer combination Eagc X Mcaa. (B). Lanes 1 to 14 represent: SAK-AK, SAK-AB, BRT-AK, BRT-AB, MLK-AK, MLK-AB , GND-AK, GND-AB, SIW-KH, SIW-DK, SIW-HB, SIW-TZ, FRA-HB and FRA-TZ. M: DNA molecular weight marker (100 bp Ladder).
24. Advantages of AFLP's ¬ Very sensitive ¬ Good reproducibility but technically demanding ¬ Relatively expensive technology ¬ Discriminating homozygotes from heterozygotes ¬ Requires band quantitation (comparison of pixel density in images from a gel scanner) ¬ Bands are anonymous
25. SCOT (Start codon targeted Marker)
26. Start Codon Targeted (SCoT) Polymorphism analysis SCoT is a novel method for generating plant DNA markers. This method was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT uses single 18-mer primers in polymerase chain reaction (PCR) and an annealing temperature of 50°C. PCR amplicons are resolved using standard agarose gel electrophoresis. This method was validated in rice using a genetically diverse set of genotypes and a backcross population. Diagram showing principle of SCoT PCR amplification
27. SCoT Analysis SCoT analyses were performed as described by Collard and Mackil (2009). Table : SCoT primers and their sequences
28. SCoT profiles of the two parental genotypes as revealed by different primers M P1 P1 P2 P2 P1 P1 P2 P2 P1 P1 P2 P2 P1 P1 P2 P2 P1 P1 P2 P2 P1 P1 P2 P2 P1 P1 P2 P2 P1 P1 P2 P2
29. ESTs (Expressed Sequence Tags) Single-pass sequencing reads from randomly selected cDNA clones 3’EST sequence 5’EST sequence cDNA Clone dbEST May 7, 2003 21,265,083 ESTs from 611 species
30. Steps for EST’s •cDNA libraries (containing many of the expressed genes of an organism) •pick cDNA clones randomly •rapidly determine some of the sequence of nucleotides from the end of each clone. •These ESTs could then be compared to all known sequences using a program called BLAST.
31. An exact match to a sequenced gene means that the gene encoding that EST is already known. If the match was close but not exact one could conclude that the EST is derived from a gene with a function similar to that of the known gene. The EST sequences with their putative identification are then deposited in the GenBank and the clones from which they were derived are kept in a freezer for later use.
32. Overview of the EST sequencing process Clones are picked from petrie dishes into microtitre plates, and archived for later use. All subsequent manipulations (PCR, clean up and sequencing) are carried out in microtitre plates to yield medium-throughput.
33. Features RFLP PCR- RFLP DFP RAPD Microsatellite SNP Detection method Hybridization PCR Hybridization PCR PCR PCR Type of probe/primer used g DNA/ cDNA sequence of structural genes Sequence specific primers Mini satellite synthetic oligos Arbitrarily design primer Sequence specific primers Sequence specific primers Requirement of radioactivity Yes No/Yes Yes No/Yes No/Yes No/Yes Extant of genomic coverage Limited Limited Extensive Extensive Extensive Extensive Degree of polymorphisms Low Low High Medium to High High High Phenotype expression Co dominant Co dominant Co dominant Co dominant/D ominant Dominant Co dominant Possibility of automation No Yes No Yes Yes Yes
34. DATA ANALYSIS
35. 765432 000110 011000 111101 100111 111111 011000 111111 100111 111111 000111 111111 Scoring of bands
36. 653742 1002 10094.14 10087.593.37 10080.094.187.53 10053.371.462.566.75 10092.357.161.553.357.16 Genetic Similarity matrix calculated according to Jaccard’s coefficient based on marker data.
37. Dendrogram constructed with UPGMA cluster analysis of marker data showing the genetic relationships among the different samples. 0.50 0.70 0.80 0.90 1.00 2 3 4 55 6 7

38. ▪ Fingerprinting . ▪ Diversity studies . ▪ Marker-assisted selection . ▪ Genetic maps . ▪ Gene tagging . ▪ Novel allele detection . ▪ Map-based gene colning . ▪ F1 identification . ▪ Comparative maps . ▪ Bulk segregant analysis . ▪ Seed testing . DNA marker applications

- **LECTURE 15 .** QTL analysis
- A quantitative trait locus (QTL)
- is a section of DNA (the **locus**) which correlates with variation in a phenotype (the quantitative trait).
- QTLs are mapped by identifying which molecular markers (such as ***SNPs*** or ***AFLPs***) correlate with an observed trait.
- This is an early step in identifying and sequencing the actual genes that cause the trait variation.
- A **quantitative trait locus** (**QTL**)
- **is a region of** *DNA* **which is associated with a particular phenotypic trait, which varies in degree and which can be attributed to polygenic effects.**
- **Polygenic effects is the product of two or more genes, and their environment.**
- **These QTLs are often found on different chromosomes.**
- **The number of QTLs which explain variation in the phenotypic trait indicates the genetic architecture of a trait.**
- **It may indicate that plant height is controlled by many genes of small effect, or by a few genes of large effect.**
- A **quantitative trait locus** (**QTL**)
- Typically, QTLs underlie continuous traits (those traits which vary continuously, e.g. height) as opposed to discrete traits (traits that have two or several character values, e.g. red hair in humans, a recessive trait, or smooth vs. wrinkled peas used by Mendel in his experiments).
- Moreover, a single phenotypic trait is usually determined by many genes.
- Consequently, many QTLs are associated with a single trait.
- **Another use of QTLs is to identify candidate genes underlying a trait.**
- **Once a region of DNA is identified as contributing to a phenotype, it can be sequenced.**
- **The DNA sequence of any genes in this region can then be compared to a database of DNA for genes whose function is already known.**
- **Quantitative traits**
- **Polygenic inheritance** is to inheritance of a ***phenotypic*** characteristic (trait) that is attributable to two or more genes and can be measured quantitatively.
- **Multifactorial inheritance** is to polygenic inheritance that also includes interactions with the environment.
- Unlike monogenic traits, polygenic traits do not follow patterns of Mendelian inheritance (discrete categories).

-
- **QTL mapping**
- For organisms whose genomes are known, one might now try to exclude genes in the identified region whose function is known with some certainty not to be connected with the trait in question.
- If the genome is not available, it may be an option to sequence the identified region and determine the putative functions of genes by their similarity to genes with known function, usually in other genomes.
- This can be done using ***BLAST***, an online tool.
- BLAST allows to enter a primary sequence and search for similar sequences within the BLAST database of genes from various organisms.
- It is often not the actual gene underlying the phenotypic trait, but rather a region of DNA that is closely linked with the gene.
- **BLAST for Basic Local Alignment Search Tool is an algorithm for comparing primary biological sequence information,**
- **such as the amino-acid sequences of proteins or**
- **the nucleotides of DNA sequences**
- **QTL mapping**
- **In a recent development, classical QTL analyses were combined with gene expression profiling i.e. by DNA microarrays.**
- **Such expression QTLs (eQTLs) describe cis- and trans-controlling elements for the expression of often disease-associated genes.**
- **Observed epistatic effects have been found beneficial to identify the gene responsible by a cross-validation of genes within the interacting loci with metabolic pathway- and scientific literature databases.**
- **Epistasis** is the phenomenon where the effect of one gene (locus) is dependent on the presence of one or more 'modifier genes', i.e. the **genetic background**.
- Originally the term meant that the phenotypic effect of one gene is masked by a different gene (locus).
- **Analysis of variance**
- The simplest method for QTL mapping is analysis of variance (ANOVA, sometimes called "marker regression") at the marker loci.
- In this method, in a backcross, one may calculate a t-statistic to compare the averages of the two marker genotype groups.
- For other types of crosses (such as the intercross), where there are more than two possible genotypes, one uses a more general form of ANOVA, which provides a so-called F-statistic.
- **Analysis of variance**
- The ANOVA approach for QTL mapping has three important weaknesses.

- 1) we do not receive separate estimates of QTL location and QTL effect. QTL location is indicated only by looking at which markers give the greatest differences between genotype group averages, and the apparent QTL effect at a marker will be smaller than the true QTL effect as a result of recombination between the marker and the QTL. 2), we must discard individuals whose genotypes are missing at the marker.
- 3) , when the markers are widely spaced, the QTL may be quite far from all markers, and so the power for QTL detection will decrease.
- **Interval mapping**
- Lander and Botstein developed interval mapping, which overcomes the three disadvantages of analysis of variance at marker loci. Interval mapping is currently the most popular approach for QTL mapping in experimental crosses.
- The method makes use of **a genetic map** of the typed markers, and, like analysis of variance, assumes the presence of a single QTL.
- In interval mapping, each locus is considered one at a time and the logarithm of the odds ratio (LOD score) is calculated for the model that the given locus is a true QTL.
- The odds ratio is related to the Pearson correlation coefficient between the phenotype and the marker genotype for each individual in the experimental cross.
- **Interval mapping**
- The term 'interval mapping' is used for estimating the position of a QTL within two markers (often indicated as 'marker-bracket'). Interval mapping is originally based on the maximum likelihood but there are also very good approximations possible with simple regression.
- The principle for QTL mapping is:
- 1) The Likelihood (*Вероятность*) can be calculated for a given set of parameters (particularly QTL effect and QTL position) given the observed data on phenotypes and marker genotypes.
- 2) The estimates for the parameters are those where the likelihood are highest.
- 3) A significance threshold (*порог*) can be established by permutation (*перестановка*) testing.
- **Interval mapping**
- **Conventional methods for the detection of quantitative trait loci (QTLs) are based on a comparison of single QTL models with a model assuming no QTL. For instance in the "interval mapping" method the likelihood for a single putative QTL is assessed at each location on the genome. However, QTLs located elsewhere on the genome can have an interfering effect.**
- **Interval mapping**
- As a consequence, the power of detection may be compromised, and the estimates of locations and effects of QTLs may be biased (LANDER and BOTSTEIN 1989; KNAPP 1991). Even nonexisting so-called "ghost" QTLs may appear (HALEY and KNOTT 1992; MARTINEZ and CURNOW 1992). Therefore, it is obvious that multiple QTLs could be mapped more efficiently and more accurately by using multiple QTL models. One popular approach to handle QTL mapping where multiple QTL contribute to a trait is to iteratively scan the genome and add known QTL to the regression model as QTLs are identified. This method, termed composite interval mapping determine both the

location and effects size of QTL more accurately than single-QTL approaches, especially in small mapping populations where the effect of correlation between genotypes in the mapping population may be problematic

- Introduction
- QTL analyses are specialized techniques that construct genetic linkage maps to locate loci (QTLs) that affect a quantitative trait and estimate the genetic effect of QTLs on the trait.
- These techniques range from the relatively simple Single Marker Analysis, to the more complex Composite Interval Mapping, where multiple linked markers are taken into consideration.
- JMP Genomics has three different methodologies for quantitative trait loci (QTL) analysis:
- Single Marker Analysis
- Interval Mapping (IM)
- Composite Interval Mapping (CIM).