BIOTECHNOLOGY

Biotechnology per se is a confluence of a variety of techniques applied to biosystems. Biotechnology encompasses a broad area of biology involving all areas of science and techniques. It is only in the past 5-8 years it has been hailed as the ultimate technology which provides panacea for the all the ills and miseries of health and agriculture. Development of molecular techniques like gene engineering, its application and the impact on society and its culture has brought the name and fame for this term. It is true that any technique related to any biosystems can be called biotechnology; no body can raise objections to it. A farmer can himself be called a biotechnologist for he sows seeds, grows the crop and harvest the crop and sells. Since the day he wandered among trees and started living in groups, he has used biotechniques like domesticating a variety of animals and plants for his own use. He is a pioneer and a par excellent Biotechnologist.

In the present context, instead of dealing all sundry biological methods, it is apt and appropriate to deal with molecular aspects of biotechnology, call it if you like

"**Molecular Biotechnology**" because it is the technology that can be applied to solve variety of biological problems that present society is in. The methodology though it is in its infancy, it has great potential and the time for achieving solution will be shorter and faster. For example, an agricultural scientist wants to develop a plant variety with a desired character and to put into commercial use; by classical breeding methods, he may take a minimum of 12 to 16 years. But genetic engineering and tissue culture techniques can achieve this in about 3-4 years of time.

A variety of biotechniques, including gene-engineering and gene editing techniques have been developed, some are being in development, and some are perfected to experiment and use. In this text only few gene engineering techniques and application of the same is given in its simple form. For greater details one can refer few advance books and reviews like Gene-VIII, Molecular biology of the Gene by Watson et al 2005, Molecular biology of the cell by Albert et al 2005. Cloning techniques in its simple form will be discussed. Application of gene engineering in the improvement of crop plants, improvement of dairy farm and related animals, human

disease diagnosis and cure for genetic disorders and opportunistic diseases will be briefed.

In gene engineering techniques basic principle of cloning and the required tools and techniques will be explained.

Gene cloning:

When the first gene was cloned about 35-36 years back, it was considered as an achievement and a big achievement in the field of Molecular biology. It is now so common thing in the labs, even undergraduate students in US of America, do cloning, PCR, sequencing, blotting techniques and many others; this is to full fill their lab assignments. This is done under the supervision of professors.

Tools required for cloning:

Restriction enzymes, a source for obtaining the gene, vectors for carrying the foreign gene, some enzyme like polymerases, lipases and some radioactive isotopes or non-radioactive compounds for labeling, host cells for propagating the cloned material.

Restriction Enzymes:

There are a variety of endonucleases, which recognize certain sequences and cut the ds DNA in a variety of ways. Among them one class of enzymes used are called class-II. They use a sequence of 4, 6 or 8 base pairs long, which are palindromic in nature and cut the strands, within the sequence or at the border, in such a way they produce sticky tail ends or blunt ends. However, each enzyme binds to a specific sequence and cuts the DNA in a specific way. Restriction enzymes can be considered as molecular scissors.

Ex. Alu I (4bp AG*CT), E.co-R1 (6bp; G*AATTC), is the enzyme derived from E. coli strain-R.

Hin D-III (6BP;A* AGCTT) from Hemophilus influenzae strain D-III,

Dra-I (6bp:TTT*AAA),

Hapa-I (6BP:GTT*AAC),

Sac I (6BP:GAGCT*C)

Bgl-I (12BP: GCCNNNN8NGGC),

Not I (8BP:GC*GGCCGC).

The astrich mark indicates the position of cutting, which reveals whether the cut ends have blunt ends or 3′ sticky ends or 5′ sticky ends.

DNA ligases:

They can be considered as molecular stitchers. DNA ligases have the ability perform ligating the nicked ends, provided they are next to each other. E. coli DNA ligase is NAD/P dependent and the viral T4 DNA ligase is ATP dependent. T4 ligase is very efficient in ligating sticky ends and it can also perform ligation of blunt ends but with little efficiency. However, its efficiency can be enhanced in the presence of RNA ligase. This can be furthered by molecular crowding by the addition PEG -800 (polyethylene glycol) to 15%.

DNA polymerase-I: This is a versatile enzyme which has the ability to perform 5′>3′ polymerase activity and 5′>3′ exonucleases and 3′>5′ exonucleases activities. However, the genetically modified DNA pol-I is available as Klenow fragment with out it's %′>3′ exonucleases activity. This is extensively used in the second strand of cDNA synthesis from poly(A)-

RNA. This enzyme can also be used for end modifications, random primer labeling and nick translation protocols.

Alkaline Phosphotase:

Alkaline phosphotases are extracted from bacteria or calf thymus sources. This enzyme has the ability to remove 5'end phosphate groups. Many a times the vectors are treated with AP for the removal of 5' phosphates, otherwise the vectors are capable self ligating.

5' Polynucleotide Kinase:

These enzymes are capable of adding radio active label from the gamma phosphate of the ATP. 5' Phosphate is very much essential for ligation.

Reverse transcriptase:

This enzyme is available form Avian viruses or from Melony Murine leukemia viruses. The enzyme is called RNA dependent DNA polymerase. These enzymes are unique for they are capable synthesizing a cDNA on RNA templates, so one can use this enzyme in combination with DNA-pol-I for producing cDNA library.

Vectors:

Vectors are DNA molecules of various sizes and they act as transport vehicles for carrying foreign DNA segments from one source to the other source. They come from variety of sources; they have different characteristic properties. The vector source can be from bacterial plasmids, lambda viral DNA, or combination of lambda sequence with bacterial plasmids called Cosmids, bacterial artificial chromosomes called B-acmids, Pi phage derived P-acmids, yeast episomal expression vectors or yeast artificial chromosomes, they are shuttle vectors. They can be constructed to the desired needs. Whatever may be the source, all should have at least some of the following features.

- Most of the ds DNA vectors should be as small as possible.
- One has to decide whether one wants the vector to be replicated as episomal plasmid DNA or one wants the plasmid to be incorporated in to the host DNA for integrated expression.
- One has to select a vector to the desired needs, for example, do you want just clone a single gene fragment or

one wants to generate cDNA library or one wants genomic library and where do you want them to be propagated, whether it is in bacterial cells, or in yeast cells or in animal or plant cells.

- What ever may be the goal, all of them should contain an 'origin' for efficient replication to generate multiple but high copy numbers. The origin should be suitable for the host where the vector is allowed to multiply. Bacterial origins that are used are from Col E1 which is very efficient in bacterial hosts.
- Eukaryotic origins are mostly from animal viruses such as SV 40 origin, or Epstein Barr viral origin or Bovine papilloma viral origin or one can use origin derived from yeast autonomously replicating sequence called ARS or origin from DHFR source, which are very efficient origins in eukaryotic cells. For plant one has get hold of Ti plasmid derived origin or Cauliflower mosaic viral derived origin, for they are good for plant systems.
- They should have good marker for selection like an antibiotic marker, in the sense the vector carries an antibiotic gene. In some case one can use color producing gene in the presence of a substrate, such as beta

galactosidase with X-Gal. If a foreign gene is inserted into such a gene, it does not produce color, but if a foreign gene is not inserted it produces color, thus one can easily detect which are recombinant and which are not.

- Glucorunidase gene and jelly fish fluorescent color producing gene or fire fly's luciferase gene for fluorescent color producing, can be incorporated as reporter genes. One has to have clear concepts of Reporter genes and selection marker genes.
- One has to select antibiotic markers which works in bacterial or works in eukaryotic systems. Ampicillin is a good selection marker gene for bacterial systems and Hygromycin or Kanamycin are good marker gene for eukaryotic systems.
- All should have a good and efficient promoter compatible for the host cell and for efficient expression. The kind of promoter depends upon in which host cells you want to express the cloned gene.
- There should be unique multiple cloning sites (MCSs) next to the promoter, the sites can slightly overlap the promoter without hindering its function.

- It is also essential, if one wants the gene to be expressed one should have a leader sequence containing ribosome binding site called shine Dalgarno sequence for bacterial expression or Kozak sequence for eukaryotes for proper translational initiation.

  Multiple cloning sites multiple cloning sites (MCS) means such sites are found in specific region, mostly next to the promoter, in the plasmid and they are unique in the sense they are not at any other region of the plasmid.

These sites are useful in cloning DNA fragments generated by any of the Restriction enzyme found in MCS. It is also easy to release the fragment from the cloned sites.

- Next to the MCS one can include transcriptional terminator sequence or Poly(A) signal sequence for foreign gene's transcription terminater.
- It is also possible to produce fused protein from the cloned DNA by including segment of DNA coding for a required amino acid in the proximal region of the MCS. The fused protein can be purified easily.

The most commonly used plasmids, though derived from pBR322, are from the labs of university of California are called pUC plasmids. Small 'p' represents the plasmid. The other alphabets refer to the person's name designed them or the university or the organization that has produced them or a company that is designed or the symbols may represent the functions of the vectors.

The pUC plasmid vectors have been variously modified, today it is difficult to recognize any semblance of its original features. Some of the pUC plasmid DNA has been added with the DNA segments containing origins from filamentous viruses either to generate (+) or (-) single stranded DNA, which is very useful in sequencing or creating site directed mutations.

pUC plasmid originally referred to as pUC8 and pUC9 have the following features:

1. The plasmid DNA is circular and 2685 (can vary) base-pairs long.
2. It contains an efficient Origin segment derived from Col E1 plasmid, which produces high copy numbers in E. coli strains.
3. It has an Ampicillin (+) marker gene for selection.

4. It has a lac-Z promoter and a coding region for the lac-Z alpha fragment.
5. The lac-Z gene codes for Beta galactosidase enzyme. The Lac-Z alpha is left part of the gene and produces a part of the Lac -z protein. If such plasmids are introduced into bacterial strains containing the other part of the gene called beta fragment and if both are expressed in the same cell, each of the fragments of proteins complement each other and function as complete enzyme, which cuts beta Galactosidase. If the cells are provided with X-Gal, which is also similar to Beta Galactoside, cleaves the X-Gal resulting in blue color. For this reason, this gene is used as marker gene or reporter gene.
6. It is in the proximal part of the coding region multiple cloning site or linkers have been introduced.
7. In pUC 8, the cloning sites are in this order EcoRI> SacI.KpnI> SmaI> BamHI> XbaI> SalI/HincII> PstI> SphI> HindIII; the direction of transcription is in the same direction but starts earlier to the MCS and even translation starts at two positions just earlier to the Eco RI in pUC 8 and Just earlier to Hind III in pUC 9. pUC18

and pUC 19 are built on the same structure as that of pUC8/9 but more restriction sites are added.

8. In pUC 9 the position of MCS is same but the orientation of mcs is from Hind III> to>>>>> EcoR I and the direction of the transcription is in the same order i.e. from Hind III towards Eco R I.
9. If any cloned DNA is located in any one of the cloning sites, if the transcript is in proper reading frame the translated product is a fused protein.
10. A modified pUC plasmids containing an additional fragment derived from f1 or M13 viral origins, they are called phasmids or Phagmids. Depending upon what origin has been introduced and how it is oriented, it can generate either (+) single stranded phages or (-) single stranded phages. They are very useful in generating ssDNA that can be used for sequencing.
11. Often depending upon the commercial company that generates and sells such Phagmids are differently called, based on the variations they have introduced.

pUC plasmid map- General:

~~I--ori->I---I-promo-I-mcs-I-lac-Z-alpha--->I-I-amp------>I~~

mcs of pUC 8:

I-- EcoRI>SmaI>BamHI>HincII>PstI>HindIII>

Mcs of pUC 9:

I-HindIII>PstI>HincII>BamHI>SmaI>EcoRI>--I

mcs of pUC 18:

[--ATG— EcoR1-------HinD3---]

EcoR1EcoR1>SacI>KpnI>XmaI>BamHI>XbaI>HincII>PstI>SphI>HindIII>

Mcs of pUC-19:

HindIII>SphI>PstI>HincII>XbaI>BamHI>XmaI>KpnI>SacI>EcoRI>

Note:

Amp = Ampicillin Gene.

prom = promoter.

mcs = multiple cloning sites.

Lac-Z-alpha = alpha subunit of beta galactosidase.

Ori = ColE1 origin.

~~~~ = the ends of circular plasmid.

Lambda DNA based vector:

Lambda phage is temperate phage exhibits both lytic cycle and lysogenic cycle and can perform transduction function. The genome is 48502 and odd base pairs long. It is linear inside the phage head with terminal sticky tails of 12 ntds long which are complementary to each other, so they have cohesive ends. When the genome is injected into bacterial cells the cohesive ends base pair and the genome becomes circular and super coiled. The genomic end that is base paired is called 'Cos" site which can be cleaved with phage enzyme called Terminase, which generates cohesive ends. The neighboring sequence has sequence acts as packaging sequence for the DNA into viral heads.

cos>> head.tail.recombination.lysogeny.DNA repl.Ltr.lysis<<cos

~~I------I----I---------------I---------I-------I------I----I~~

In the central region there few genes which are not essential fo the survival of the phage. Such segments are called stuffer regions, which can be removed and, in its place, a foreign DNA can
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be inserted. Thus, the lambda DNA can used as a vector. But this cloned DNA has to be propagated by the virus itself. One can clone into poly-linkers with a promoter for expression in a suitable host. After screening the desired clones can be picked and they can be recloned into plasmid-based vectors. This can be used for obtaining cDNA library or genomic library. The insertion size can between 7kbp to 14 kbp.

A general model:

~~~I----------I---------stuffer ---------I ----------I~~

I-pro->I mcs >I---lacZ-a->I

OR

--I-Amp-I pro-I- mcs-I-stuffer-I mcs-I-

Cosmid Vectors:

It is the combination of a plasmid and cos sequences from lambda DNA. The plasmid contains an origin for high copy numbers. A selection marker gene like Ampicillin gene can be added for screening. One can use lac-Z promoter with suitable MCS for
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genomic library, and similar to that of a pUC plasmid. This can be used for genomic library construction, where the insert size can be any where between 30 to 40 kbp. The recombinant DNA has to packed into lambda packaging extracts. Once it is done the packed phages are used for E.coli infection and screening.

~~cosI----Amp-I-pro-I mcsI----lac-Z -a subunit---Icos~~

Yeast Artificial Chromosomes (YACs):

Large sized genomic DNA of 10^6 to 10^7 bp long DNA can be inserted and the vector is used for the transformation of yeast. The vector now acts as a chromosome that replicates and segregates similar to other chromosomes. In general the vector contain a CEN region, a selection marker for yeast cells, origin for bacterial cells, an origin for eukaryotic cells, an eukaryotic promoter if one wants and suitable **mcs** and importantly segments consisting of Telomeric DNA as ends. Yeast YAC vectors have been extensively used for preparing Human genomic library.

~I-Tel-I--pk Ori-I- marker-gene-I CEN-I-I-mcsI-ARS-Ori-I-Tel-I~~

Vectors for animal cells:

Vectors for animal cells can be designed to the kind of animal system and the tissue in which it has to be expressed. If one wants the vectors to be expressed as episomal one can use Epstein Barr viral derived plasmid for high copy numbers or one can use retroviral derived vectors for integration into host chromosomes. Depending upon the tissue in which the cloned gene has to be expressed one should use tissue compatible promoter; or if one wants the gene expression to be regulated,, one has to use regulatable regions. Most of the vectors have Kazak sequence for efficient initiation of translation and transcription termination region for addition of poly-A tail for again efficient translation. A good antibiotic marker gene is essential and a reporter gene is also essential.

Vectors for plant cells or tissues:

Depending upon the characteristic features of host cells one has to choose the vectors. The most common vector used for plant transformation is Agrobacterium Ti plasmid derived vector. This vector contains promoters such as CAMV 35S promoter for general expression; otherwise one can design the promoter to the user needs. Most of the vectors have Kozak sequence for efficient initiation of translation and transcription termination

region for addition of poly-A tail for again efficient translation.
A good antibiotic marker gene is essential and a reporter gene is also essential.

Genetic Engineering:

Transformation of Animal Cells

Animal Cell Culture and Media:

Animal cell culture and maintenance require expertise and experience. These cell lines cannot be maintained for a long time, in some cases fresh batch of cell cultures have to be initiated and some has to be transferred to fresh media for some period of time (8to 10 passage can be OK for some cell lines). Some of the cell lines are genetically transformed. One has to use all his ingenuity to keep them intact and functional.

Mouse connective tissue,
M.fibroblast,
M.embryonic stem cells,
M.monocyte,
M. macrophages,
M. spleen cells,

Mouse 3T3 NIH cell lines,

Rat fibroblasts,

Rat hepatomas,

Human lymphomas,

Human keratinocytes,

H.small cell lung cancer cells,

H. lymphocytes EBV transformed,

H embryonic kidney cell HEK293 cell lines,

Chinese hamster ovary cell lines (CHO),

Cat kidney cell lines,

African green monkey kidney cell lines,

SV 40 transformed African monkey kidney cell lines (COS),

Dog's primary hapatocytes,

Chick embryonic fibroblast cell lines,

Hela cells (Henrietta Lock),

Myeloma cell lines,

Bovine fetal heart cells,

Some of the egg cells and other cells cultured for Transgenic and animal cloning experiments:

Human egg cells,

Mouse eggs,

Xenopus eggs,

Cow eggs,

Pig eggs,

Sheep eggs,

Sheep udder epithelial cells,

Sheep embryonic epidermal cells,

Mouse blastocysts,

Many stem cells from variety sources have been cultured.

A list of cell lines commercially available:

3T3: Mouse, 2n=40, endothelial fibroblast, show heteroploidy cannot grow in suspension media.

L: Mouse, 2n=40, connective tissue –fibroblasts, grow in suspension media, show heteroploidy.

CHO: Chinese hamster ovarian cell, epithelia, grows in suspension cultures and show pseudo diploidy.

BHK-1: Syrian hamsters, kidney cell-fibroblasts, grow in suspension and show diploidy.

BSC: Monkey, 2n=42, kidney epithelial cells, don't grow in suspensions, show diploidy.

MPC: Mouse, 2n=40, bone marrow, Myeloma-lymphoid, grow in suspension, show heteroploidy.

RHP: Frog, 2n=26, egg-epithelial, don't grow in suspensions, show haploids.

Hela: Human, 2n=46, cervical tumor-epithelial, show growth in suspension and show heteroploidy.

KB: Human, 2n=46, Nasopharengial tumor-epithelial, grow in suspension culture, and heteroploidy.

Cell Type	Animal	Tissue origin	Type of tissue	Growth in suspension	Chromosomo number
3T3	Mouse (2n=40)	Connective	Fibrobla	No	Heteroploidy

L	Mouse, 2n=40	Connective	Fibrobla	Yes	Heteroploidy
CHO	Chinese Hamster	Ovary	Epitheli	Yes	Pseudodiploi
BHK-	Syrian hamster kidney	Kidney	Fibrobla	Yes	Diploid
BSC Monk	Kidney	Epithelial	No	Diploid	
MPC	Mouse	Bone marrow Meyloma	Lymphoi	Yes	Heteroploidy
RHP	Frog 2n=26	Egg	Epitheli	No	Haploid
HeLa	Human fe	Cervical tumo	Epitheli	Yes	Heteroploids
KB	Human 2n=46	Nasopharynge tumor	Epitheli	Heteroploid	

Some promoters used for expression in animals:

Promoter	Response Element	Gene cloned	Animal system Used
Metallothionin		HGH	Mice, Rat
BPV		Bovine growth Hormone	Bovine
Avian leucosis v Promoter			Chick
MMtr	DRE, GRE	HGH, b-Gal	Fish
MMtr MuMLV		HGH, porcine Growth hormone, Rat growth Hormone, Bovine GH, Thimidine Kinase,	Pig, Sheep
Ovine b-lactogl Promoter,		Human factor-X, Human antitrypsir	Ovine
Prolactin promo		Tissue type plasm	

		nogen activating factor	
SV40 early Promoter	SV40 enhancer		
Beta Actin			
DJHFR			Increases copy Numbers with Methotrexate

Mouse: Promoter Metallothionin gene:

Genes cloned- Human growth hormone, rat GH, bovine GH.

Chick: Promoter- ALV (Avian leucosis viral promoter.

BPV- Bovine Papiloma viral promoter.

Fish- MMtr- cloned genes are HGH, b-Gal.

Pig- MMtr promoter- genes cloned are- HGH, BGH, porcine GH, rat GH.

MuMLV promoter-gene cloned is rat GH.

Rabbit: MMtr promoter- genes cloned are HGH,

hMT promoter- genes cloned are -HGH.

RbEu promoter- gene-rbc-myc.

Sheep: MMtr- genes cloned are –HGH, TK, b-GH, hGRF (growth releasing factor), oBLG- human Factor-X, oBLG- human XI antitrypsin gene, Factor IZX.

Goat- La promoter- genes cloned are Prolactin, tissue specific plasminogen activating factor.

GH = growth hormone,

H = human,

MuMLV = Murine leukemia virus,

B = Bovine,

O = Ovine,

TK = Thymidine kinase,

General procedure for isolation of animal cells:

Dissected out tissues are Trypsinized (0.05% Trypsin plus 0.53mM EDTA in buffered saline).

Monitor the cell shape. When cells become spherical, they are filtered through 4-layered cheeses cloth.

Then the cells are washed with serum containing growth medium

Plate them in growth medium and allow them to grow as single layers or what is called monolayer.

The most common medium used is GIBC-BRL's Dulbecco's modified Eagle medium (DMEM). To this Glutamine is added as additive to 2mM concentration.

In many cases fetal bovine serum (heat killed and filtered) is also added to 10 % (V/V). This supports growth of cells.

Antibiotics such as Ampicillin or penicillin 100ug per ml are added to prevent bacterial contamination.

The pH of the culture medium at 7.2 is maintained by adding bicarbonates (2.0 to 3.7gm per liter).

During Transfection the cells have to be prepared in serum free state.

When cells are added to plates, they adhere to the surface and divide and redivide and grow to density called confluence at which time cell-to-cell contact is maximum. This contact inhibits them further growth. In cancer cells contact inhibition is lost so cells pile up one another.

The number of passages is limited to 8-10 times, and then the cells have to be extracted from fresh tissues and cultured.

During Transfection cells should be in 70-80% confluence. Such cells are repeatedly sub cultured once in every 4-5 days.

Cells at the density of 1-4 x 10^4 cells/cm^2 before the cells are used for Transfection.

Viable cell concentration can be accounted by treating a sample of cells with Trepan blue stain. Stained cells are considered as dead cells and unstained are living cells.

The cells used for the said purpose should be competent, whose efficiency can be estimated by using control experiments.

Embryonic Stem cells:

Cells are obtained from embryonic blastula stage.

Blastocysts can be cultured in Petri plate with suitable culture media providing primary embryonic cells from fibroblast as feeder layer.

When blastula embryos are grown on feeder layer of cells, the ectoderm spreads out and inner embryonic stem cells come out and now they are exposed to feeder layer of cells.

Such stem cells can be expanded and maintained for a number of generations by reculturing. Precaution should be taken about the change in chromosomal number.

Such cells can be used for developmental studies for they have potentiality to develop into different types of tissues, which depends upon the kind of stimulants you provide.

The feeder layer prevents stem cells from differentiating. Addition of Leukemia inhibitor factor (LIF) also prevents stem cells from differentiating.

The number of passages for keeping stem cells in active state is possibly 12-14.

Transfection Protocol:

Transfection by Calcium Phosphate method:

Take the desired recombinant DNA and linearise them.

Then take about 10-20ug in 225 ul of H2O

Add this to 25ul of 2.5M Ca Cl2 drop wise and mix. Then add 250ul of 2xHEPES buffered with saline.

A fine ppt. develops. Add the ppt. to ES cells drop wise, incubates for 4hrs.

Remove the liquid.

Give glycerol shock by adding glycerol to DMEM 15%.

Incubate for 4 minutes and remove glycerol.

Add DMEM and incubate for some time and remove DMEM.

Then add DMEM incubate overnight.

Select the cell colonies on specific antibiotics after 12 -24 hrs of culture.

Liposome Mediated Transfection:

Cationic liposomes are available in many companies, which provide molecular materials for research activities.

- Transfectum from Promega are called DOGs.
- Boehringer Manheim provides them as DOTAP.
- GIBCI-BRL provides lipofectine as DOTMA.
- GIBCO-BRL also provides another lipofectamine as DOSPA

General Protocol:

The above said lipid contain quaternary amino group. Such cationic lipids are blended with neutral but natural lipids.

When such materials are mixed with water they form a monolayer of vesicles where hydrophilic part faces water and hydrophobic part faces inside of the vesicle.

Such mixture of lipofectamine is mixed with recombinant linear DNA in 10:1 ratio. The phosphate backbone spontaneously reacts with cationic head of lipids and form a complex and remain as the complex.

Dilute DNA to 100ul in 150mM NaCl, 20mM HEPES pH 7.5.

Dilute cationic lipofectamine to 100ul in NaCl/HEPES buffer.

Then add serum free medium and mix.

Prepare cells with washing in serum free culture medium

Then lipofectamine-DNA complexes are added to competent animal cell culture.

The positive charged liposomal components react with negatively charged lipid membrane and they fuse with the cell membranes and deliver the DNA into the cell.

Incubate cells 1-24 hrs at 37^oC.

Remove the medium and add growth medium.

Grow them and allow for transient expression of the selection marker gene.

Then plate on selection medium.

For ES cells:

Add 10ug of linearised vector DNA to 2ml of OPTI-MEM serum reduced medium.

Then add 100ug of cationic liposome.

Mix and set at 30^oC.

Add this final preparation to cells suspended in OPTE-MEM-1 medium.

Incubate for 4 hrs.

Plate them on feeder layers.

Allow cells to recover and express the selection marker gene.

Remove the medium and plate the cells on regular growth medium containing proper antibiotic or the drug.

Electroporation Protocol:

Prepare the cells with culturing and reculturing and finally grow the cells free from serum.

Take cells in PBS pH 7.5, swirl for 1-2 minutes decant.

Then add Trypsin dilution, incubate for 1-3 minutes at 37^oC.

When cells detach from the surface of the glass container add DMEM-H medium, mix gently to create single cell suspension.

Pellet the cells and remove the supernatant.

Then suspend cells in 200ul of the same medium.

Add purified Recombinant DNA to 3nM concentration.

Mix the contents and transfer cells into the cuvette.

Pulse at 270 volts, 50 u Faraday and 360-ohm resistance for 50 to 100 milliseconds.

Remove the cells and decant and add DMEM-H media and plate cells on a feeder layer and allow cells to recover and express a marker gene.

Then plate the cells on a growth medium containing proper drug for selection.

Plant Cells:

Vectors for Plants:

Expression of genes in plants requires components that are rarely required for animal systems. Most of the vectors are shuttle vectors. The following are the important components. Transfer of genes in to plant system is more difficult than animal systems.

Origins for replication in E.coli and Agrobacterium:

- If one wants the plasmid to replicate in plant cells as episomal DNA, then one requires an origin similar to that of free replicating plant viruses.
- Require a selection marker gene.
- Require a strong promoter with regulatory elements.
- Require a cloning site or sites.
- Require a transcriptional terminator signal.
- Require T-DNA left and right borders, it is with which selection marker gene and expression cassette is included.
- Require a reasonable small sized plasmid having all the above features.

Few plant vectors used:

pBI 101.

pBI 103.

pBI 121.

pBIN.

pGA 472.

pGK 100.

pGPTV.

Some promoters used for expression in plants:

CAMV 35S (Cauliflower mosaic virus):

PRS (pathogen response sequence).

Osmotin: stress controlled.

Chl a/b (chlorophyll A and B gene). Light controlled.

RUBISCO light chain (Ribulose bi phosphate light chain gene).

NOS (Nopaline synthase gene).

OS (Octopine synthase gene).

Hordein promoter.

Glutenin.

Legumin.

PRS: pathogen response promoter.

Cecropin: response to bacterial infection

Promoters can chosen for tissue specific or stage specific expression in embryo- early or late, cotyledons, leaves, anther, leaves, ovary, fruit and other tissues

Selection marker genes:

Glyphosate (herbicide resistant gene).

Phosphonithricin (inhibits glutamine synthesis).

Some of the reporter genes used are Glucorunidase and Luciferin, and Protein-fluorescent green (PFG).

pGK 100:

(This vector was developed by G.R. Kantharaj):

This vector was modified from pGA472 (from Galvin) provided by Dr. Veluthambi.

Size is 11.6 kbp.

T-DNA left border (LB) and right border (RB).

Ori-T (Ti plasmid origin).

Ori V.

Ori Coli E I.

Tet +.

An over drive sequence to the right of the RB.

Osmotin promoter.

Cloning sites.

Poly (A) signal.

Hygromycin resistant gene with Nos promoter.

--LB—P/Osmotin- gene---Ttr---P/Nos—Hygromycin—Ttr-RB-O/Drive— (circular mode)

P/O = Promoter Osmotin.

LB = Left border.

RB = Right border.

O/Drive = over drive.

Nos = Nopaline synthase gene promoter.

Few bacterial strains used:

Agrobacterium tumefaciens LB 4404.

Agrobacterium tumefaciens C 58 (virulent).

The said strains have plasmids, which lack in tumor inducing genes, but they do contain genes, which can provide components for transferring DNA segments that are found in other plasmid DNA, if located in between the LB and RB. Genes such as Shi, Roi and opine producing

genes are deleted. The said strains also lack in endonuclease activity (Rec ^-- RE ^ - and they are sensitive to some antibiotics such as carboxylin.

Agrobacterium tumefaciens:

It is a soil bacteria, causes tumors in plant especially in Solanaceae members. These bacteria can also cause tumors in some members of liliaceae and few other monocots. They are gram-negative bacteria. These bacteria contain a plasmid, which is capable of inducing tumors, so the plasmid is called Tumor inducing plasmid (Ti-plasmid). Depending upon the kind of tumors they produce they are classified as Nopaline synthesizing plasmids called Nos plasmid.

Nos-Plasmid (Nos):

Nos plasmids synthesize Nopaline, an Arginine derivative. Nopaline is N-alpha dicarboxy ethyl-L Arginine. This provides both Nitrogen and carbohydrate as a source for the bacteria. This Ti-plasmid produces teratomas, which can differentiate into roots and shoots.

Octopine plasmids (OCS):

They generate Octopine- N-2-1-3 dicarboxy propyl-arginine. The tumors don't develop differentiated organs such as roots and shoots.

Agropine plasmids (Ag S):

They also produce substance called Agropine. They produce poor tumors and they don't differentiate.

Agrobacterium rhizogenes:

They contain Ri plasmids and induce hairy roots on infection. However they do contain Agropine genes.

The most remarkable feature of these bacteria is that they infect the wounds, but never enter into the cell, instead they transfer a segment of its plasmid DNA into the cell, which on integration into host nuclear DNA, transforms the cells into tumors. Perhaps this is the only one of its kind of prokaryote that has the ability to transform a plant cell and generate tumor with out entering into cells. We all know that a virus on binding to the bacterial or eukaryotic cells they transfer their genetic material, there is no other evidence to show a bacterial cell transferring it genetic material into a eukaryotic cell.

The DNA that is responsible for inducing tumor is called Ti DNA. The segment of Ti-DNA that is transferred and the DNA that is actually responsible for inducing tumor is called T-DNA (Tumor inducing DNA). The Ti plasmid is 200-230 kbp long, circular and double stranded. With in the Ti-DNA it has T-DNA.

T-DNA:

The T-DNA is about 20-23kbp long and it is bracketed by 25 base pair long imperfect direct repeats called left border and right border.

LB = TGGCAGGATATATTCGNNG/ATGTAAC/A.

RB = TGACAGGATATATATTG/CNNGA/GTAAC/A.

The T-DNA also contains an Origin called Ori-T at the right border. It also contains four more genes, two of them are involved in the synthesis of Indole acetic acid and one gene is responsible for the synthesis of cytokinins. Auxin-I gene, Tryptophan mono oxygenase (IAM), converts Tryptophan into Indole acetamide. Auxin-II called Indole acetamide hydrolase converts Indole Acetamide into Indole Acetic acid (IAA). Together thy are called root-inducing genes, Roi-

genes (IAA) and the shoot inducing genes are called Shi-genes (Cytokinins).

Cytokinin synthesis is performed by Isopentinyl Transferase, which transfers Isopentinyl pyro phosphate to 6' of Adenine monophosphate to generate Iso Pentinyl Adenine (IPA- it is one of the Natural cytokinins).

All the said genes have their own specific but efficient promoters and transcriptional terminator regions.

Tryptophan----→Indole Acetamide-------→ IAA.

Isopentinyl pyro phosphate + AMP------→ IPA.

T-DNA:

I---Vir—**LB—I<--IAAH—ITMO→---IPT→--←Nos—RB**—O/D----I

Blue colored region is T-DNA

LB = Left border.

RB = Right border.

iaaH = (aux2 and tms 2), Indole-3acetamide hydrolase gene which converts IAM to IAA. iaaM = (aux 1, tms 1, Tryptophan-2mono-oxygenase, which converts Tryptophan into indole3-acetamide (IAM).

IPT = Isopentinyl transferase gene.

Nos = Nopaline synthase gene.

O/D = Over drive sequence.

ViR = Cluster of virulence regulator genes.

The said genes are similar to bacterial genes, which are involved in Auxin and Cytokinin synthesis. But Agrobacterium rhizogenes don't have Cytokinin genes; instead they have genes like rol-A, rol-B and rol-C. These are responsible for developing hairy roots and they can regenerate plants.

The Ti plasmid, outside the T-DNA region, also contains several other genes such as opine catabolizing genes such as Nopaline catabolizing gene (NOC), Octopine catabolizing gene (OCC) and Agropine catabolizing gene (AgC). On to the left of the T-DNA there is a segment of DNA, 36kbp, consisting of a cluster of genes called ViR genes. On the right flank of the RB of T-DNA abutting to it is 25 bp long sequences called Over- Drive

(5'TAApuTpyNCTGTpuTNTGTTTTGTTTG3'). This region is recognized by ViR-C gene products and facilitates ViR-D gene products to cut the strands in the RB.

Along with there are regions for the replication Origin called Ori-V and Ori-T (Tra) for replication and transfer of T-DNA. There are many other genes such as Chv for chromosomal virulence (Chv-A, Chv-B, Chv-D, Chv-E, cel, exoC and Psc I involved in recognizing the host, which actually determine host bacterial compatibility. Genes such as exo-C and att are involved in the binding of bacteria to host cells.

L-DR-I------I--iaaH->I<---I-iaaM—I—Ipt→--I→tml-→--I--←Nos---I-R-DR

Arrows + indicate the direction of transcription.

Numbers indicate = number of transcriptional units.

L-DR and L-DR = left and right direct repeats.

iaaH = Indole acetamide hydrolase.

iaaM = Tryptophan -2-monooxygenase.

ipT = (tmr, Cys) Iso pentinyl transferase.

Nos = Nopaline synthase.

Tml =large tumor locus

Gene	Function	--

Chv-A	Codes for i membrane pr essential for transport of b-1, glucan from bact cytoplasm into peripl	
Chv-B	An inner membrane protein likely to be involved in the synth of b-1, 2 glucan	
Chv-D	Required for express of Vir genes	
Chv-E	Required for the expression of Vir ge	
Exo-locus Exo-C	Exo-biosynthesis of attachment polysaccharides Exo-C is involved required for the synthesis of b-1, 2 glucan	
Cel	Cellulose fibril synth	

	to enable bacterial c to be firmly adhere plant cell walls	
Psc-I		

ViR Genes:

It is a cluster of several genes and each of which have their own hexa nucleotide boxes in the promoters. Each of the operons is inducible. They are involved in activation and transfer of the T-DNA from the bacterial cell into plant cell. The ViR region consists of seven operons called ViR-A (2kbp, 1 gene), ViR-B (9.5kbp, 11 genes), ViR-G (1kbp, 1 gene), ViR-C (2kbp, 2 genes), ViR-D (4.5kbp, 4genes), ViR-E (2kbp, 2genes) and ViR-F (? 1 gene).

Organization of Vir-genes from left to right:

-I--Vir-H→I-I-ViR-A→I-I--ViR-B->I-I-ViR-G->I-I-←ViR-C--I-ViR-D-→I-I-E→I-I-F->IRB

ViR-A: It is constitutively expressed at low level. The protein is localized on the plasma membrane of bacterial cell and it acts as a receptor. It has an autokinase activity and phosphorylates ViR-G with

the activation of ViR-G; receptors are produced in large numbers. It acts in conjunction with Chv-E (a sugar binding protein).

ViR-B: It consists of 11 genes called ViR-B1 to ViR-11. Their location is plasma membrane. They perhaps organize into channel like structure and involved in the transport of T-DNA in single strand form. The B-11 has ATPase activity.

ViR-G: Its location is cytosol. Before activation the gene products are at low level, but once they are activated, they in turn activate its own gene and also activate all other ViR genes. In super virulent strains this product is over produced. When this gets phosphorylated by activated ViR-A it becomes a dimmer and now it acts as a DNA binding protein. This gene not only activates its own gene, it also activates all other operon. Thus all ViR gene operons can be considered as a regulon for they all get activated by ViR-G

ViR-C: This consists of two genes called viR-C1 and viR-C2. Location of these proteins is cytosol and they bind to over drive region at the right end of the right border of T-DNA, acts as a helicase and helps in unwinding the ds DNA. It also helps in activating cleavage of DNA at the right border. They help ViR-D products in this process.

ViR-D: It consists of viR-D1, -D2, -D-3, and D-4. ViR D1 is considered as Topoisomerase and it binds to the right border of the T-DNA. The vir-D2 is considered as an endonuclease. They recognize the RB sequence ACC and cuts at that sequence to generate – ACC 5' The 5' of the nicked T-DNA gets covalently bound to D2. Binding to the 5' end it prevents 5' exonuclease activity. Perhaps this protein is also involved in recombination events in the host cells.

ViR-E: It consists of two genes called E1 and E-2. They act as single strand binding protein (ssBPs), which in turn bind to the peeled of single strand of T-DNA region from right border to the left border. This protects from the exonuclease or endonuclease digestive activity. They may also help in transporting the DNA across the channel produced by ViR-B products. This protein is also believed to be involved in recombination in the host.

Vir-F: It consists of few F genes. The ViR-F genes help in DNA transfer. It is believed that some ViR-F-pin genes activate several host cell genes.

ViR-H: Not much is known about this gene.

Most of the said genes are silent when the bacteria are in Free State. But once the said genes are transferred into the host cell and when they integrated into host cellular genome the said genes i.e. Auxin and Cytokinin genes are activated by host cellular factors. The other genes such as OCS NOS or Agropine gene products are secreted out of the host cells. The Agrobacterium feed on these opines and it is an important nitrogen and carbohydrate source for the bacteria.

Plant Oncogenes:

Any gene or genes, which are capable inducing tumors, is called Oncogenes. In Agrobacterium system the genes such as Auxin synthesizing and Cytokinin producing genes when get integrated into host genome, they are activated by cellular factors. These gene products produce Indole acetic acid from Tryptophan, and Isopentinyl adenine is produced from Isopentinyl pyrophosphate and Adenine mono phosphate respectively. The concentration of Auxin and cytokinins produced their concentration is balanced so as to transform the cell into actively dividing undifferentiated cells. This produces a mass of undifferentiated cells, which is called callus, or this can assume the shape of tumor, so the genes responsible for inducing callus are called as plant Oncogenes. It is not unusual to see such tumor on various plants.

Mechanism of infection and gene transfer:

- Agrobacterium infects plants through external wounds.
- The bacterial infection and compatibility depends upon certain gene products such as Chv-A, B and C and Exo-C. They have a role in the attachment of the bacteria to the host cell. Mutations in the said genes affect the attachment of the bacteria to the host cells. Certain mutations and variation in ViR-A exhibits limited host range (LHR). On the contrary super virulent strains have wider host range capabilities.
- However host range compatibility and incompatibility lies in bacterial factors and host plant factors as well; a situation similar or akin to Rhizobium infection of certain legume members during the development of rood nodules.
- Phenolic compounds released to the surface of the host cells during injury act as signals for the Agrobacterium cells to bind to host cells. Some of the glycoprotein on the surface of the bacterial cells may play a pivotal role in the recognition and binding.
- Tight binding between the bacterial and host cells is very important for cell-to-cell communication. Host cell signals may

precede the attachment, because the signals binding to bacterial receptors may activate certain bacterial genes located in the bacterial chromosome and their activation may facilitate or actuate the process of binding.

- Actively dividing cells at surface of the wound is an important contributory factor for the ensuring the binding of the bacterial cell to the host.
- However, incompatibility may be due variety of host responses such as- hypertensive of the host cells to infection, host cells in adequacy in forming callus at the wounded surface, inhibition of the gene activity of the T-DNA may be due to methylation, or and not producing signaling molecules as inducers. Not all plant types are compatible for the bacterial infection. Some families of plants such as Solanaceae are high susceptible to other, which means the hosts also contribute some chemicals for the bacteria to bind to their surface area.

- When plants are wounded, they secrete a variety of substances in response to injuries as defensive mechanism. Some of the compounds like aceto Syringone and hydroxyl aceto Syringone if secreted and in a place where the bacteria happen to be present, Aceto Syringone bind to ViR-A receptors found on plasma

membrane.

- Binding of the ligand activates the cytosolic side of the ViR-G protein. This activation leads a series of phosphorylation and dephosphorylation reactions. In their process ViR-G proteins are activated. The activated G- proteins in turn bind to several of the ViR- gene clusters through their operons. Primarily this leads to the production of more ViR-A products and ViR-G products. As more and more of ViR-G are produced most of the ViR genes are activated and products are produced inside the bacterial cell.
- Gene products such as ViR-C bind to the overdrive sequence found on to the right flank of the Right Border of the T-DNA. This may exert some influence on the DNA structure, this facilitates the binding of ViR-D products, where ViR-D1 and D2 as dimmers they bind to the right border sequence facilitated by ViR-C prot5eins. The D2 protein acts an endonuclease and cuts the lower strand at a sequence that generates ----ACC5' to which the D2 complex covalently binds. Then from the point of the nick the lower strand is peeled off till it extends beyond the left border.
- As the single strand is generated, it is bound by ViR-E proteins, which are similar to ssDNA binding proteins (ssB) of E.coli.

Binding of the proteins to ssDNA stabilizes the DNA into rigid rod like structure but also prevents the SSDNA from any nuclease digestive activity.

- At the same time the ViR-B products organize at the interphase of bacterial and plant cell and may organize into channel like structures or similar conjugation tube like structures for the transfer of ssDNA across the membrane into host cell cytoplasm. One of the ViR-B products has ATPase activity, which may provide energy for the physical movement of the DNA into the host cell across the cellular barrier.
- Another contributory factor perhaps is plasmodesmata sites. It is known that several viral genomes are transported why even the entire viruses are transported across the cells via plasma demotic channels aided and augmented by "Transport "proteins, which also possess ATPase activity.
- Once the DNA is in the cytoplasm, it will find its way into the nucleus. Before the DNA enters into the nucleus whether the ssDNA gets circularized or it is converted into double stranded and circularized and then enters into the DNA is not clear.
- Some of the RNA viral genomes generate ds DNA, which contain LTR sequence at the ends and then they are circularized by integrase before they enter into the nucleus.

- Even in the case of T-DNA integration circularized DNA is preferable than the linear DNA. In the nucleus it gets integrated into host genomic DNA, perhaps using T-DNA s border sequences for the some of the border sequences are invariably found in the host DNA.
- Using the left and right border sequences of the T-DNA, it is now known that the DNA gets circularizes before it enters, as the left and right border sequences are well preserved within the host DNA. Especially the right border sequence i.e ---ACC5' and the left border sequences are found. Right border ACC5' is very well conserved, but not all the left border sequences conserved.
- The site of Integration of the T-DNA into host cellular DNA is random and there is no sequence bias and again depends upon cells mitotic activity. Yet the site of integration is more or less AT rich.
- Replicating DNA is more amenable for the T-DNA integration. Mitosis provides nuclear membrane barrier free access to host chromosomal DNA.
- Integration of T-DNA at multiple sites is very common and the integrated DNA is very stable, and it is propagated along with its host DNA.

Design of vectors for Plant transformation.

To introduce any foreign gene into plants one has to construct a plasmid, which has the ability to transfer the DNA and ability to integrate into host genome. Generally, such vectors are binary vectors. They contain the following features:

- A plasmid containing RB and LB and an overdrive sequence at the RB.
- A suitable promoter with regulatory sequences such as response elements or enhancers.
- Contain cloning site and a transcription terminator signal.
- Contain the desired gene and a selection marker gene towards RB.
- For manipulation in both E. coli and Agrobacterium it should contain origin and a gene for antibiotic resistance.
- Such constructs can be transferred into plant tissues through Agrobacterium or through gene gun.
- For transferring the gene through Agrobacterium, one has to get a bacterial strain that is disabled, it means the entire T-DNA is deleted and all other features are retained in the Ti-plasmid.
- The strain should be Rec^- and RE minus. And it should be compatible for the host cells.

Agrobacterium mediated plant transformation;

- Use your recombinant construct with desired gene and selection marker gene.
- Transfer the construct into disabled (for the said characters) Agrobacterium.
- Grow them to sufficient density.
- Transfer the culture into plant culture medium with stimulant like aceto Syringone.
- The transfer the desired callus and allow the bacteria to infect overnight.
- Then wash the tissue with carboxylin or Carbamycin. The Carbamycin kills all agro bacterial cells.
- The transfer to regular differentiating medium contain in 100ug/ml of Hygromycin.
- Select the tissue that grows.
- From that one can generate shoots and check for the gene or gene produce by western blotting or PCR amplification.

Gene Gun method:

- Principle of gene gun is simple.
- It has an inlet for inert gas and a meter showing the pressure of the gas that allowed into the gun.

- At the base of the gun is a small slit into which one can insert a devise having a window, which is covered with a nitrocellulose membrane.
- Before inserting the devise into the window the DNA coated with the desired DNA laid and dried.
- Place the required tissue just below the gun.
- Allow the inert gas into the gun as the gas fill up the pressures builds up.
- At a particular pressure the membrane burst and the gold particle coated with DNA are shot into the cells with out harming the tissue.
- Take out the tissue and grow on culture media containing Hygromycin or any other antibiotic you are using.

Electroporation method:

The required tissue such as small embryos and small pieces of callus or protoplast can be used for electroporation. However one has to determine the input voltage, resistance and the time. Many times if dry embryo is soaked with water containing the required DNA, the DNA is efficiently taken into the embryonic cells. The expression or the presence of the gene can be checked either by western bolting or by PCR.

Detecting the gene and its expression in the transgenic plants:

Any tissue grows in the culture media is definitely transgenic tissue for the tissue can grow only when it has the antibiotic resistant gene. As the media contains the antibiotic it is certain the tissue that grows is a transgenic tissue.

In order to identify the gene of interest, whether or no it is inserted, isolate small amount of DNA from any tissue and amplify the gene or a pert of the genetic DNA using specific set of primers.

To find out \whether or not the gene has expressed, one can perform western blotting using specific antibody or one use set of primers for amplifying specific mRNA.

Nutrient medium for plant tissue culture:

Murshige / Skoog medium:

Basically, this entire medium contains basic components such as macronutrients, micronutrients and vitamins and carbohydrate sources such as sugars. Addition of hormone such as Auxin and Cytokinin vary depending upon what one requires. For the development of shoot and plant regeneration one uses higher concentration of cytokinins and on

the other hand if one wants only callus formation one uses more of Auxin in relation to cytokinins. Manipulation of hormonal concentration is important in obtaining regeneration. Regeneration of explants that one uses for transgenics has to be standardized. Each plant and the tissue used require some changes in the media and it has to be determined by trial-and-error methods.

Basic salts:

Name	mg/L
NH4NO3	600.
KNO3	1900.
KH2PO4	170.
KCl	300
MgSO4	300
CaCl2	600
MnSO4	010,
ZnSO4	002,
Na2MOO4	0.25,
CuSO4	0.025,

H3BO3	003,
KI	0.75,
Sequestrene	028,

Sugar sources:

Sucrose	250,
Xylose	optional
Mannose,	" "
Sorbitol,	" "
Mannitol	" "

Organic acids:

Na Pyruvate	20,
Malate	40,
Citrate	40,
Fumerate	40,
Cassaminoacids	250
Coconut water	20,

Vitamins:

Thiamine HCl	10,
Riboflavin	0.2,

Ascorbic acid 002,

Folate

Pyridoxine HCl,

Inositol 100,

Biotin,

Vit-A,

Vit-D,

VitB12,

In addition one has to add hormone to the desired concentration.

Protoplast culture:

Use Cellulase 2gm (1%),

Pectinase 1%,

Pectolyase 0.1%

Macerase 0.1%

Sucrose 13.7%.

Surfaces sterilize the tissue. Use the very young growing leaves or meristems and chop them into small pieces. Place the tissue into the liquid mentioned above containing the culture medium. Allow the tissue over night. Hold the tissue in a forceps and shake so that all cells

freed come into solution. Filter in cheesecloth. Gently centrifuge at 500 rpm for 5 minutes. Discard the upper liquid continue foe another here time so as to get rid of the enzyme. Then suspend the cells in regular nutrients media. Now count the cell number using Hemocytometer. The plate the same on nutrient agar plate or use them for liquid culture.

Good protoplasts show no cell wall and all cell are spherical. To finds there is any remnant of cell wall one can use fluorescent whitener, which actually binds to cellulose fibers. If there is any remnant of cell wall one can observer the fluorescence at the surface of the cell. Protoplasts should be free from cellulose.

To find out how many cells are living one can stain with Triptan blue. Those cells take the stain are dead and those don't are living.

Application-Plant Biotechnology:

Plant Disease Diagnosis:

In general, most the plant diseases can be diagnosed by physical symptoms. However, if there is any mixed infections or some rare infectious agents detection can be made by using ELISA techniques.

For this kind of investigations, one has to have the required antibodies for each and every infectious agent.

Similarly one can use PCR as the diagnostic test. To use PCR one has to have specific set of primers for each of the disease causing organisms. Combination of both will certainly provide the information on infectious agents.

Disease Resistant Plants:

In the past agricultural scientists used selective breeding methods for developing disease resistant varieties. This process is time consuming and lot of manual work and waiting. If the breeder is lucky, he may get a disease resistant plant in about 12 to 20 years. With the knowledge of molecular technology, it is possible to introduce a specific gene to combat a particular disease.

For example, disease resistant against ToMV and ToLCV in Tomato, disease resistance against fungal infection, disease resistance to bacterial infection or and disease resistance to pests.

Resistance to plant viruses:

Disease resistance against plant viruses one can use capsid-mediated resistance, or RNA mediated resistance or one can employ Antisense techniques.

Capsid mediated resistance: Example: Tomato plants resistant to tomato mosaic virus and tomato leaf curl virus. Isolate viral particles. Identify genes for capsid proteins and clone them into a binary vector in such a way the cloned DNA is in reading frame.

Using the binary vector transform the tomato tissue either by Agrobacterium or by gene gun method. Regenerate the tissue into plantlets. Different constructs are used separately to develop two specific Transgenics. Check for the expression of the capsid protein by western blot.

Transgenic plants thus obtained are resistant to ToMV and similarly other plants are also resistant to ToLCV. In order to combat mixed infection, which usually the case, hybridize the two transgenic to obtain resistance to both viruses. It is also very important to check the stability of the insert, which can be done using PCR protocols. Production of capsid protein provides protection against invading viruses by the binding of capsid proteins to newly introduced viral RNAs, thus the multiplication of the viruses is prevented.

RNA mediated: In this case the capsid gene is truncated in such a way, when this piece of the gene is cloned; the expressed mRNA does not translate to proteins. So only RNAs produced. Continuous synthesis of foreign RNA induces some RNase that cleaves all the incoming viral RNAs and newly produces viral RNAs. Thus, it gives protection against plant viruses. The methodology can be employed for other plant viruses. Plant viruses cause devastating disease and the crop production is reduced in many cases from 60% to 70%. The most important agricultural crop plants are Rice, Sorghum, Potato, wheat, Tomato and few others. To day it is possible to generate another class of RNA called siRNAs and miRNAs, where one can selectively knock out viral or any other cellular RNA.

Resistance to fungal diseases: Clone specific Chitinases or and beta-Glucanase genes specific to fungi, into binary vector in proper reading frame. Transform the desired plants. When the genes express the enzymes attack the fungal cell walls and destroy the infected fungi, thus one can obtain resistance to fungi. However these constructs have to be standardized.

Resistance to Bacteria: Bacterial cells have peptidoglycans, which can be degraded by Lysozyme. Similarly Cecropin is another, which punches holes into bacterial membranes and destroys bacterial cells. By cloning such Lysozyme genes form bacterial or viral source one can develop plant resistant to bacteria. Plants such as citrus members are the most important crops, which are devastated by bacterial agents

Resistance to pests: Cotton, Coffee, Rice and many other important crop plants are infected with pests, which actually attack plants as larvae and bore into plants and kill them. In many situations nearly 50-70% of the cotton crop is lost because stem borers. The stem borers are species specific. For example cotton larvae are killed by some bacterial proteins, which are in crystal form. Bacillus thuringianesis has many strains and each of the strains produce specific proteins. When the larvae eat such proteins, they die. Bacillus thuringianesis mediated cotton is now in production all over the world and cotton GM plants are grown and farmers have been greatly helped and their crop production increased and this benefits them. Only thing that is important is the bacterial strains used should be specific to the peas, other wise many innocuous insects will be killed and it is disastrous to environment.

Terminator Gene:

This gene construct was constructed by Delta and Pineland (USA), patent number 5723765, March 3rd 1998 and now owned by Monsanto, a giant Biotech firm in USA. This technology was designed to produce hybrid seeds for a particular crop and in the second generation one cannot use the seeds by farmers because the seeds if used don't germinate. Thus farmers are forced buy hybrid seeds from the company, which produces them. Though this technology has been banned from production, the scientific technique that is employed in this is very fascinating.

In this technique three gene constructs with three different promoters are employed and some of the gene constructs are regulated.

One gene construct contained a Toxin gene called Cholera toxin. Even an RNase gene or a Barnase gene or Ribosomal inhibitor genes can be used as lethal genes that can kill the cells where they are expressed.

Lethal gene construct:

A lethal gene, any one of them mentioned above is cloned under the promoter, which is active only during late embryogenesis. But the expression of this gene is blocked by a blocking sequence inserted between the promoter and the lethal gene. The blocking gene is bracketed by lox-P sequences, which can be exercised only

Recombinase coded for by the gene called Cre. In a transgenic plant this gene is expressed very late in the embryogenesis. Even if it is expressed the Toxin gene is not produce because of the presence of blocking sequence.

But if the tissue also contains a Cre gene and if it is expressed, at this stage of development, a Cre product recombinase uses the lox-P sequences and removes the blocking sequence in such a way the Toxin gene is placed next to the late promoter in reading frame.

Interestingly the Cre gene is cloned under a Tet operon promoter, which is blocked by Tet repressor proteins, which is also expressed in the same tissue. The Tet repressor protein binds to Cre- recombinase promoter and inhibits the expression the gene.

If Tetracycline is added to the tissue, the antibiotic binds to the Tet repressor protein and makes it inoperative and it does not bind to the promoter, so the recombinase gene expresses and the recombinase acts on the lox-P sequences and produces a functional Toxic gene. The Tet repressor protein is expressed under the control of CaMV35S promoter as constitutively expression mode.

I---L-P----I>>>>> Blocking<<<<I-----Toxin gene-----I Ttr--

Note the promoter in this is late embryo promoter and this will be active only at late embryo development. Other wise it remains silent. Ttr means it is poly (A) signal for transcription termination.

I---Tet P/O—I-------Cre-gene---------I-Ttr-

Tet-P/O; it is Tet-promoter operator, which can be regulated by the Tet-repressor protein. The repressor is from Tn10-tet. This when present it binds to the operator promoter and prevents the expression of Cre-gene.

I—CAMV35S-P---I-----Tet-repressor-----I-Ttr—

CAMV35S-P is a promoter derived from Cauliflower mosaic viral capsid promoter. This promoter is used for constitutive expression.

I—L/P---I------Toxin gene----I—Tr-tr-

When all these constructs are added to the plant tissue and the transgenic plant, which is a hybrid for a specific trait, grows and produces the desired product. In this tet-repressor is constitutively expressed and it binds to the Tet-o/p and prevents the expression of recombinase. In the absence of recombinase the blocking segment remains intact and the functional Toxin gene product is not produced. If such first generation seeds are obtained and treated with tetracycline, during the development recombinase is obtained and the

recombinase acts on the Lox-P sequences found at either ends of blocking sequences and cleaves and removes the Blocking sequences. Thus the toxin gene is placed under the control of late embryogenesis promoter.

When such seeds are grown the plants develop well and the harvest is obtained. In this the seeds also produced. But while the seeds develop the late promoter becomes active and during late embryogenesis the toxin gene is expressed and the tissues are killed. When such seeds are sown the embryo won't develop. So the farmer is forced to go back to the hybrid seed producer every time.

This very fascinating and brilliant technology, but look at the company's intent behind it. Interestingly the same technology can be used for making a gene inactive at specific stage of development. For example most the GM plants have antibiotic genes. When the plants develop and seeds or fruits produce the KAN^+ or $Hygromycin^+$ genes are present. Consumers won't be happy and certainly it is not desirable. If gene constructs are made to eliminate the antibiotic gene late in the development, the plant products are desirable and bring no harm to the consumers.

Post Harvest Technology:

This post harvest techniques are virtually non-existence not in practice by our Indian farmers. However, they have developed few protocols such as storing food grains in deep pits in the earth or in large wooden containers. At the time of storing farmers used mix the grains with lot of Neam leaves. Now scientists have found that Neam leaves and seeds have insecticidal property.

Greatest flaw in storing fleshy fruits is that they cannot stored for a long time and they either have to be disposed or they have to be consumed before they rot. But our farmers have developed certain post harvest technology of hastening the ripening process of raw fruits. Toady we know the relevance and scientific mechanism involved in this process which was used by plantation owners (small and big) since thousands of years.

Farmers at eve of village shanty or the market, they used to harvest plantains, what is called Banana bunches. Then they are kept in a big oval shaped earthier containers which a kiln like opening at the bottom. In the evening the used fire some cow dung both dry and semi dry type. This burning produces sufficient smoke. When the smoke fills up the earthen container to the brim, they used to close the kiln and the top with wet mud.

When the earthen pot opened in the morning, one finds all green colored fruits have turned into beautiful yellow color. Even harvested green mangoes were buried in paddy or Eleusine or sorghum. This was also found to hasten the process of ripening and producing beautiful color.

Today the knowledge of fruit ripening is known. As the raw fruits, fleshy and green, as age start producing ethylene by gene activation. Ethylene activates the ethylene producing genes; so more and more of ethylene are produced. Ethylene in turn activates gene responsible for degrading chlorophylls and genes responsible for the synthesis of flavones and color pigments. At the same time few more genes are activated for degradation Cellulase and hemicelluloses. Another set of genes that are activated is responsible for converting stored food materials into sweet sugars. In their process of ripening hard fruit becomes soft, sour or tasteless content becomes sweeter and green color changes into lovely orange or reddish orange color. The biochemical process of ripening reaches a crescendo resulting in a climacteric process and has triple effects.

Almost all fleshy fruits are perishable and cannot be kept for a long time, so they have a very short shelf life. Farmers do suffer from

this. Longer shelf life is advantageous for the farmers to keep them for longer period and they can sell their products as and when they get good prices, otherwise they are all in the mercy of commission agents and Dallas; they money suckers and bloodsuckers.

Ethylene synthesis multistep biochemical pathway where several enzymatic and steps are involved. To start with L-Methionine is converted to S-adenosine Methionine. Then it is converted to 5' Methyl adenosine and 1-aminocyclopropane -1-carboxilic acid, which when oxidized liberate CH3=CH3 and CO2 and Ammonia. The enzyme responsible for the synthesis is ACC synthase. Plant molecular biologist have constructed a gene for ACC synthase and cloned into a binary vector under a promoter that is activated only when the fruit is ripening. The gene is cloned in such a way its orientation is reversed. When this gene is expressed the RNA that is transcribes has nucleotide sequences complementary to that of ACC synthase mRNA. When the anti sense RNA is expressed in sufficiently large amounts the antisense RNA hybridizes with ACC mRNA and makes it untranslatable. So no ethylene is produced. Thus, automatically fruit ripening is delayed. This is because; ethylene production is not completely stopped. This has a great advantage for one can keep the fruits for a long period of time and also allow the fruit to ripe slowly.

Such fruits were first produced in USA of America and introduced into Chicago and Los Angeles market as Flavor-Savor fruits. People actually lapped up the fruits. The same technology can be applied to banana and other fleshy fruit plants.

Antisense technology can also be applied to delay senescence of cut flowers. Cut flowers some how rot in their stacks, this is due to ethylene effect. If the same anti sense mRNA for ACC synthase is used it is possible for delaying life of cut flowers?

Post harvest technology can also be applied to protect food grains from insect attack. Similar to Bt gene products one has to find such toxins to specific insects. Their genes can be cloned into the required plants. So, as they are over expressed late in at the time of grain filling. When such grains are stored, even if insects feed upon, they die. One has to take care of such proteins are not harmful to humans and other animals that eat them.

Transgenic Male Sterile Plants:

Production of male sterile lines of specific species is an invaluable tool in the hands of plant breeders. Such male sterile lines are always being the females and the hybrids with other pollen donors can be selected and the desired genes can be transferred. And the genes transferred will be known. The offspring's of such breeding with male sterility will also be male sterile. In such situation one has to resort to restorer lines for making them bisexual.

Technically one has to be a toxic gene like cholera toxin, RNase or Barnase genes. Any one of them can be used. Such genes have to be cloned into a binary vector under the control of anther specific promoter. If the gene is expressed where the anther is maturing the expressed products ablaze the tissues and no pollen grains are produced. Thus the plant becomes male sterile line.

Improvement of Timber yielding plants:

Phytohomones such as Auxins and Cytokinins control growth of plants in height and thickness of stems. Gibberellins also control the height of the plants. Gene for the synthesis of Auxins and Cytokinins has been isolated. Certain transgenic plants with IAA and IPA genes have been obtained. But unfortunately the level of hormone synthesis was not under the control. In spite of this, plants showed very bushy

characters. If the level of hormone production is properly controlled, it is possible control the growth of the plant. Thus many tall tree plants can come to maturity in short period of time instead of taking 50 to 75 years characters and the character of timber can also be improved.

If such timber yielding plants also contain antifreeze genes the plant do not suffer from frostbite. Such transgenic plants have been produced and perhaps they are in use. The gene for anti freezing protein has been isolated from certain fishes.

By genetic manipulation it is possible to generate new color or a combination of colors in horticultural plant, which are in demand for their colorations. It has been done in the case of petunia.

Pharma-Plants:

Plants can produce a variety of organic compounds if their genes are properly regulated. With the introduction specific genes one can make the plants to produce a desired compound, which can be made either secrete out of tissues or it can be made to store in vacuoles or chloroplasts. Many biopolymers such as poly hydroxyl butyrate (PHBs) are produced by gene manipulations.

- Edible vaccines have been produced against cholera toxin genes. The said bacterial gene is cloned into a binary vector and placed

under a general promoter or it can be placed under inducible promoter. Here the toxin gene is not cloned in its entirety, but only specific segment of the gene is chosen which gives maximum immunity. Such vaccines are called subunit vaccines.

- Cholera toxin is a multimeric protein consisting of one 27KD protein and 5 11.6KD subunits, which are non-covalently linked to one another dough like structure). This multimeric binds to gangliosides that are present on epithelial cell surface. Antibodies against such proteins prevent the binding of toxins to cell surfaces.

- When such gene fragments, which produce a part of the protein, which act good inducers of immunity, can be introduced into plants, so as to express in a particular tissue, such as a fruit or a tuber. In the Texas A & M University, cholera toxin gene has been introduced into potato plant and the gene was made express in the tuber. By eating the tubers, by Galt system one can develop immunity against cholera disease by a process called passive immunization. People are working on developing a variety of subunit vaccines in plant and the product to be expressed in fruits such as tomato, apple or Banana, for these fruits are more palatable for eating than the tubers.

- Plant scientists have developed transgenic plants, which produce specific antibodies, which are called plantibodies. Advantage of producing antibodies in plants is one can scale up the production of the tissue by single cell culture or callus culture. If the genes are clines as inducible, it will be great. It is not only Antibodies; one can produce many many important medicinal products in plants. One can use plants as biopharmaceutical living factories.

- In Scrip's Institute, Lajolla, California scientists have developed antibodies when added to tooth pastes against specific bacteria one can prevent tooth infection and decay in children. Jack Johnson etal has reported from Perdue University that they have cloned gp4 of HIV viral gene into cowpea mosaic viral genome and introduced into cowpea plant. The produce was capable of neutralizing HIV infection.

- Certain strains of E.coli produce heat labile enterotoxins, which cause Diarrhea. If such enterotoxins gene is used to produce proteins they can act as active oral antigens or immunogens. These are called efficacious vaccines have the potentiality the mucosal immune system leading to the production secretary

Immunoglobulin type A (Ig-A) this kind of immunization is achieved by oral than prenatal antigen delivery. So the delivery of this enterotoxins protein or any other pathogenic proteins (including subunits) orally makes it as a very potent oral vaccine against many such diseases causing organisms.

- Gene encoding LTB or LTB-fusion protein with SKDEL signal sequence for targeting to microsomal membranes is cloned into a binary vector and then such genes are transferred into potato and Tobacco plants. ELISA has detected the expressions of such proteins. The amount of proteins accumulated is found to be 14ug per gram of total soluble Tobacco leaf protein and 100ug per gram of soluble protein in potato microtubular proteins.

- When such protein extracts of LTBs were orally administered to human trail specimens with a dosage of 12.5ug for 4, 21 and 25 Th day, the mucosal antibodies were detected at 30 to 35 days. This clearly demonstrates that plant derived proteins have immunogenic properties.
- In recent years people have developed techniques to use plastogenome for genetic engineering to inserted into plastids using biolistic methods or transfection method. Proper vectors

are constructed with marker genes.

The above said examples are typical paradigms for a vast variety of recombinant DNA products that can be produce in plants in large quantities which have an applied value on large scale.

Plant based Biopolymers:

Poly R-3 Hydroxy Butyrate called PHBs and other related polyhydroxy alkanates are aliphatic polyesters. These have Thermoplastic properties of industrial applications.

Acaligenes entrophus and few other bacteria produce polyhydroxy alkanates as carbon source when grown on excess carbon diet. The PHBs are synthesized and accumulate as granules of 0.2 to 1um size. Often accumulation is up to 80% of the dry weight the cell protein.

The pathway of the synthesis is: –

Acetyl Co-A --E1→ --E2--→ ---E3→ PHB

E1 = 3-Ketothiolase (PHB-A gene).

E2 = acetyl Co-A reductase (PHB-B gene).

E3 = PHB synthase (PHB-C gene).

The said genes were isolated and cloned independently into PBI 121 binary vectors under the control of CAMV35S promoter. However the genes are cloned in such a way the gene product was targeted into

chloroplasts. The signal sequences used for this were from small subunit RuBP Case (Ribulose bi phosphate carboxylase). The said genes were used obtain transgenic plant for each of the genes. Then they are crossed to each other to combine all these genes to be in the same plant. Such plants showed the expression of the cloned products accumulated in chloroplasts of older leaves. More to it was the enzymes were able to synthesize PHBs to such an extent that 14% of the dry weight of the chloroplasts was found to be PHBs. PHBs are not utilized as carbon source by plants. Such PHBs can be used as thermoplastics and they are environmentally friendly and they are biodegradable.

An advantage against cotton fibers is that cotton fibers exhibit wrinkles, but if the PHBs are expressed in such a way they are to be with the core of the cotton fibers. Such fibers are free of wrinkles. Agricetus Inc in Middleton, Wisconsin, has worked on such polymers. Maliyakal Ejohn in Agricetus has succeeded in gene engineering of cotton plants and produced cotton plants, which produce PHBs along with cellulose fibers.

Alpha Trichosanthin: Alpha Trichosanthins (THS) are inhibitors of ribosomes. They are produced by Trichosanthin kirilowii maximowicz. The product is monomer 27KDs. It binds to 28s RNA and it inhibits

translation when administered in higher doses. This protein is synthesized as pre-protein with 19 amino acid residues signal sequence and secreted after the cleavage of signal sequences. The secreted product is an n active protein. It is believed that it inhibits HIV replication in CD4 lymphocytes as well as macrophages; hence this protein has great potentiality as therapeutic agent.

This gene has been cloned under the control of a promoter of sub genomic coat protein of TMV. By genetic manipulation a hybrid of TMV RNA and an mRNAs for Trichosanthins was obtained. When this hybrid RNA was inoculated into Nicotiana banthamiana species, recombinant viral particles were produced. These viral RNAs produced more viral particles and they were found spreading to other parts of the plant. At the same time they also generated Trichosanthins in plenty. The amount of this protein produced in the plant in this mode was found to be 2% of the total soluble proteins. This production was estimated to be highest amount of protein produced by a transgenic plant.

Genetic Engineering:

Application-Animal Biotechnology:

Transgenic animals:

Ever since the modern human specie arrived on the surface of this planet and started walking around and started living in groups, he collected animals and plants of his liking for his own use. He also reared them and stock piled them under his care. This is the beginning of animal and plant farming. He also observed variation among the same animal and plants cross bred them to develop new varieties, this is yet another technology innovated by him called reproductive breeding, where he used transfer of an entire genome from one form to another. This is nothing short of "Transgenic" methodology. In 20th century one uses techniques where one or two genes are transferred into animal cells and obtain a complete animal called transgenic animals; so also transgenic plants are obtained.

In fact animal cloning experiment s started early 1960s and by sixties experimenters were able to get a large number of amphibian clones. The technique was simple; frog eggs were laid outside the body. The eggs were large enough to be seen by naked eye. One can obtain such eggs in large numbers whenever he like by injecting certain reproductive hormones. Enucleation of eggs was a simple technique,

which was perfected at this time. Then they have sucked 2n nucleus from somatic tissues such as intestine, and injected the same into enucleated eggs and prodded the cells to grow and actually they grew into normal frogs. Several hundreds of such amphibians were grown for experimental purposes. In fact fishes were also used for the same purpose. Some people have perfected culture medium for regrowth of organs such as frog palm or limbs by using special medium containing Vitamin-A.

The first transgenic animal produced was from Ralph Brienster in Univ. Penn, Lippincott, Philadelphia, in 1980-82. They introduced human growth hormone gene into mice eggs.

Donor strain mice were reared and females were injected with pregnant mare serum. Or one can use porcine follicle stimulating hormone.

After 48 hrs human Gonadotrophin was injected. Hybrid mice are better than inbreed mice. ?

Such females were mated with selected male studs.

Each of the female will produce 18-20 fertilized eggs.

After 12 hrs of post coitum, oviducts were taken out and the eggs squeezed out into specific nutrient M16 media.

M16 Media:

NaCl,

KCl,

KH2PO4,

MgSO4,

Glucose,

NaHCO3,

Na pyruvate,

CaCl2,

HEPES, pH 7.5,

Phenol Red.

Wash eggs in M16 media and then they are transferred to M2 media micro drops.

Use powerful dissecting microscope equipped with microinjecting facility having very thin and narrow glass tips which can hold only few µl of liquid.

Inject linearised plasmid DNA containing Growth hormone gene into the nucleus of the egg.

The amount injected is about 2picoliters containing at least 200 copies.

Prepare the set of mice by injecting hormones and mated with vesectomiced males when the females are in estrous stage or what is called animal in "Heat"

These animals behave or rather feel as pseudo pregnant and act as foster mothers.

All these operations have to be done in anesthetic condition and the job requires skill and deft hands.

Under anesthetic conditions these females are operated and the oviduct and uterus is pulled out.

Then eggs injected with a gene are transferred into the uterus via infundibulum.

Stitch the animal and inject with antibiotics (heavy dose).

In about 19-22 days pups will be delivered.

When they are 10 to 15 days old one can peal off tail skin, for it grows back, and homogenize and isolate DNA and do southern blot, or isolate RNA and do northern blot or isolate proteins and do western blot and find out whether or not the injected genes are integrated and expressed their gene products.

Transgenic animals can also be obtained by another method where blastocysts derived stem cells are transfected with the desirable gene placed under specific promoter to express under either induced

condition or expressed in tissue specific manner. They are selected against an antibiotic. They are further amplified and the same are transfused into freshly isolated blastocysts, which are then transplanted into uterus for further development. Many mice cell lines have been created in this manner. One such cell line is used for gene knock out experiment.

Molly and Polly were obtained as transgenic animals with gene Factor-IX under the promoter beta lactoglobin for it expression in the mammary tissue during milking period. A thirty-day fetal cell was used to produce "Transgenic Bull" in 1997 by ABC-Global at Wisconsin. In Honolulu scientists obtained cow.

Animal Cloning:

Animal cloning was new technique for it was already achieved in 1960s. Recent years have shown new techniques employed in developing animal cloning. In fact any cell having the full complement of genome is endowed with potentiality to regrow into a complete animal if the cells are provided with proper media and the required vitamins and hormones and stimulus. This is what is called as totipotency of cells. This phenomenon is easily demonstrated plants, where the plants are

grown from one or two nucleated cells. This technique of Micropropogation is now employed in agriculture and horticulture.
A celebrated animal that was obtained, in 1996-97, by modified cloning technique was a sheep called Dolly named after popular singer Dolly Parteon. British scientist Keith Campbell from Rosalyn institute had field days of glory and rejoiced in spite of some diehard critics. Americans were not far behind for they have also achieved animal cloning especially monkeys. They used different techniques.
Fusing enucleated egg cell with cultured udder epithelial cell at Go stage produced dolly and electric shock activates them. Cells were obtained from six-year-old female sheep. They have made 227 attempts before they were able to get this sheep. Dolly has the distinction of becoming a mother. There were some claims that the Dolly was obtained from fetal cells. Unfortunately, after all these years of glory, Dolly is believed to be suffering from arthritis. The disease Arthritis may be due to any other reason not because it is a cloned animal.
Monkeys were developed by growing an embryo to 8-16 cells and then the cells were separated and grown individually into embryos, which were then implanted into pseudo pregnant mother.

Human cloning was attempted by culturing embryos to 8-cell stage under in vitro conditions and for apparent reasons the embryonic cells are frozen for posterity.

The clonal sheep Meg, Morgan and Tracy were obtained from embryonic cells. Tracy has genetically manipulated to produce sodium channel proteins involved in cystic fibrosis.

Honolulu people in 1997 also obtained a clone by implanting 2n nucleus into an enucleated egg of cumulina mice. Since then many animals have been obtained as clonal animals. In Japan many cows have been developed as clones. Scientists have attempted to produce pig clones without surface antigens belonging to MHC class.

People have claimed that they have cloned humans and their birth has been heralded all over the world, but genetic proof for the said claims. Cloning of human beings does not require great technological protocols; in fact with the existing methodologies one can do this in any labs, which have the facility for in human in vitro fertilization and implantation.

13. Hybridoma Technology:

This ingenious technique was developed around 1976. Monoclonal antibodies were once considered as magic bullets that can target a cell, a tissue, molecule and or an infectious agent.

Polyclonal antibodies are produced by B-lymphocytes. When an antigen comes in contact with the receptors of B-lymphocytes, the genes within the cells get activated and start producing antibodies against each of the epitopes that are presented to the lymphocytes. Epitopes are the antigenic determinants of the same antigen.

Antigens consists of different motifs, different domains derived from amino acid sequences which generate different secondary structural features. Each of these specific domains is a distinct motif. They are recognized by different B.cells, for they carry specific receptors for a specific epitopes. If an antigen has twenty different epitopes twenty different species of B-cells get activated and twenty different antibodies are produced in one-to-one fashion. Production of antibodies to each of the epitopes is not equal, some elicit weak response and some elicit strong response. Thus, the antibodies produced are Polyclonal in nature.

Many a time's Polyclonal antibodies are not useful for specific purpose. Yet Polyclonal antibodies are used on large scale for it is easy to develop such antibodies and cost of production is relatively low. Monoclonal antibodies are for a specific epitope. Under certain situation one wants to target only one of the epitopes and in such circumstances monoclonal antibodies are very useful; that is why they are called as magic bullets. Production of monoclonal antibodies is possible by Hybridoma technology.

B-Lymphocytes:

B-Lymphocytes are capable of producing antibodies in response to specific antigens. These cells cannot grow and multiply in an in vitro culture medium. They can survive for some time and use salvage pathway if they are grown in the presence of Hypoxanthine, Aminopterin and Thymidine (HAT) medium; cells cannot multiply in culture conditions. They can use hypoxanthine for purine synthesis even though Aminopterin blocks DHFR they have salvage pathway. The reason is the cells have enzymes called Hypoxanthine Guanosyl Phosphor Ribosyl Transferase (HGPRT).

They can use Thymidine even in the presence of Aminopterin.

HGPRT defective Myeloma cells:

1. They are incapable of producing antibodies.
2. They have the ability to multiply under culture conditions by cell division.
3. They cannot utilize Hypoxanthine and Thymine in the presence of Aminopterin for the cell lines are mutants for HGPRT.

Inject the required antigen subcutaneously to a rabbit. After three such injection (two boosters), sacrifice the animal and take out spleen tissue and isolate B-lymphocytes. These lymphocytes are activated to the injected antigen. The cells are capable of producing Polyclonal antibodies.

B-lymphocytes are then fused to HGPRT (-) Myeloma cells. The hybrid cells now can grow and multiply in culture medium even in the presence of Aminopterin. At the same time, they also secrete respective antibodies to specific epitope. Such cells are called Hybridoma.

Such cells are diluted and dispensed into multi titer plates in such a way that each well contains one such Hybridoma cell. Such cell lines divide and redivide, grow and produce antibodies to one specific epitope.

Using a specific epitope from the antigen one can screen cells from each well and pick whatever clone from the titer plate one desires. The same cell line can be propagated and maintained.

Monoclonal antibodies are very useful tools in research:

1. One can trace the position of an antigen.
2. Scarce proteins can be purified.
3. Used for disease diagnosis.
4. Can be used for treating patients infected with a particular infectious agent.
5. It is possible to target a specific malignant tumor cell or cells and kill them by directing T-killer cells to them.
6. many more

21. Molecular diagnostics:

Infectious diseases are generally identified by symptoms, culturing and microscopic examination. And many of the investigations are also done by biochemical analysis. These methods are still in vogue. But certain infectious diseases take a long time to manifest the symptoms and often-early diagnosis leads to miss interpretation. Example malarial infection takes a month or so to fully blown manifestation. On the contrary HIV infection may take a year or so. By the time theses are diagnosed, treatment will be delayed and it can be disastrous. Of late molecular diagnostic tools have been evolved One such technique is ELISA (Enzyme Linked Immuno S-orbant) technique has been developed, either using Polyclonal antibodies or monoclonal antibodies.

This is certainly very effective, but not all diseases can be identified by this technique and expensive. Yet ELISA is a powerful and highly sensitive diagnostic method, which is used in medicine, research and industry. Definitely a monoclonal antibody technique is very very costly. Along with ELISA protocols, another technique called PCR has been developed to identify not only diagnosis of infectious agents but also to detect any suspected genetic diseases. For example HIV infection can be positively diagnosed by PCR method. Combined with ELISA the diagnosis is 100% efficient.

Antibodies as diagnostic tools:

Antibodies have to be raised for every antigen bearing infectious agent. Most of the antibodies are polyclonal. Some times Polyclonal antibodies cannot distinguish between pathogenic and nonpathogenic germs. Hence people started using monoclonal antibodies, which was found be costly and not within the reach of common folks.

A List of antibodies employed in diagnosis (just few examples):

IgGs for Polypeptide hormones:

Chorionic gonadotrophin.

Growth hormones (GH).

Luteinizing hormones (LH.

Follicle stimulating hormone (FSH).

Thyroid stimulating hormone (TSH).

Pro lactin.

Cytokines:

Interleukin 1-8.

Colony stimulating factor (CSF).

Tumor markers:

Carcinoma embryonic antigen,

Prostrate specific antigen,

Interleukin 2 receptor,

Epidermal growth factor (EGF).

Drug monitoring:

Theophylline,

Gentamycin,

Cyclosporine.

Others:

Thyroxin,

Vit-B12,

Ferretin,

Fibrin degrading factor,

Few examples IgGs against disease causing organisms:

Chlamydia,

Herpes simplex,

Rubella,

Hepatitis-B,

Hepatitis A,

Hepatitis-C,

Shigella,

Mycobacterium,

HIV,

HPTLV,

Cholera toxins,

Botulins,

Polio,

Nematodes,

Malarial antigens

A large number of viral antigens and bacterial antigens and protozoan surface antigens have been used for developing diagnostic kits. The above list is to give an idea.

In recent years instead of preparing IgGs for every antigen, scientists have used total lymphocytes and prepared a composite but combinatorial cDNA library for both Heavy chains and light chains. Lambda DNA has been used to prepare such library. This method can generate 10^6 to 10^8 clones, each distinct from the other. Proteins expressed can be screened with diagnostic antigens. Now days the antibodies are generated using genetic manipulation in such a way the antibodies when injected to human, they don't elicit immune response and such antibodies are called humanized antibodies.

DNA as the diagnostic material:

This has become a sine quo non technology when everything has failed. This technique has undergone lot modification and sophistication. The method is simple and fast. This technique can be used to detect not only disease-causing pathogens but also due to Genetic defects.

Most important requirement for PCR based diagnostic methods is knowledge of the pathogen's DNA and its sequence. From the sequence one can generate a set of primers for 5' and 3' ends. Known primers can employed to identify. The source of material required is tissue sample, fecal material, urine, blood sample, throat smears or throat

wash, a hair follicle. The amount DNA required can be as small as few picograms. In order to combat disease all over the world, WHO has a program to develop 300- 500 diagnostic kits.

Diagnosis of Malarial parasites:

Full-blown disease manifestation takes 15 days to one month. Malarial disease is caused by plasmodium falciparum. Elisa is effectively used, but the parasite often camouflages by withdrawing its surface antigen and projecting another antigen. Here the probe used is highly repetitive DNA sequences. Such sequences when used they are distinguishable from P. falciform with other P. vivax, P. cyanomoglia and other related parasites. Blood sample is more than enough diagnosis of the disease.

Similar highly repetitive sequences have been used to detect disease-causing parasite such as Trypanosome cruzi (chagas disease). Amplification of the said DNA using specific primers it is possible identify this disease with accuracy and other related forms are not detected. It is very specific. This disease has devastated South American population.

Diagnosis of DMD:

Duchene's muscular Dystrophy, a sex-linked disease, generally appears at greater frequency in male children than the female for the females are the carriers. Here the diseased children suffer from muscular wastage for the surface protein Dystrophin is non-functional. This protein is found in the myelin sheath at the inner surface covering the axons. The DMD gene is located in the XP21 arm.

The fully processed DMD mRNA is 14 000 nucleotides long. The pre mRNA consists of `60 Exons. The whole gene extends to an area of 2000kbp. The protein has a molecular mass of 600 KD; perhaps it is one of the largest proteins known in eukaryotic organisms.

When the DMD gene is cuts with Taq-I enzyme it generates seven fragments. Sites for the Taq-I enzyme is found in few introns. Scientists have developed primers for each of the fragments and the same can be used for PCR amplification. Control DNA generates 7 fragments of the size 10KB, 7.8 kbp, 4kbp, 3.8kbp, 3 kbp, 1.8kbp and 0.6 kbp and they can be discerned on an Agarose gel. If a suspected patient's DNA is used one will find some segments of the Gene are missing using multiplex PCR it is possible to diagnose the disease in a short time. And that too with certainty.

The same technique can be employed in identifying phenyl Ketonuria, Sickle cell anemia, Hemophilia, Cystic fibrosis and others.

Diagnosis of Hemophilia:

Hemophilia polymorphism is due to change in restriction sites such as Xba-I and Bcl-I. This is due to mutation in the sequence of these sites. Hemophilia X factor has 26 Exons. Abnormality was found in the 18th introns. A pair of primers were developed for amplifying a 142 bp long DNA involving the introns. After PCR amplification of the segment, it was cut with Bcl-I enzyme and separated on a gel. A patient, whether he or she is heterozygous and homozygous can be discerned from the gel pattern.

Control (Homozygous	Control (Heterozygo	Patient-1	Patient-2	
	-----	------	-----	(-) Bcl-I 142
------	-----	------		(+) Bcl-I 99b
------	-----	-----		(+) Bcl-I 43b

Multiplex PCR is used to detect mutations in b-Globin genes. Primers are used are fluorescent labeled. Thereby they were able to detect

mutations in b-Globin genes and identified one mutation in alpha Globin gene.

Human Genetic Diseases:

It has been estimated that human genetic disorder and their manifestations in the form of diseases is about 3000 and odd. The diseases can be autosomal or X-linked. Mutations can be dominant, negative dominant, recessive, can be incomplete penetrance, may show variability in expression, show heterogeneity, and or exist in alternate forms of the same gene. Genes are pleotropic.

A list of Human chromosomal DNA Sizes:

Chromosome number	Size of the DNA (MB)
1	263
2	255
3	214
4	203
5	194
6	183
7	171

8	155
9	145
10	144
11	144
12	143
13	114
14	109
15	106
16	98
17	92
18	85
19	67
20	72
21	50
X	164
Y	59

1 map unit = 1 Centi Morgan (Cm) = 10^6bp

23. Therapeutical Agents.

Any chemical or biochemical compound designed to combat diseases fall into the category called Therapeutical agent. Since the days of Edward Jenner, early 200 years ago, scientists are in hot pursuit of discovering drugs against pathogen. Vaccination against pathogens worldwide is the most effective preventive therapeutical agent next only to antibiotics. Discovery of Antibiotics is a great event in the history of mankind, but over use and abuse of antibiotics resulted in the pathogens have become or becoming resistance to drugs. It is constant and eve going struggle against pathogens and drug discoverers, who dominates that is the question is like survival of the fittest.

Vaccines:

Edward Jenner was an incredible country doctor, using folklore knowledge; he injected liquid from the cowpox pustule into an eight-year-old boy called James Philips. It was a history unsurpassed of any discovery the mankind knows in centuries.

Most of the infectious agents infection spread of some of them in epidemic, rarely in pandemic proportions can be prevented by vaccination. Any immunogenic injected into human body, elicits immune response against that antigen. The efficiency of response depends upon the epitopes found on the presenting surface of the antigen.

According to WHO the number of communicable disease against vaccine to be developed by 2000 was 400. And the target now can be much more. Conventionally vaccines were prepared by using heat killed or formal in killed or inactivated organisms as inoculants. Though maintenance of them and obtain them as pure forms endowed with some difficulties, it is still the cheapest for these have to be prepared in large quantities for global market.

With the advent of Molecular and gene engineering techniques, recent trend is to develop recombinant vaccines and many such products are made available for large-scale vaccination, ex. Vaccination against Hepatitis-B virus and recently for Cowpox virus. The modern methods have been developed to overcome drawbacks of traditional methods.

Methods employed for developing recombinant vaccines:

1. Virulent genes of an infectious agent is deleted or made nonfunctional or dysfunctional and such organisms can used as live vaccines. It has to be made certain that the organism does not regain that gene or function deleted or inactivated.
2. Live nonpathogenic organism can be used as carriers. These organisms can genetically be manipulated in such a way an antigenic determinant gene is introduced into the organism where it presents the antigen to immune system, thus such organisms can be sued as vaccines. But the antigenic determinant used should elicit strong response.
3. Develop alternate host or forms for those organisms cannot be grown and maintained on large scale, ex. Mycobacterium lepro(i). These have to be grown and sustained in the sub epidermal tissues of Armadillo, which itself is rare to find in nature. In such cases the target gene has to be cloned into either bacteria or into yeast cells so large-scale preparation are made easy.
4. Some times infectious agents do not kill host cells, instead host immune system attacks infected cells and kills them. It now possible to create targeted and cell specific killer

systems so as to destroy the infected cells.

Subunit Vaccine:

Instead of using the whole organism a specific component of infectious organism is used, such as viral capsid protein, surface glycoprotein, cell wall antigenic protein or glycoprotein can be effectively employed for eliciting strong immuno-response. The said genes can be cloned into expression system and the same can be purified and can be used vaccines. Such vaccines are stable, safe and the antigens are precisely defined and free from extra cellular contaminants. But the process is costly and many a times immune response is good as expected, but that can be overcome by certain modifications.

Subunit vaccine against Herpes simplex virus:

The viruses cause deadly sexual diseases not only in males but also in females, where they act as reservoirs and males as transmitters. Great many people belonging to higher social status have succumbed to these viruses and died with remorse. The virus is an enveloped type and the protein used is protein-D (gD) is a glycoprotein; it elicits good immune response per se. The said protein does not cause any disease or any other side effects.

The gene for this protein has been cloned into transfer vectors or episomal vectors and transfected Chinese Hamster Ovarian cell lines (CHO) produced properly folded and glycosylated proteins which are as good as the typical viral protein. When the gene was cloned in secretory mode the proteins are secreted out of the cell into the media.

Foot and Mouth Disease Virus (FMDV):

This virus has the ability spread across the continents and cause very severe damage to live stock especially Cattles and Swines.
This is very severe in South America; even Indian animal population also suffers but Indian medicine has taken care of the problem. The medicine is a concocted extract of Garlic, Oscimum, Gingiber offcianale and Leucas aspera leaves. If this given a week or so the disease is cured. However, the world over requirement of vaccine against this virus is not less than more than 2 billion doses.

The surface protein that is very antigenic is the coat protein called VP1. This gene has been cloned but the protein per se was found to be very poor antigenically. The virus possesses

~8000ntds long RNA genome and the cloned was expressed as fused protein with MS2 Replicase as a carrier protein. Even this was not effective in eliciting a good response.

Scientist looked into different motifs of the protein and the same were selected and cloned; such as- from C-terminal 141-160=21aa, 151-150=11, 200-213=13aa, from the N-terminal sequences such as 9-24aa, 17-32aa, 25-41. These subunits were used with a carrier protein and all of them were found to be very immunogenically. However fused segments from141-158 to 200-213 was found to be stimulatory but1000x weaker than killed viruses. But when the subunit from 142-160 was fused Hb-surface antigen it was found it was highly immunogenic.

Live recombinant Vaccines:

Methods employed- one is use of nonpathogenic organism and genetically modify, so it expresses the desired antigenic protein in proper perspective. The second alternative is the use of pathogenic organism and deletes the virulent gene responsible for causing the disease and retains its antigenic characters.

Live cholera vaccines:

Live vaccines are believed to be more advantageous than subunit vaccines. Cholera is a deadly disease recurs periodically in tropical countries and kills loot of people. Symptoms are fever, abdominal pain, vomiting, diarrhea, which if not take care of will result in death. In many part of the world it is an endemic disease caused by a bacterium called Vibrio cholerae, for on infection it colonizes small intestine, which is the target tissue and the bacteria secretes lot of hexameric enterotoxins.

This is the causative agent consists of subunits-A and the other 5 subunits called B. The sub unit-A has ADP-ribosylation activity and stimulates Adenyl cyclase. The A-subunit has A1 domain, which contains functional domain the other part help in joining A to B. They bind to intestinal mucosal receptor and activate intestinal problem.

First deleting the A1 subunit part of the gene by homologous recombination method, in this process tetracycline gene is introduced in the place of A1 subunit segment has developed recombination live vaccine.

Plasmid containing homologous segments and a tetracycline gene id transferred to bacteria Cholera vibrio. This leads to recombination and deletes the A1 subunit. But this cannot used for it has tetracycline gene. So, the enterotoxin gene was isolated and the A1 subunit deleted and plasmid is created containing the deleted enterotoxin gene with homologous segments at both flanking regions. This plasmid is transferred to bacteria with deleted enterotoxin gene. By recombination method the defective enterotoxin gene incorporated and the Tetracycline gene is removed. Such genetically manipulated bacteria are used as live vaccine. It is incredible feet indeed

HB-sAg vaccine:

HB-Ag gene form hepatitis was cloned into a plasmid contain vaccinia strong promoter. The flanking region of this construct contained homologous segments of Thymidine Kinase gene. When this plasmid and wild vaccinia viral DNA is co-infected into desired cell lines, by homologous recombination process the Hb-Sag gene is incorporated in the place of Thymidine kinase. This can be screened by Glycover for the absence of Thymidine kinase. This results in the expression surface antigen of Hepatitis, which can be used for vaccination purpose.

Employing the above methods, a wide variety of genes have been cloned to produce vaccines, ex. Rabies viral G-protein, Sindbis surface antigen, Influenza Np and HA protein, HSV glycoprotein, Vesicular stomatitis N & G protein and many others and used them for vaccination purpose.

Similarly, adenoviruses can be used as live viruses. This achieved by deleting replicative function of viruses and desired gene is cloned under the control of early genes. Living viruses is generated using helper viruses. Such viruses have been created for curing cystic fibrosis, a deadly disease among Greek and Cypriot population, where diseased children die before 20 yrs. When recombinant virus id produced with a cystic fibrosis, channel protein gene with flanking regions such as LTR sequences, on delivery the Gene gets integrated into host cell genome. This virus can be delivered into host by nasal spray.

Genetic Immunization:

A cloned gene construct which is capable of producing an antigenically active protein, when biolistically injected into the ears of a mice, the Gene with its borders (LTR) gets integrated

and expressed a protein. The protein now acts as an antigen and animal's immune system responds and mounts antibody production against the protein. This technique has a promising future. When humans are very young as old as 2-3 years it is possible to inject such cloned DNA into body cells to make the child immune to a given antigen. However continuous production of such antigens with in the body will have repercussion effect and how the body can tolerate such continuous stimulation. Another problem is the quantity of the antigen produced, there is no regulation. I f the production of antigen is made to be regulated with specific stimulation, probably serves the system good.

Use of antibody as drugs:

Corney bacterium diphtheriae causes a severe disease faster and kill the patient fast. It first shows infected symptoms in the throat and tonsils, where it generates exotoxins, which on circulation can severely damage organs, which are distant from the site of infection.

People use passive immunity against such disease-causing agents. By raising antibody in horse against such bacteria and then injecting the serum into humans, results in developing passive

immunity. However, second time injection with horse serum will be so serious; it may give anaphylactic shock and cause death. Yet the use of antibodies against a variety of disease-causing agents and curing the disease is prevalent. New methods and new approaches to the problems have been in vogue. Monoclonal antibodies are once considered as magic bullets, but because of immune reactions to animal antibodies it became imperative develop antibodies, which are human compatible, called, humanized antibodies. Though monoclonal antibodies are expensive, they are still used in diagnostics; tissue imaging and some times they are used as immuno-suppressants.

Among lymphocytes, T cells which differentiate in Thymus cells act as immunological helper cells and also effecter cells. They are involved in rejection tissue grafting. If grafting to be successful T-cell mediated immune response to foreign tissue should be suppressed, thus one can save lives of a large number of human beings. Researches developed antibodies against T cell receptors called CD3. The first monoclonal antibody developed against CD3 in mice is called OKT3. When injected it binds to the receptor and prevents full scale mounting of immune response to tissue grafting. This kind of treatment was first approved by

United States food and drug administration. Without the approval no one can try to use trial on human beings at least in the US of A.

Such monoclonal antibodies are still in use in hospitals where bacteremia is endemic and kills lot of patients in USA. Monoclonal antibodies were used to suppress proliferation of breast cancer cells by raising antibodies against specific cell surface receptors on tumor cells. When such antibodies were used the cell surface receptors were blocked and growth of tumor has become stagnant. Further more once the growth of tumor cell is halted, some factor should be added to cause apoptosis of cancer cells only.

Targeting MABs to specific sites:

Blood clotting in vessels leading to brain and heart can lead to stroke and heart attack. The blood clot in vessels is called Thrombus; it consists of a network of fibrin, one of the primary blood-clotting agents. A defect in the endothelial layers of blood vessel is the cause. Thrombus kills millions of people all over the world, especially in developed world.

Plasminogen(86KD) a precursor protein for plasmin, which is a serine protease. The precursor plasminogen is activated by plasminogen activating factor (70KD). Once activated the plasmin acts on fibrinogen and fibrin and degrades the clot.

Raise antibodies to human fibrin and conjugate it with plasminogen activating factor in such a way the enzyme's active site is not disturbed. When such conjugated composite factor is injected, it homes to the site wherever fibrin is present. As most of the clots contain fibrin, the plasmin a serine protease acts and hydrolyses these proteins using serine sites in the protein. Plasminogen is also activated by Urokinase (54KD). Even Streptokinase takes part in dissolving blood clots. It is important to note that using monoclonal antibodies are used to deliver the required enzyme to specific site in the body. This has great implications in curing several diseases. While treating patients additional factors are also added with t-PA to inhibit t-PA activity, other wise it can lead to internal bleeding.

Abzymes':

By genetic manipulations it is possible to convert antigen-binding site into an enzyme active site. This product has the ability to recognize the specific substrate as well as to perform enzyme activity.

Use of MABs in treatment has created problems for they themselves act as antigens, so they have modified the IgG in such way they act and behave like human antibodies (humanized). In most of the cases monoclonal antibodies were obtained from mice, and mice antibodies are not human antibodies and they develop resistance to the use of antibodies as therapeutic agents. So scientist started to look for human cell lines to generate good amount specific kind of IgGs against a specific antigen.

Developing humanized IgGs:

Several strategies were employed. First, they isolated human B-lymphocytes by tagging fluorescent antigen and then they are separated by flow cytometry and got reasonably enriched population of specific B-lymphocyte. Then they tried human antibody producing lymphocytes fused with human myeloma cells. Unfortunately, the hybrid cell lines were found to be unstable. Instead, they transformed lymphocytes with Epstein Barr viruses. Such transformed cells were viable, they divided and secreted IgGs, but alas! The yield was very very poor.

The other strategy was to clone a human IgG gene, both L and H chains and transform embryonic cells of the mouse and obtain the transgenic mouse and stimulate mouse for specific antigen and get specific antibodies.

In another method, they obtained specific antibody gene for a specific antigen. By genetic manipulation it is possible to replace human Fc fragment in the place of mice Fc fragment. Similarly, even the antigen binding hyper variable region can also be replaced. Such genes were transfected to known mammalian cell

lines and expressed IgG were found to be human compatible. But it is yet to be realized to produce such Abs on large scale.

Use of Bispecific Antibodies:

Produce two different but specific antibodies; say one for tumor cell surface receptor and the other for specific T-cell receptor. When such antibodies were denatured and mixed to renature appropriately, one obtains antibodies containing two specificities called Bispecific antibodies.

Another way to generate Bispecific antibodies is to genetically manipulate hyper variable sites to the designed purpose. When such Bispecific antibodies are employed one can target two systems simultaneously. Bispecific antibodies, say one for specific T- killer cells and another for tumor cell, T-killer cells can be targeted to tumor cells.

In the case of malignant Hodgkin's melanoma there are a large number of tumors infiltrating T-cells. And tumor cells have their own specific receptor. Raise anti bodies separately for these two types of cells. Generate hybrid Ig Gs. Collect as much as possible Tils (Tumor Infiltrating Lymphocytes) from the tumor and treat with specific Interleukin to activate the T-cells into killer cells. Inject T-killer cells and Bispecific antibodies. They

bind to T-cell and bring them to tumor cells and bind. The activated Tils destroy tumors and clinically found such tumors were regressed.