SCREENING AND SELECTION FOR RECOMBINANTS

The art of cloning is to find the one particular transformed cell/recombinant cell that contains the cloning vector with the gene of interest (referred to as a recombinant cell).

It is thus most important to be able to select recombinant cells from transformed cells (containing the vector without insert DNA).

IDENTIFICATION OF RECOMBINANTS:

After the introduction of rDNA into suitable host cells, it is essential to identify those cells which have received the rDNA molecules. This process is called **screening or selection.**

Generally, the **selection methods are based on the expression or non-expression of certain traits such as antibiotic resistance, expression of an enzyme such as β- galactosidase or protein such as GFP (Green Fluorescent Protein) and dependence or independence of a nutritional requirement such as the amino acid leucine.**

Direct selection, which means that the cloning experiment is designed in such a way that the clones obtained are the clones containing the required gene. Almost invariably, selection occurs at the plating-out stage. Direct selection is the method of choice, as it is quick and usually unambiguous. However, it is not applicable to all genes.

Identification of the clone from a gene library, which entails an initial "shotgun" cloning experiment, to produce a clone library representing all or most of the genes present in the cell, followed by analysis of the individual clones to identify the correct one.

I) DIRECT SELECTION OF RECOMBINANTS:

Two examples will be discussed:

(i) direct antibiotic resistance screening, and
(ii) blue-white color screening.

II) INDIRECT SELECTION OF RECOMBINANTS:

(i) Screening by Nucleic acid hybridization
(ii) Screening by Colony hybridization
(iii) Screening by Immunological assay
(iv) Screening by Protein/Enzyme activity

1.) Direct antibiotic resistance screening

Most cloning vectors are designed so that the insertion of a DNA fragment into the vector **destroys the integrity of one of the genes present on the molecule** (usually an antibiotic resistance gene).

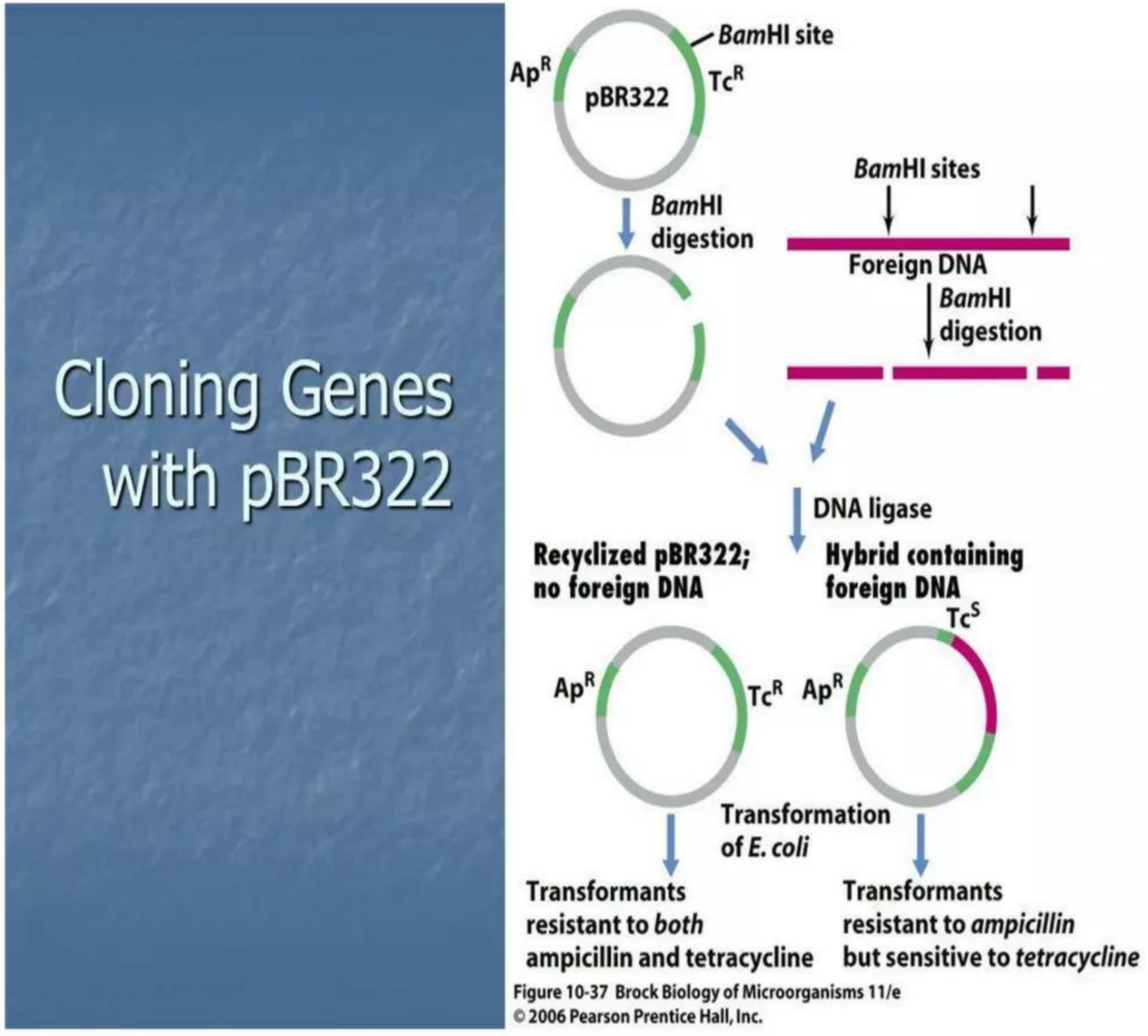
INSERTIONAL SELECTION INACTIVATION METHOD:

In this approach, one of the genetic trait is disrupted by inserting foreign DNA. **Antibiotic resistance genes act as a good insertion inactivation system.**

Plasmid pBR322 contains two antibiotic resistance genes, one for ampicillin (amp^{r}gene), and the other for tetracycline (tet^r gene).

If the target DNA is inserted into tet^r gene using Bam HI, the property of resistance to tetracycline will be lost. Such **recombinants would be tet^{s}sensitive.**

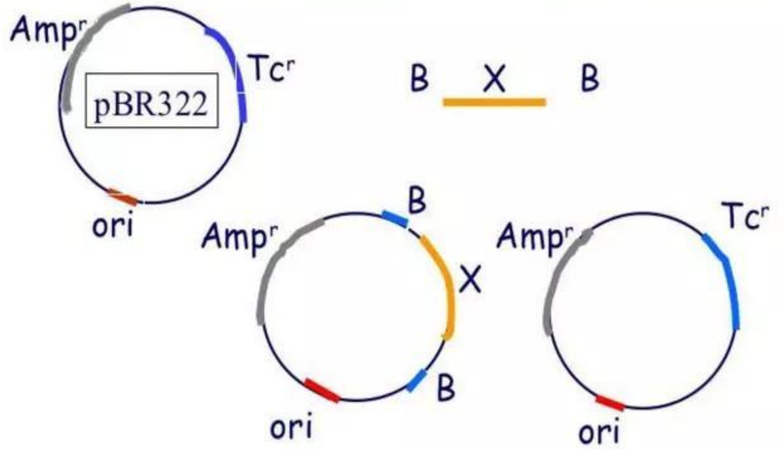
When such recombinants (containing target DNA in tet^r gene) are grown into medium containing tetracycline, they will not grow because their tet^r gene has been inactivated. But they are resistant to ampicillin because amp^r gene is functional.



On the other hand, the self-ligated recombinants will show resistance to ampicillin and tetracycline. Therefore, they will grow on medium containing both the antibiotics.

Twin antibiotic resistance screening

-Screening by insertional inactivation of a resistance gene



Ampicillin resistant? *yes* *yes*

Tetracycline resistant? *No* *yes*

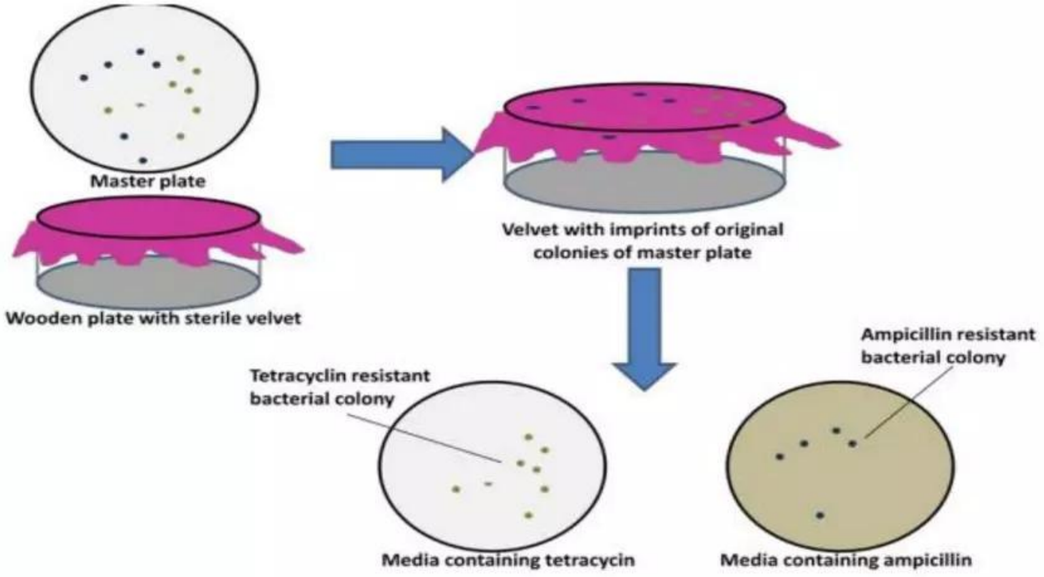
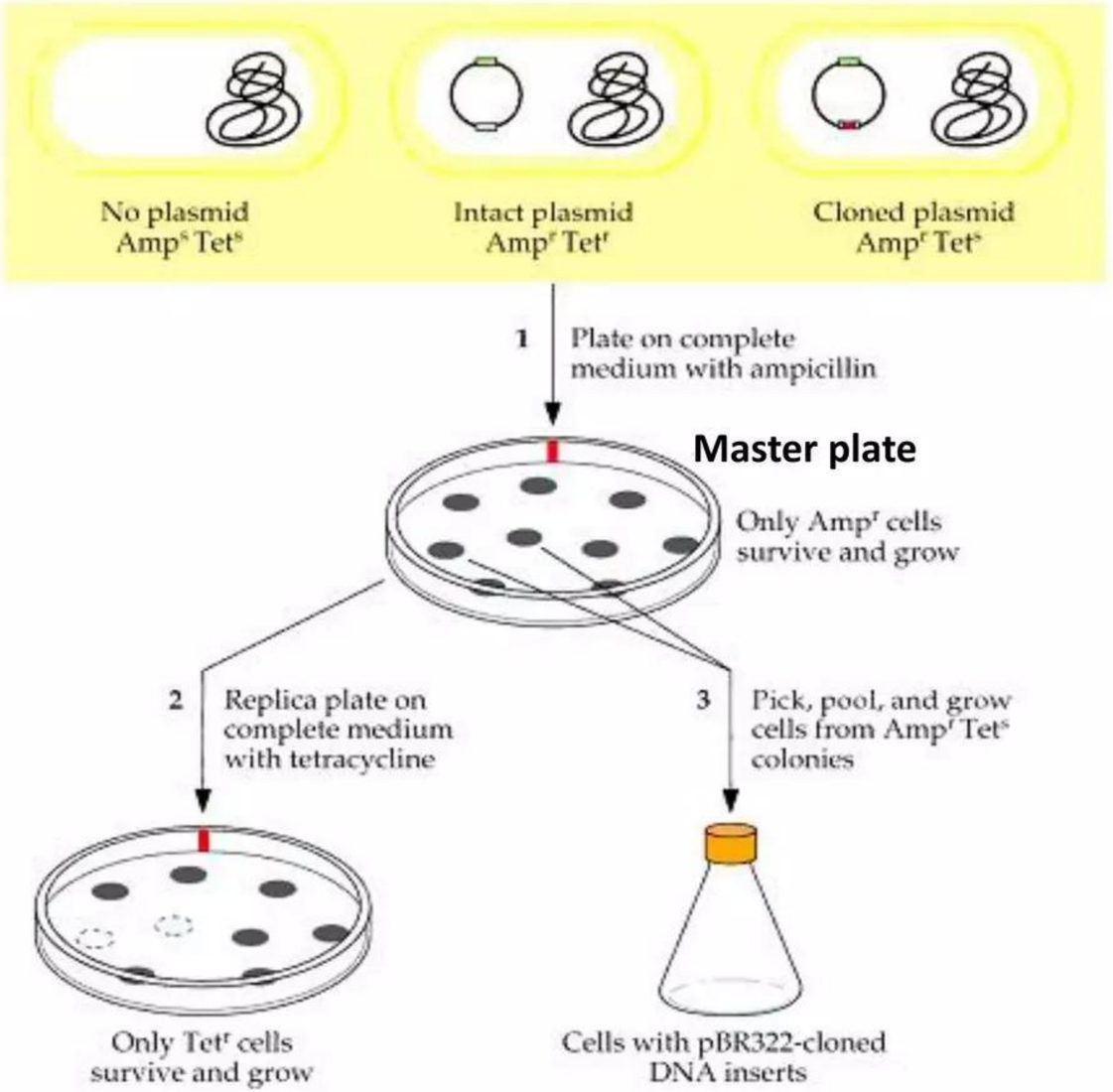


Fig 1-2.2.8.1: Selection through recombinant bacteria by replica plating



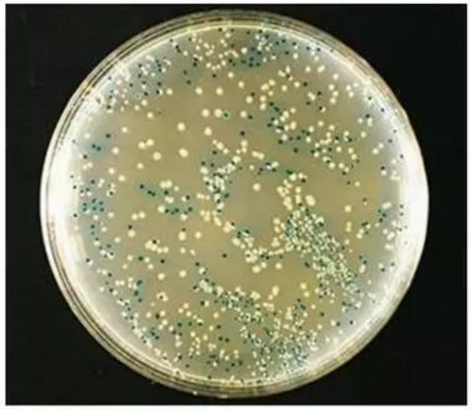
Blue white selection-

2. Visual Screening :Blue-white colour screening

A more sophisticated procedure for screening for the presence of recombinant plasmids, which can be carried out on a single transformation plate, is called blue-white screening.

This method also involves the insertional inactivation of a gene and uses the production of a blue compound as an indicator. The gene is *lacZ*, which encodes the enzyme ß-galactosidase, and is under the control of the *lac* promoter.

If the host *E. coli* strain is expressing the lac repressor, expression of a *lacZ* gene on the vector may be induced using IPTG (isopropyl- ß-D-thiogalactopyranoside), and the expressed enzyme can utilize the synthetic substrate X-gal (5-bromo-4-chloro-3-indolyl- ß-D-galactopyranoside) to yield a blue product.



pUC plasmids:

- pUC plasmids are small, high copy number plasmids of size 2686bp.

- This series of cloning vectors were developed by Messing and co-workers in the University of California. The p in its name stands for plasmid and UC represents the University of California.

- pUC18 and pUC19 vectors are identical apart from the fact that the MCS is arranged in opposite orientation.

- **pUC vectors consists of following elements:**

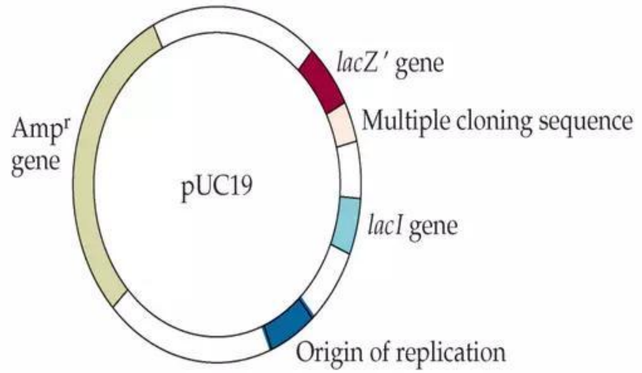
► **pMB1 *"rep "* replicon** region derived from plasmid pBR322 with single point mutation (to increase copy number).

► " *bla* " gene encoding β lactamase which provide ampicillin resistance which is derived from pBR322. This site is different from pBR322 by two point mutations.

► LacZ gene from *E.coli* lac operon system, which contains MCS restriction sites.

- pUC vectors contain a *lacZ* sequence and multiple cloning site (MCS) within *lacZ.* This helps in use of broad spectrum of restriction endonucleases and permits rapid visual detection of an insert.

- **"*rop* " gene** is removed from this vector which leads to an increase in copy number.



Copy number:

500-600 copies per cell

Alpha complementation:

The lac operon consists of structural and regulatory genes. The lac Z gene is a structural gene and is required for galactoside metabolism. The lac-Z gene product (β-galactosidase) is a tetramer, and each monomer is made of two parts - lacZ-alpha, and lacZ omega.

1. The pBSKS$^+$ / pUC18 plasmid carries the alpha fragment of the lac Z gene. The alpha fragment is the amino-terminus of the protein and is not functional by itself. The alpha fragment is denoted by lac Z'.

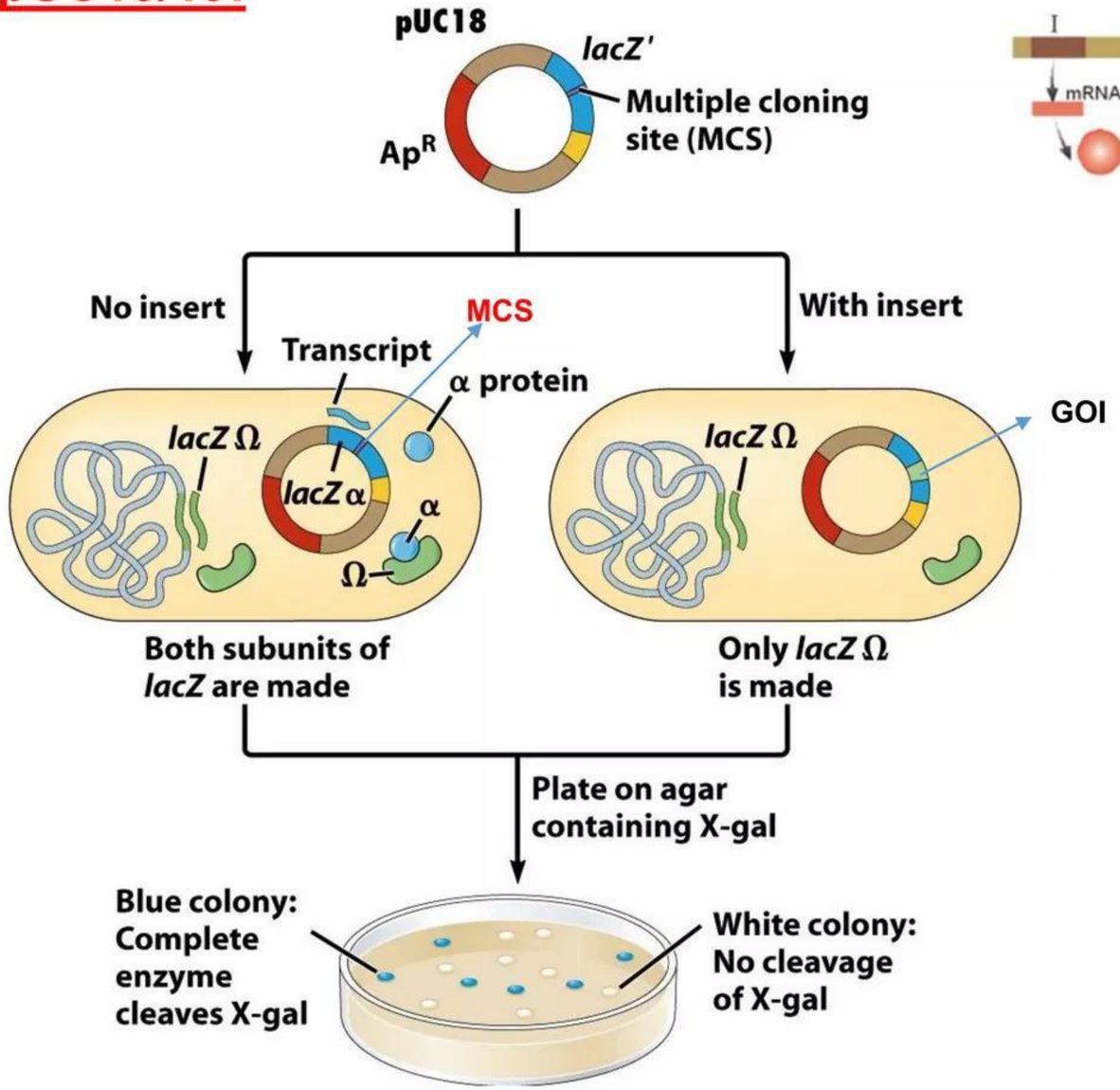
2. Typically, the *E. coli* host strain used for transformation is a mutant strain that has a deletion of the alpha fragment of lac Z. The *E. coli* chromosome carries only the omega fragment, which is the carboxyl-terminus of the protein. The omega fragment alone is also non-functional.

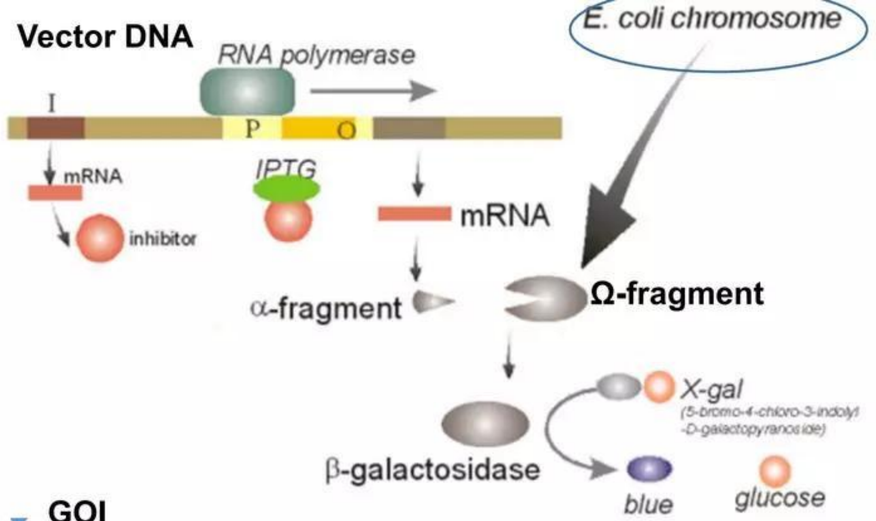
When both the alpha and omega fragments interacts, a functionally intact β-galactosidase protein are produced. This interaction is called alpha complementation.

It is determined that if the alpha fragment was deleted, the omega fragment is non-functional; however, alpha fragment functionality can be restored in-trans via plasmid.

The substrates for the beta-galactosidase enzyme are galactosides, such as lactose. Lactose is hydrolyzed by this enzyme into galactose and glucose. Artificial galactosides, such as 5-Bromo-4-Chloro-3-Indolyl-beta-D-galactoside (X-Gal), are also substrates for β-galactosidase. Isopropyl thiogalactoside (IPTG) acts as an inducer of lacZ gene expression.

Cloning in pUC18/19:

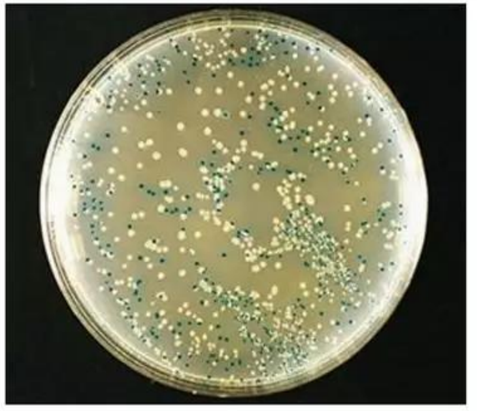




β-galactosidase enzyme + chromogenic substrate (X-gal)

↓

Blue color colonies

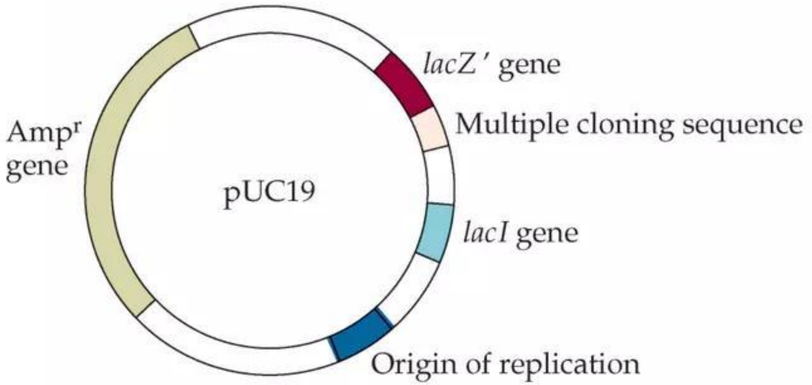


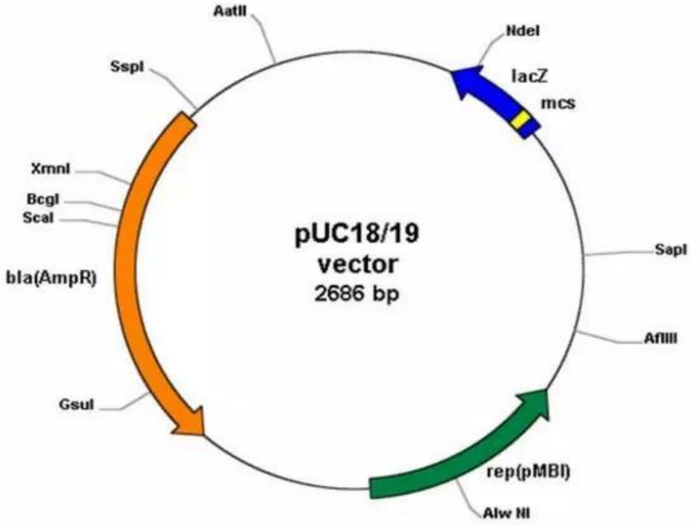
GOI- GENE OF INTEREST

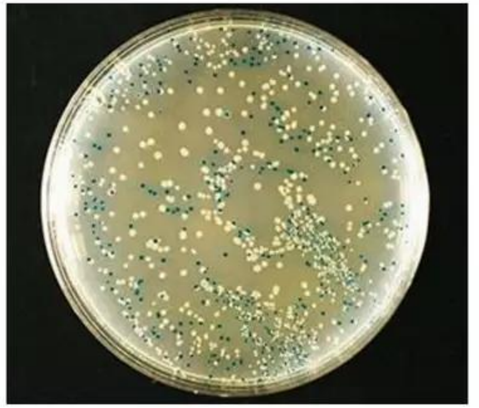
Cloning of insert DNA using β - galactosidase: as reporter gene

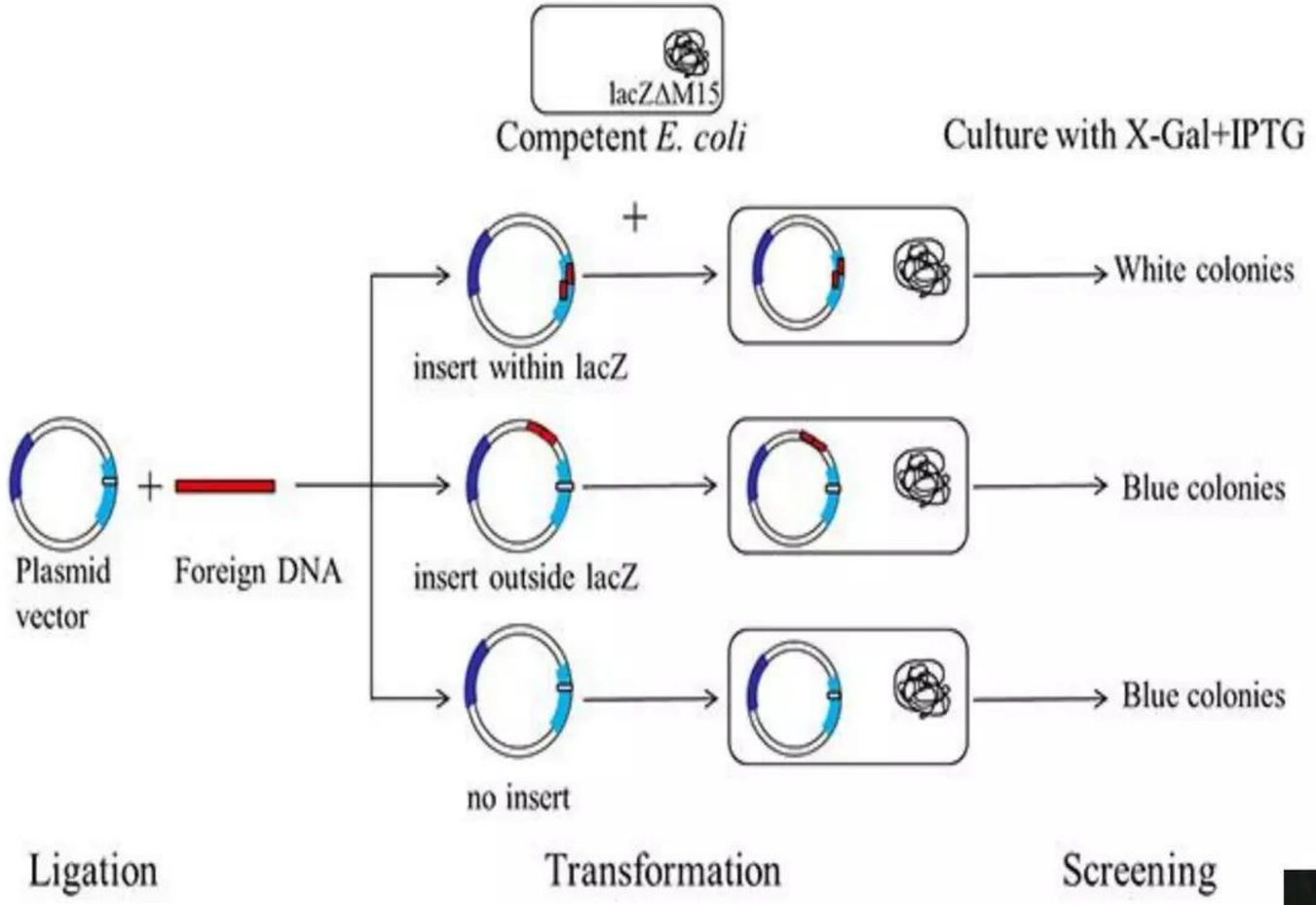
1. Multiple cloning site is inserted into the gene *lacZ'* coding for the alpha peptide of enzyme β-galactosidase. (An MCS is a short DNA sequence consisting of restriction sites for many different restriction endonucleases).

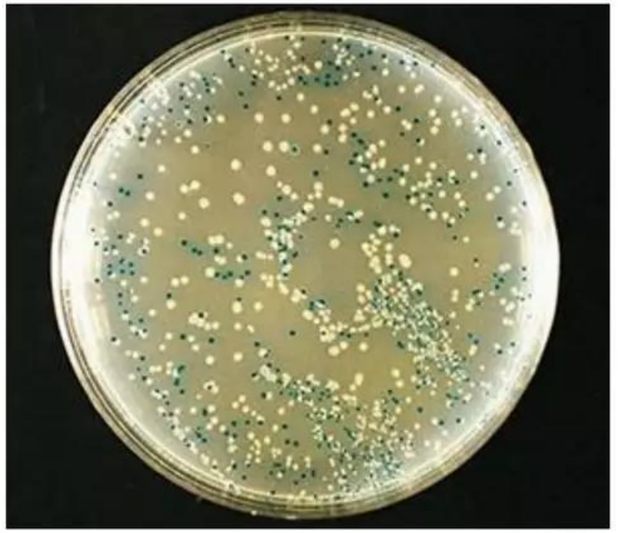
2. Insertion of the MCS into the *lacZ'* fragment does not affect the ability of the α-peptide, while cloning DNA fragments into the MCS does.
3. Clones with foreign DNA in the MCS disrupt the ability of the cells to make β-galactosidase.
4. Plate on media with a Substrate X-gal (β-galactosidase indicator) and clones with intact β-galactosidase enzyme indicate that cells containing empty vector without insert, will produce blue colonies. *Colorless (desirable) colonies indicates that cells contain the plasmid with foreign DNA*
5. Therefore, recombinants can be detected by blue/white screening on growth medium containing X gal in presence of IPTG as an inducer.








lacZΔM15
Competent E. coli
Culture with X-Gal+IPTG
+
White colonies
insert within lacZ
Plasmid vector
+
Foreign DNA
insert outside lacZ
Blue colonies
Blue colonies
no insert
Ligation
Transformation
Screening



II) Indirect methods:

Identification of the clone from a gene library:

Many different techniques are available for screening a library.

The most important approaches involve

screening by nucleic acid hybridization and

screening by Colony hybridization

screening by functional analysis- Immunological assay

- Protein Assay

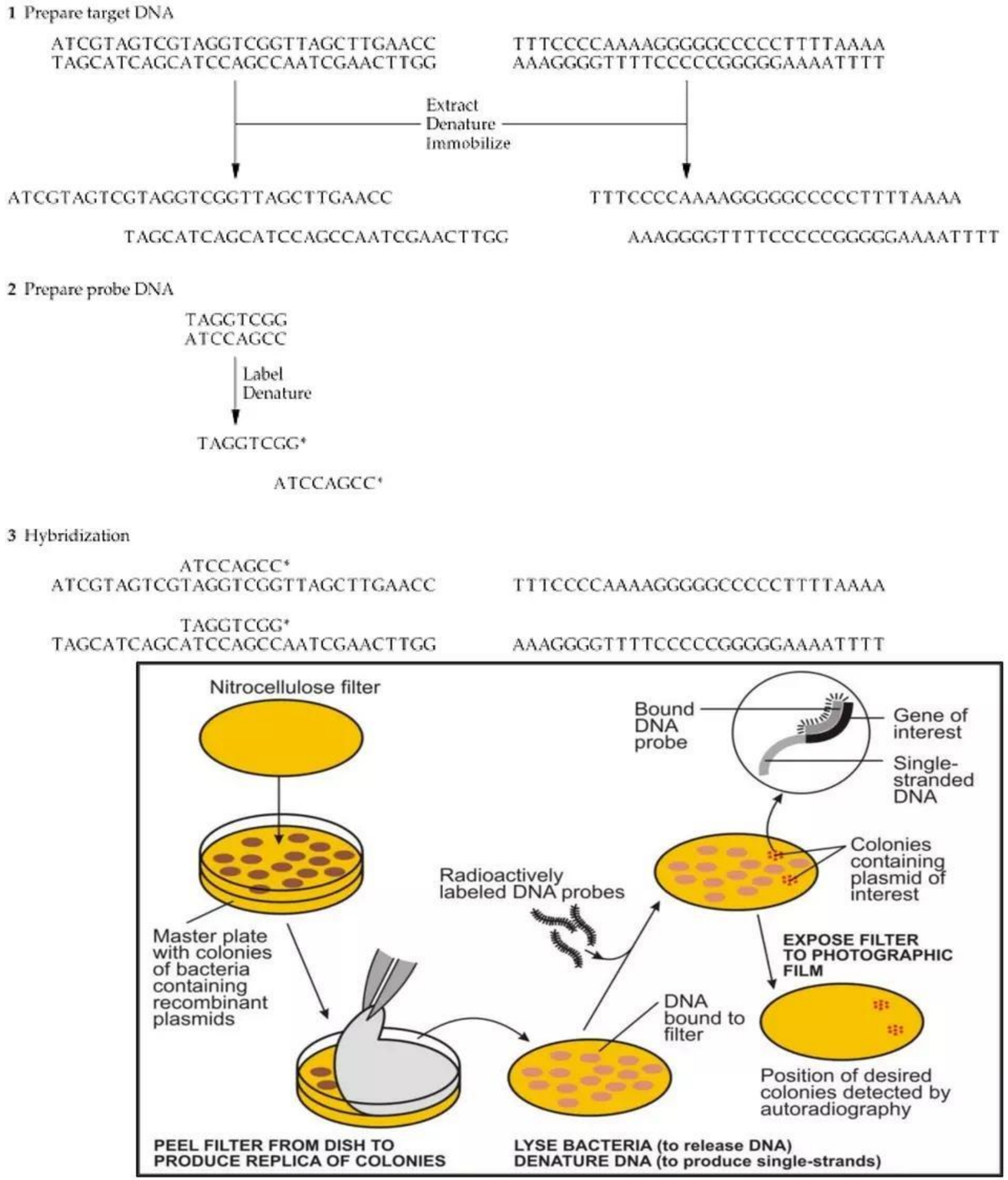
Screening by Nucleic acid hybridization

1. The first step of a hybridization screening experiment involves DNA from the clones are isolated and separated on the agarose gel electrophoresis. The transfer of the DNA from the gel to a nylon or nitrocellulose membrane is carried out.

2. The DNA is denatured with alkali to produce single strands that are bounded to the membrane by heat treatment or UV irradiation.

3. The membrane is then immersed in a solution containing a nucleic acid probe (usually radioactively labeled) and incubated to allow the probe to hybridize to its complementary sequence.

4. After hybridization, the membrane is washed to remove unhybridized probe, and regions where the probe has hybridized are visualized with autoradiography.



DNA hybridization.

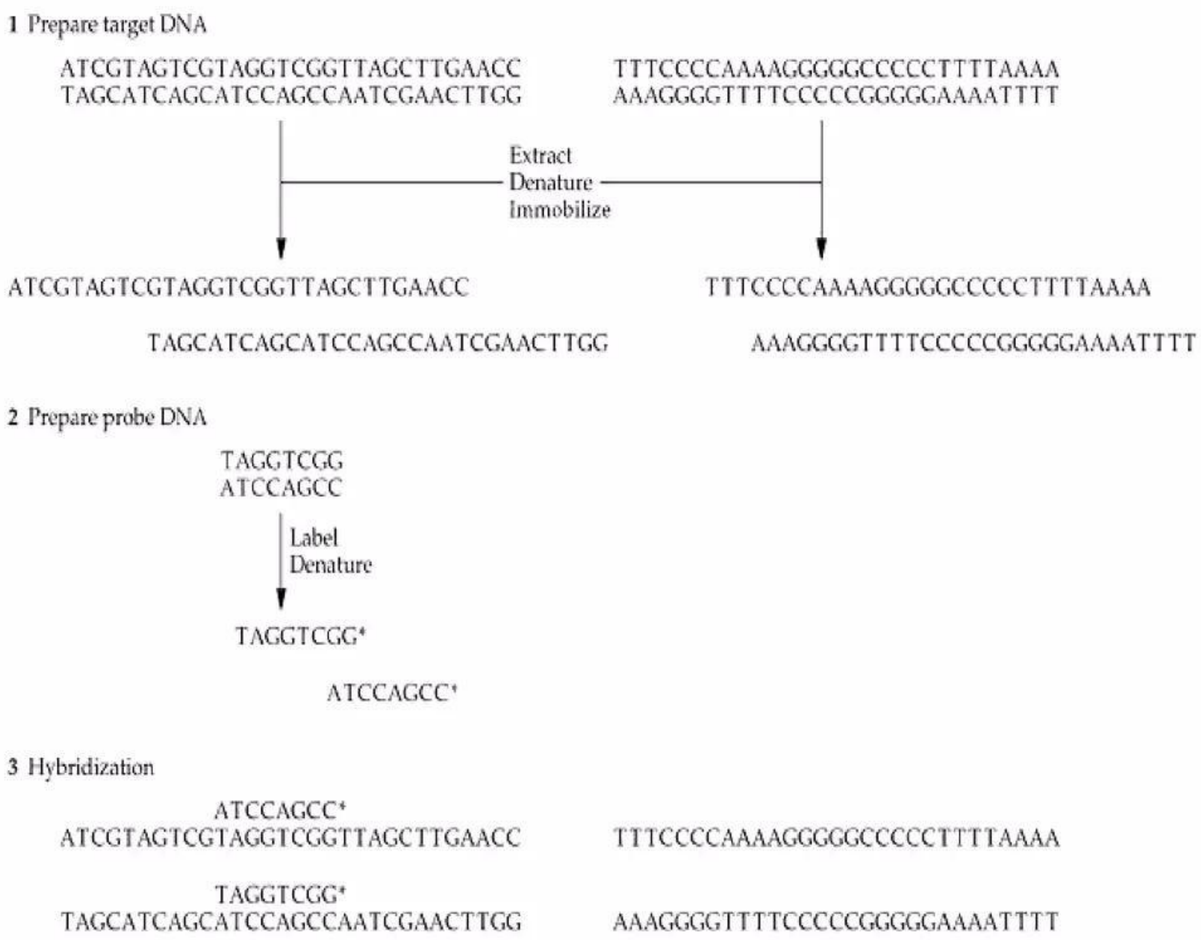
(1) The DNA of samples containing the putative target DNA is denatured, and the single strands are kept apart, usually by binding them to a solid support, such as a nitrocellulose or nylon membrane.

(2) (2) The probe, which is often 100 to 1,000 bp in length, is labeled, denatured, and mixed with the denatured putative target DNA under hybridization conditions.

(3) (3) After the hybridization reaction, the membrane is washed to remove nonhybridized probe DNA and assayed for the presence of any hybridized labeled tag.

(4) If the probe does not hybridize, no label is detected.

(5) The asterisks denote the labeled tags (signal) of the probe DNA.



Screening by Colony hybridization:

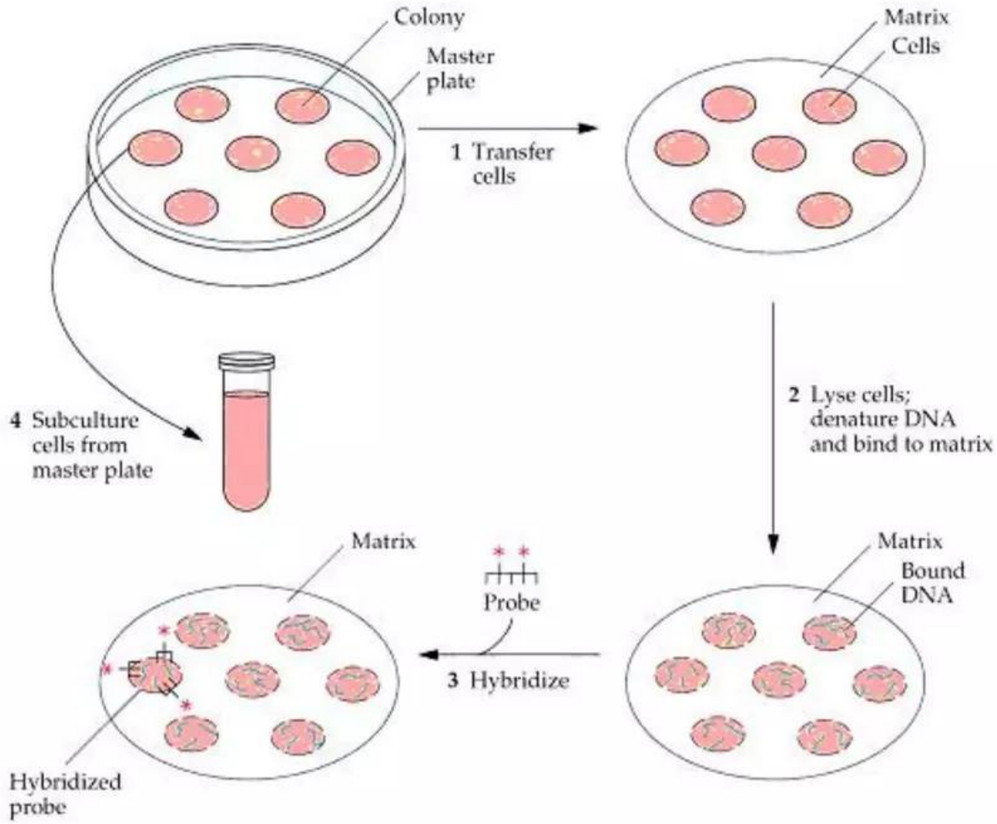
Screening a library with a labeled DNA probe (colony hybridization).

Cells from the transformation reaction are plated onto solid agar medium under conditions that permit transformed, but not nontransformed, cells to grow.

(1) From each discrete colony formed on the master plate, a sample is transferred to a solid matrix, such as a nitrocellulose or nylon membrane. The pattern of the colonies on the master plate is retained on the matrix.
(2) The cells on the matrix are lysed, and the released DNA is denatured, deproteinized, and irreversibly bound to the matrix.

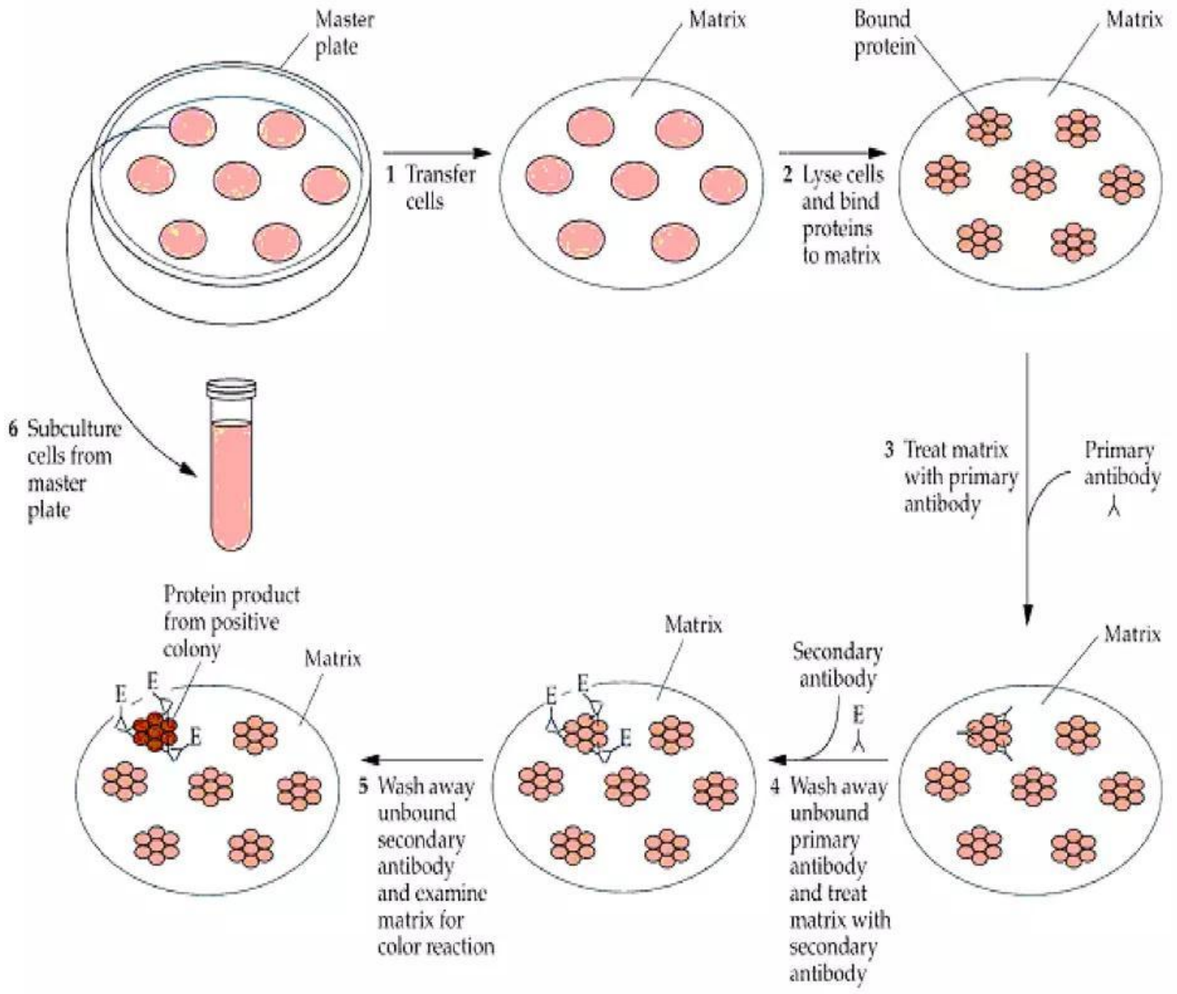
(3) A labeled DNA probe is added to the matrix under hybridization conditions. After the nonhybridized probe molecules are washed away, the matrix is processed by autoradiography to determine which cells have bound labeled DNA.

(4) A colony on the master plate that corresponds to the region of positive response on the X-ray film is identified. Cells from the positive colony on the master plate are subcultured because they may carry the desired plasmid-cloned DNA construct.



Screening by Immunological assay

The desired gene can also be identified by the activities of the encoded gene product.



1. The cells are grown as colonies on master plates which are transferred to a solid matrix (i.e., nitrocellulose).

2. The colonies are then subjected to lysis and the released proteins bound to the matrix. These proteins are then treated with a primary antibody which specifically binds to the protein (acts as an antigen), encoded by the target DNA.

3. After removing the unbound antibody by washings, a second antibody is added which specifically binds to the first antibody.

4. Again the unbound antibodies are removed by washings. The second antibody carries an enzyme label (e.g., horse reddish peroxidase or alkaline phosphatase) bound to it. The detection process is so devised that as a colourless substrate it is acted upon by this enzyme, a coloured product is formed.

5. The colonies which give positive result (i.e., coloured spots) are identified. The cells of a specific colony can be sub-cultured from the master plate.

Screening by Protein activity

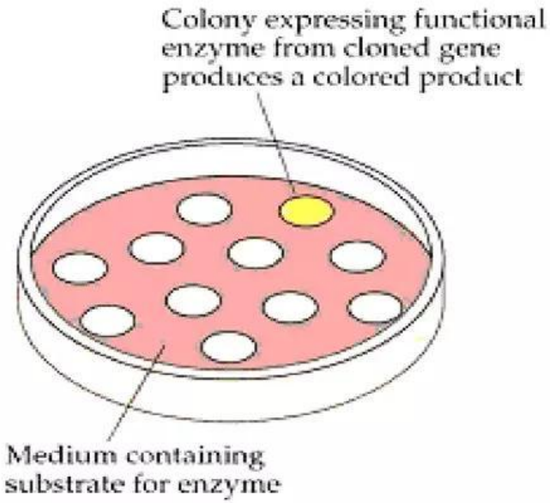
DNA hybridization and immunological assays work well for many kinds of genes and gene products.

1. If the target gene produces an enzyme that is not normally made by the host cell, a direct (in situ) plate assay can be devised to identify members of a library that carry the particular gene encoding that enzyme.

2. The genes for α-amylase, endoglucanase, β-glucosidase, and many other enzymes from various organisms have been isolated in this way.

3. This approach has proven effective for isolating genes encoding biotechnologically useful enzymes from microorganisms present in environmental samples.

4. This technique has enabled the isolation of many novel proteins with interesting properties without the need to first culture the natural host microorganism.



Screening a genomic library for enzyme activity.

1. Cells of a genomic library are plated onto solid medium containing the substrate for the enzyme of interest.

2. If a functional enzyme is produced by a colony that carries a cloned gene encoding the enzyme, the substrate is converted to a colored product that can be easily detected.

3. Note that other, noncolored colonies on the medium also contain fragments of the genomic library, but they do not carry the gene for the enzyme of interest.