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REKAYASA GENETIKA
IBD 131

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Pertemuan 5

GEN KLONING

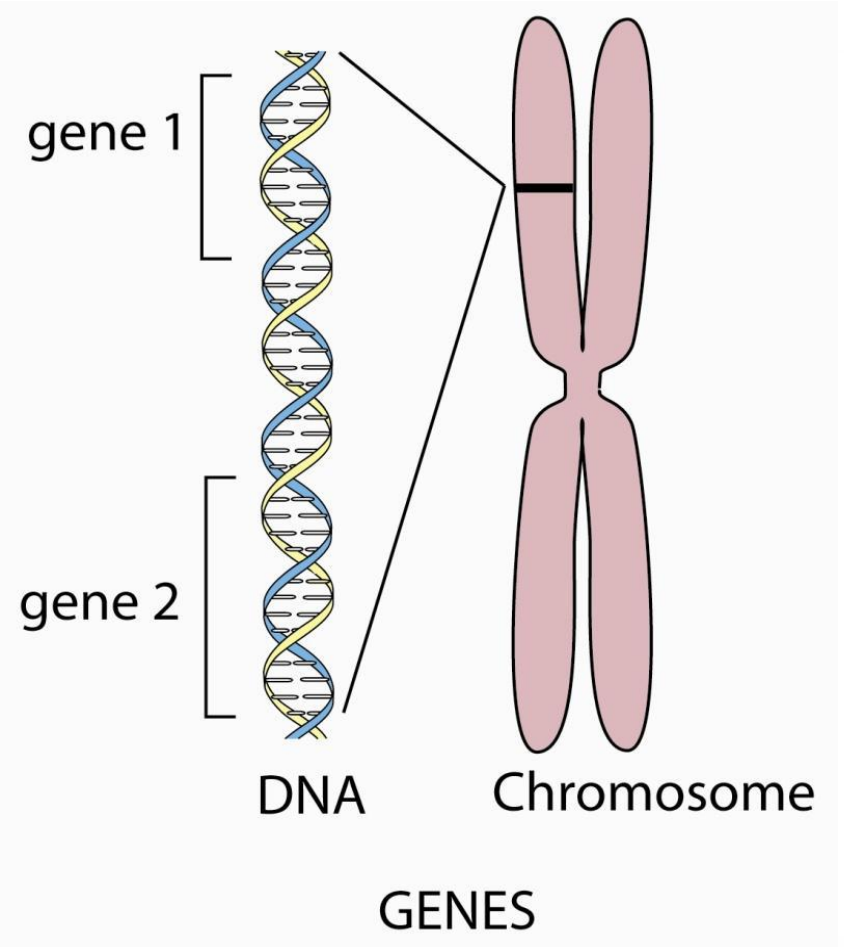
Universitas Esa Unggul

Sasaran Perkuliahan

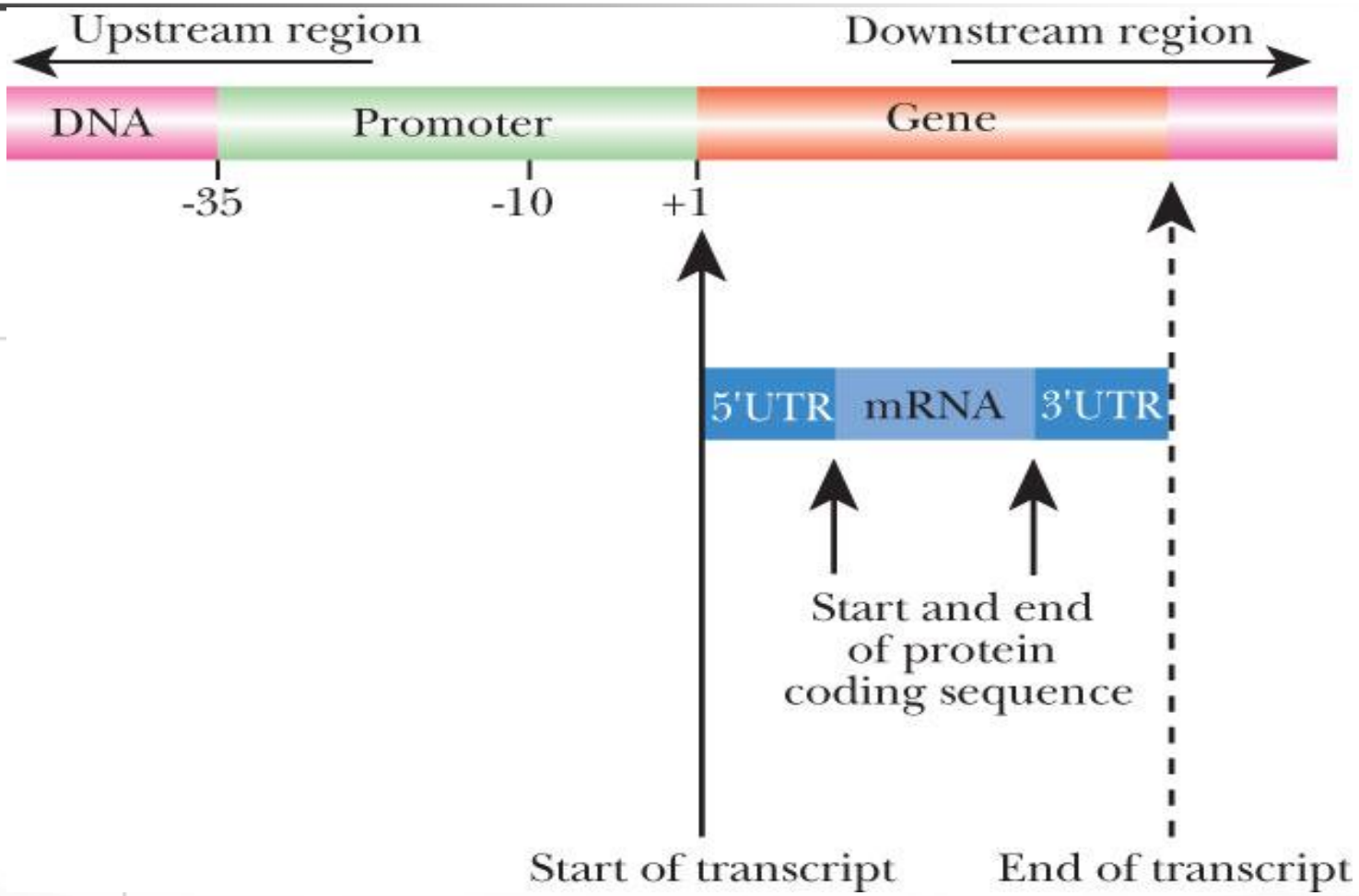
- Mahasiswa dapat Mamahami dan menjelaskan tentang prinsip Kloning Gen
- Mahasiswa dapat menjelaskan prinsip dalam sintesis cDNA
- Memahami dan mampu menjelaskan tentang TA-Cloning
- Memahami tentang metode transformasi
- Memahami metode pembuatan cell compotent

Principles and Methods of Gene Cloning

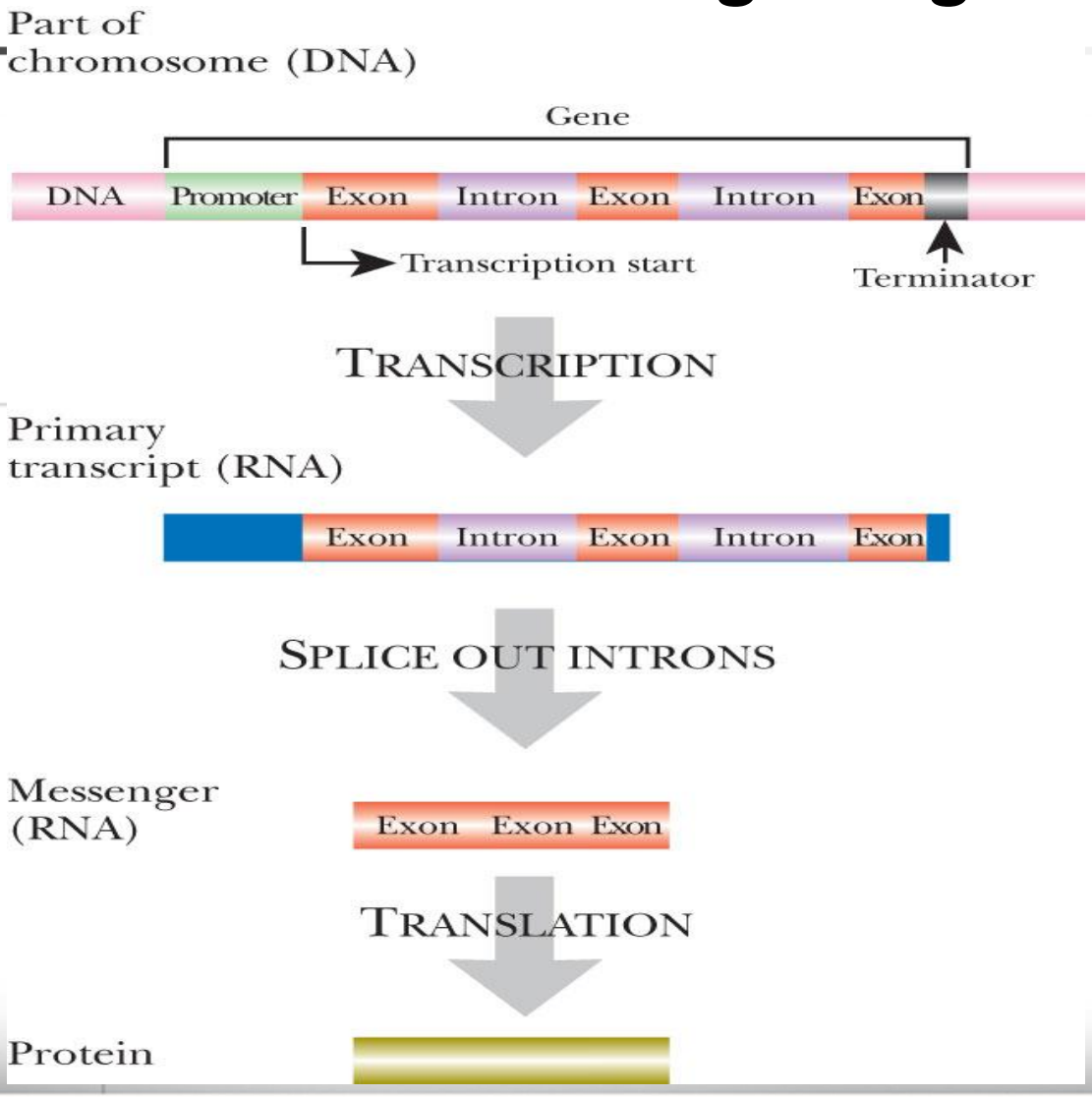
- Clone: a collection of molecules or cells, all identical to an original molecule or cell
- When DNA is extracted from an organism, all its genes are obtained
- Gene cloning: attempts to isolate identical genes from an individual



Gen dan Promoter



Bakteri tidak bisa menghilangkan intron



Conventional cloning method: General Steps

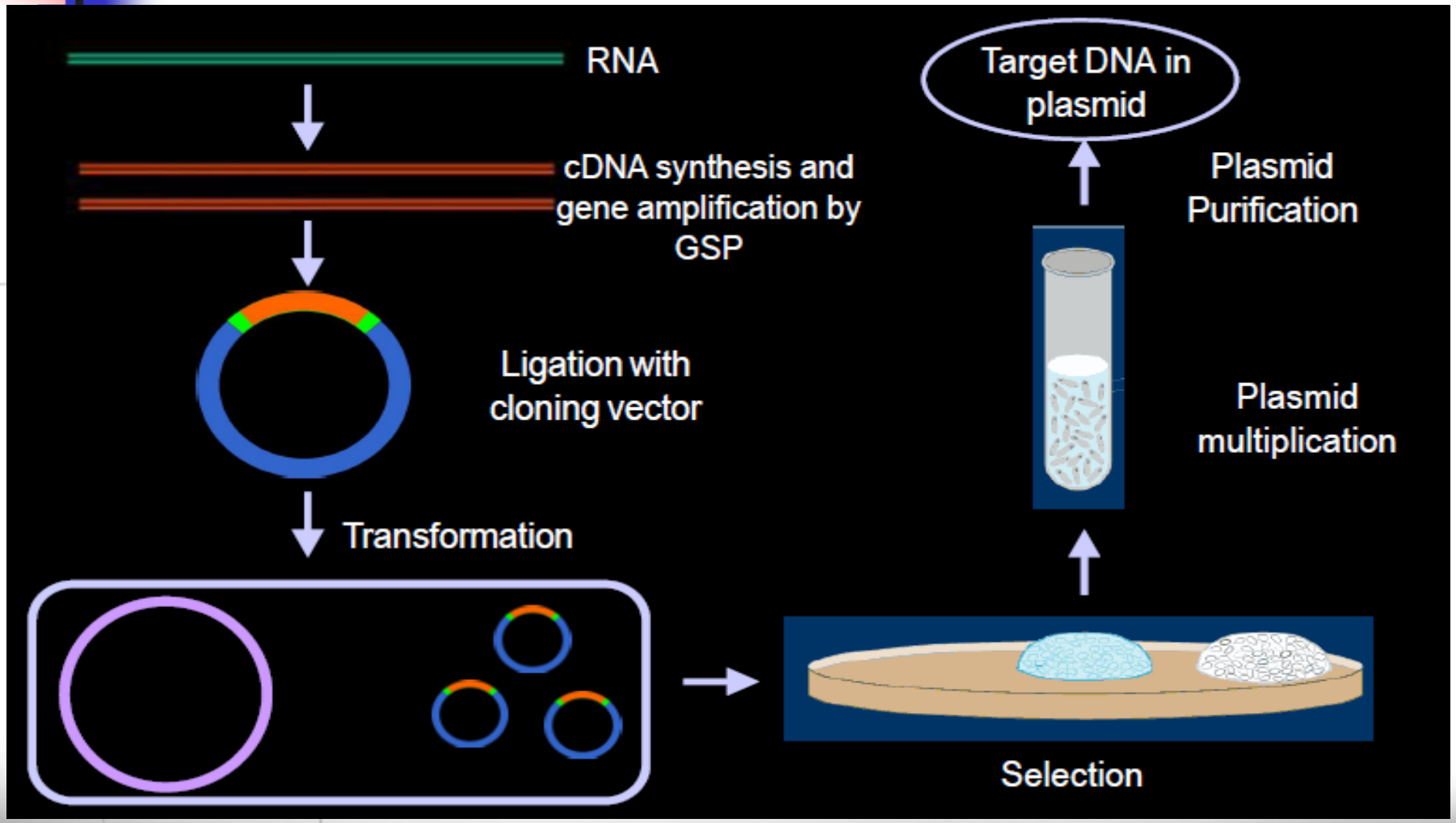
- cDNA synthesis from mRNA
- Amplification of target gene by gene specific primer ⇨ PCR
- Extraction of putative DNA band from agarose gel
- Insertion into plasmid ⇨ TA-cloning

Conventional cloning method: General Steps

Transformation into *E. coli* ➡ *lacZ and antibiotic selection*

- Selecting the "true-transformed" colonies ➡ colony PCR
- Amplification of plasmid-contained colony ➡ colony culture
- Plasmid purification ➡ to collect plasmid from *E. coli cell*

CLONING PROCESS



cDNA synthesis from mRNA

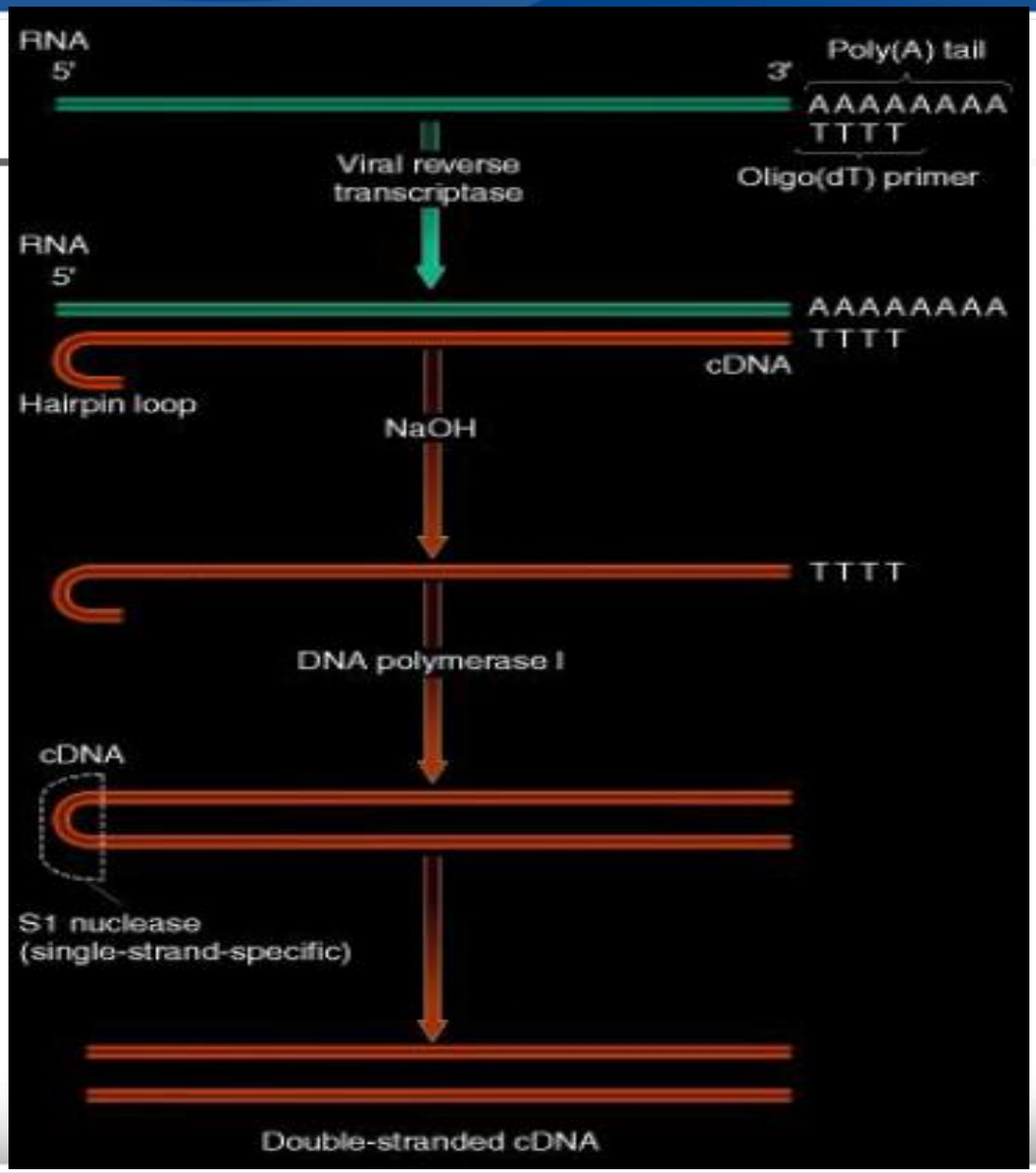
cDNA = complementary DNA.The classical steps of double-stranded cDNA synthesis from mRNA are:

- Eukaryotic mRNA's have a poly A tail. An oligo (dT) molecule hybridises to the poly A tail.
- The oligo (dT) segment serves as a primer for the action of reverse transcriptase, which uses the mRNA as a template for the synthesis of a complementary DNA strand.

cDNA synthesis from mRNA

- The resulting cDNA ends in a hairpin loop.
- When the mRNA strand has been degraded by treatment with NaOH, the hairpin loop becomes a primer for DNA polymerase I, which completes the paired DNA strand.
- The loop is then cleaved by S1 nuclease (which acts only on the single-stranded loop) to produce a double-stranded cDNA molecule

cDNA synthesis from mRNA



cDNA synthesis from mRNA

Recent common method of cDNA synthesis:

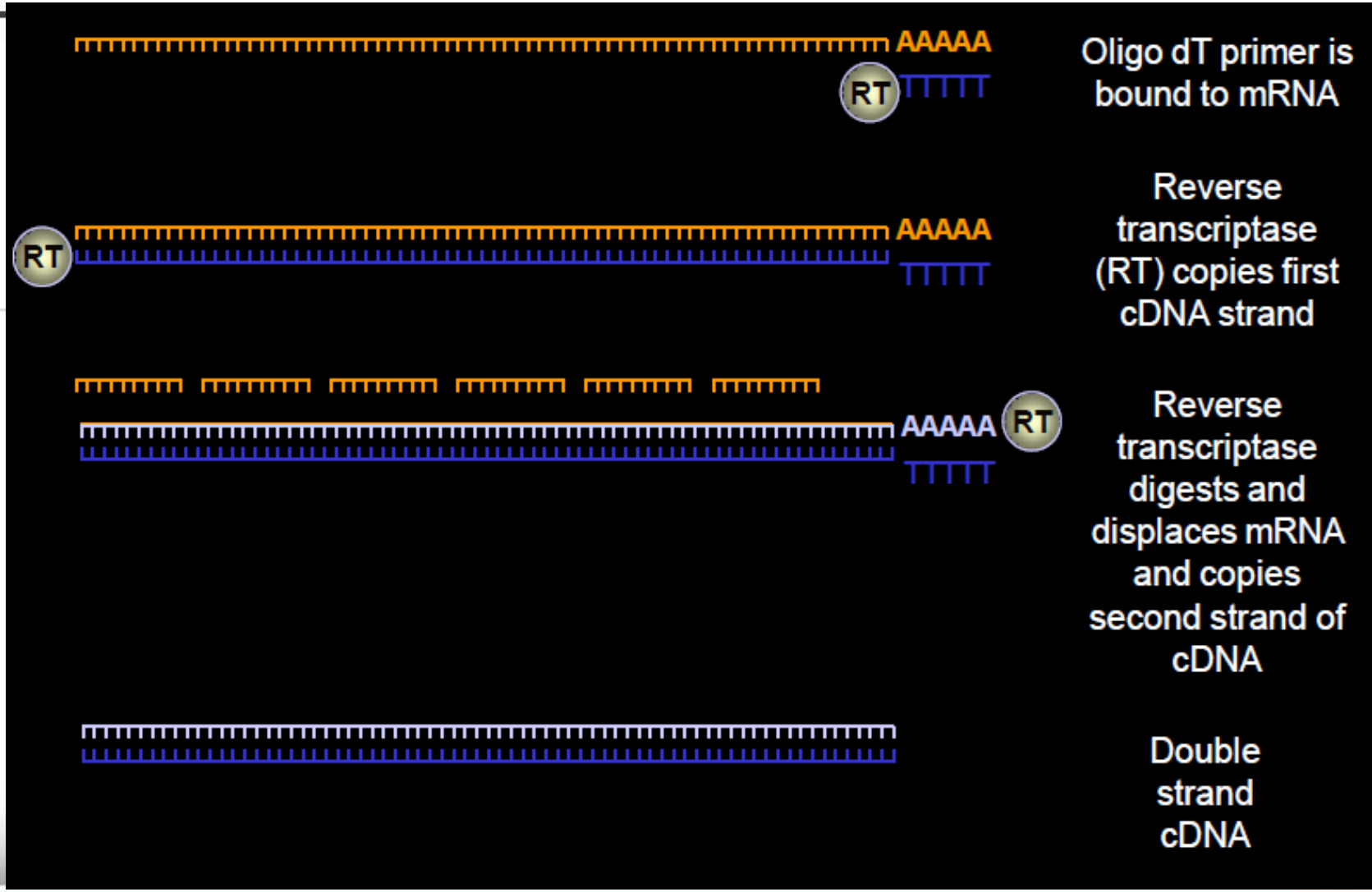
- Another strategy for cDNA synthesis employs a ribonuclease (RNase H)
- Differ from the classical method at the step of second strand cDNA-synthesis
- This method does not use S1 nuclease but RNase H

cDNA synthesis from mRNA

Recent common method of cDNA synthesis:

- The RNase H recognizes the RNA component of a DNA:RNA hybrid and cleaves the RNA at a number of non-specific sites leaving short oligoribonucleotides attached to the cDNA.
- These serve as primers for the polymerase to synthesise the second strand cDNA

Conversion of mRNA to cDNA by Reverse Transcription



Example of protocol: Superscript, Takara

- Incubation can be done using PCR, heat block, or air incubator
- The minimum amount of total RNA is 1 µg, thus you have to calculate the RNA volume base on the RNA concentration (i.e. for RNA concentration 0.675 µg/ µl, 2 µl sample will resulted in 1.35 µg RNA).

Steps (work on ice):

Mix:

a. Total RNA (1-2 µg)	2 µl	
b. Oligo (dT) primer (100µM)	3 µl	
c. Water	5 µl	
Total	10µl	

Heat at 65°C for 5 min → To denature primer

Stored on ice for 5 min → To prevent enzyme inactivation in the following reaction

Spin

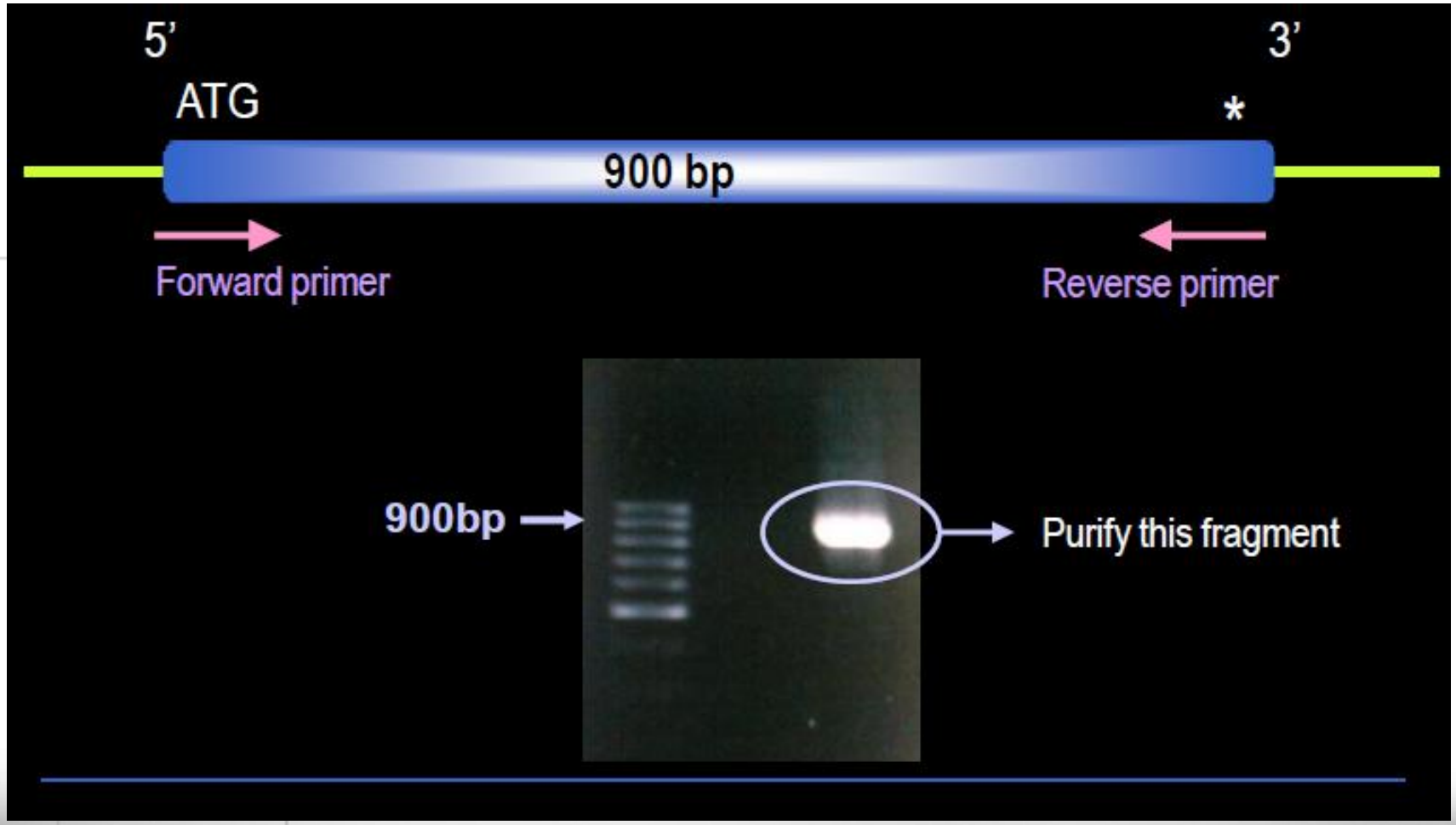
Add the below reagent to previous microtube:

a. Water	3 µl	If you have many samples, you may mix these reagents and split into your samples.
b. 5X RTase M-MLV Buffer	4 µl	
c. dNTP-mix (10mM)	2 µl	
e. RTase M-MLV	1 µl	→ Take the enzyme directly from -20°C refrigerator
Total	20µl	

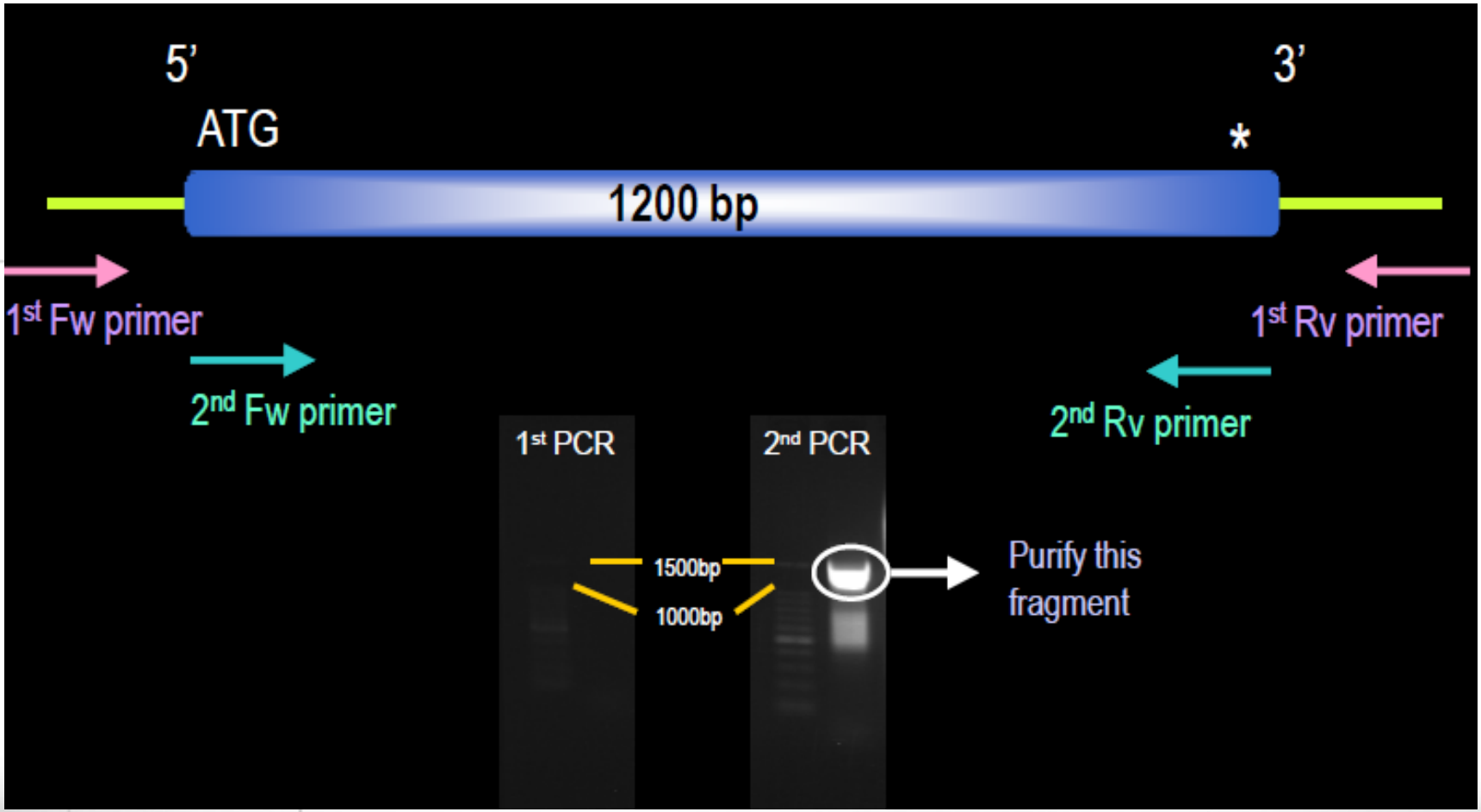
Incubate at 42°C for 1 hr → RT-reaction

Heat at 70°C for 10 min → To stop any enzymatic reaction

Amplification of target gene: one time PCR



Amplification of target gene: nested PCR



Extraction of putative DNA band from agarose gel

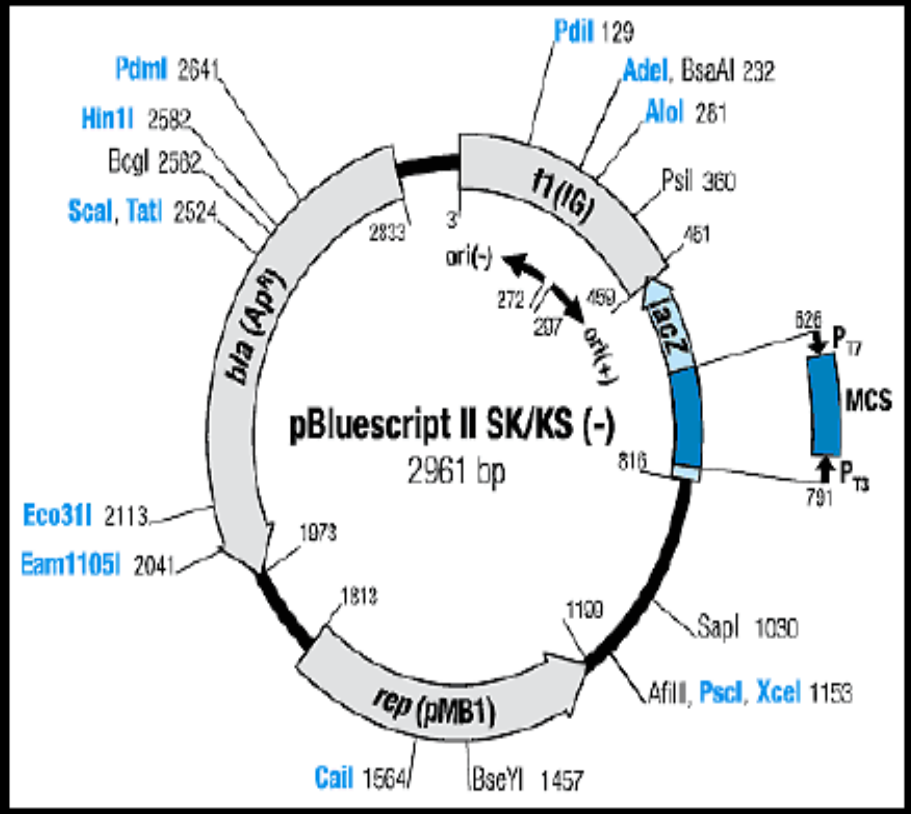
Protocols included in these kits generally consist four main steps:

- Dissolution of the gel-slice (i.e. in 3 volumes of chaotropic agent at 50 -65°C),
- DNA trapping: application of the DNA binding solution to a spin-column to trap the DNA in the column,
- Washing: a 70% ethanol wash to wash out salt and impurities while the DNA remains in the column
- Elution: elution of the DNA in a small volume (10 -50µL) of water or buffer.

Inserting the target gene into a plasmid: Plasmid

Plasmid or cloning vector

- Independent circular DNA in bacteria
- Size of <10 kb useful for cloning; natural plasmids can range up to 250 kb
- Plasmids may contain antibiotic resistance genes or other reporter genes (i.e. lacZ) that allow the selection of bacteria that contain the plasmid.

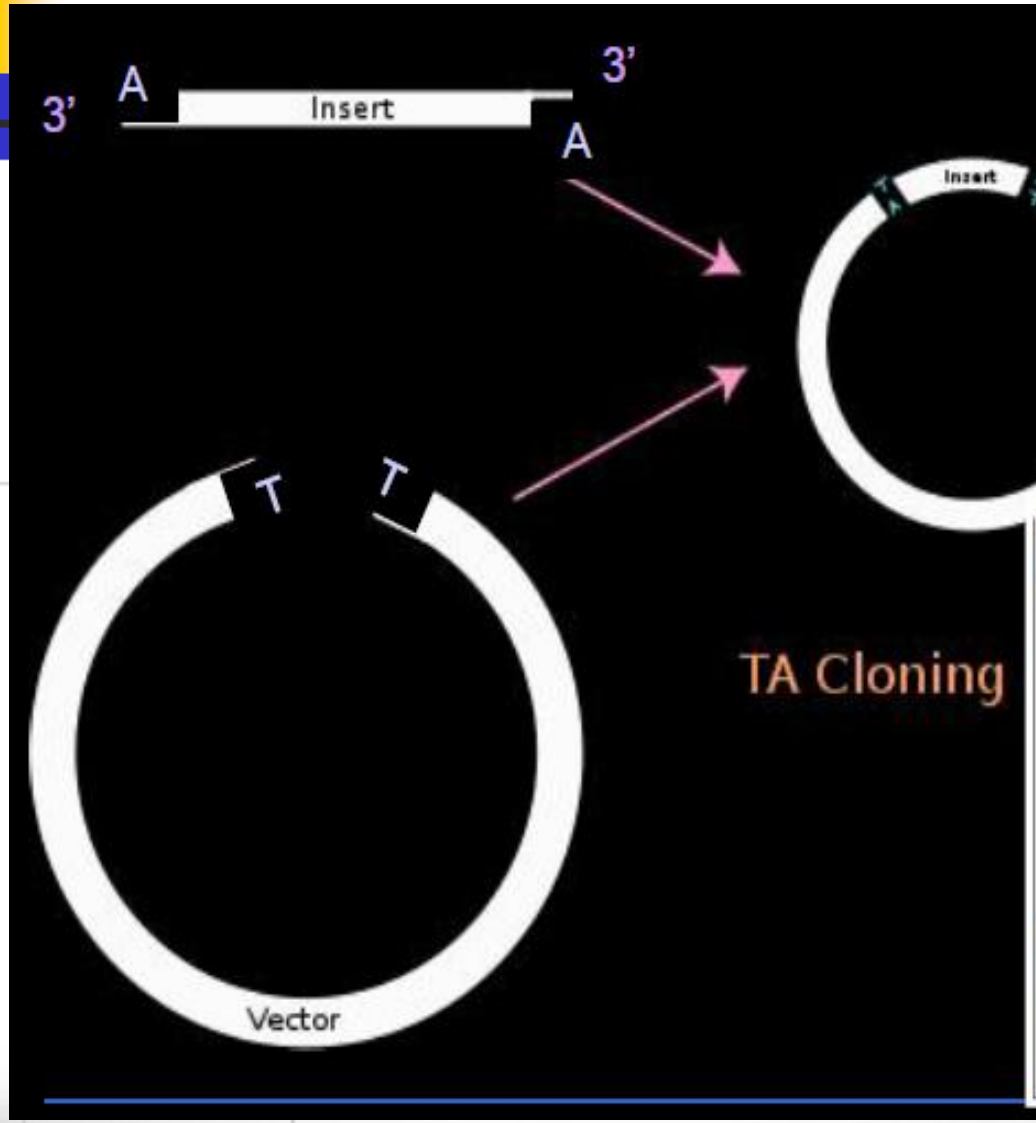


Inserting the target gene into a plasmid: TA-cloning

- TA Cloning is a subcloning technique that doesn't use restriction enzymes. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together.

- PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product.

- Such PCR amplified inserts are cloned into linearized plasmid/ vectors that have complementary 3„ thymine overhangs.



Ligation Results:

Plasmid with insert
Plasmid only
Insert only

Ligation Steps:

Mix:

TA-vector 100 ng/µl	1 µl
Ligation buffer	5 µl
DNA fragment	1.5 µl
Water	2.5 µl
Total	10 µl

Mix well by tapping
Spin briefly
Incubate at 16ºC 1h–over night.

Transformation into a host cell

- After you create your new plasmid construct that contains your insert of interest , you will need to insert it into a bacterial host cellso that it can be replicated.
- The process of introducing the foreign DNA into the bacterial cell is called transformation.
- Bacterial cells that can take up DNA from the environment are said to be competent.
- An example of *E. coli strain used for transformation is DH5α*
- *Two methods for transforming: heat shock and electroporation*

Making Competent Cells

- Washing cell with GLISEROL
- **Cells** are mixed with an equal volume of ice-cold 2 x **TSS** (Transformation & Storage Solution)
- Chilling cells in the presence of divalent cations such as Ca^{2+} , Washing cell with $CaCL_2$ (Calcium chloride) Heat-shock Methode

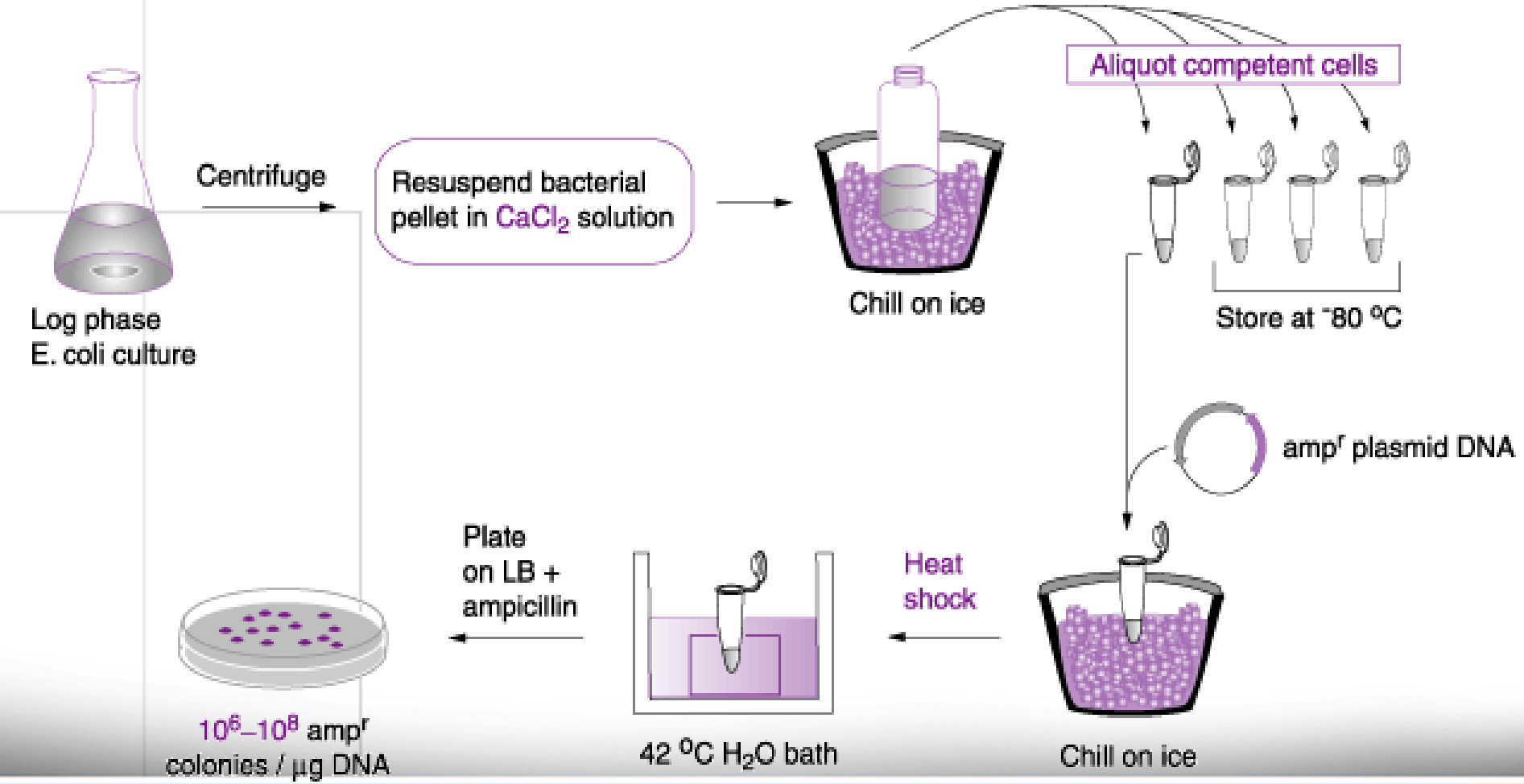
Transformation: Chemical method

- Chemical competence is conferred to *E.coliby re-suspension in CaCl2solution at 0°C.*
- Under these conditions, the Ca^{2+} ion is thought to create pores in the membrane, assist binding of the DNA to the cell membrane and mask the negative charge on the DNA, easing it's passage through the hydrophobic cell membrane.
- The DNA is forced into the cells by applying a short 42°C heat shock, which results in a thermal current that sweeps the DNA into the cells.

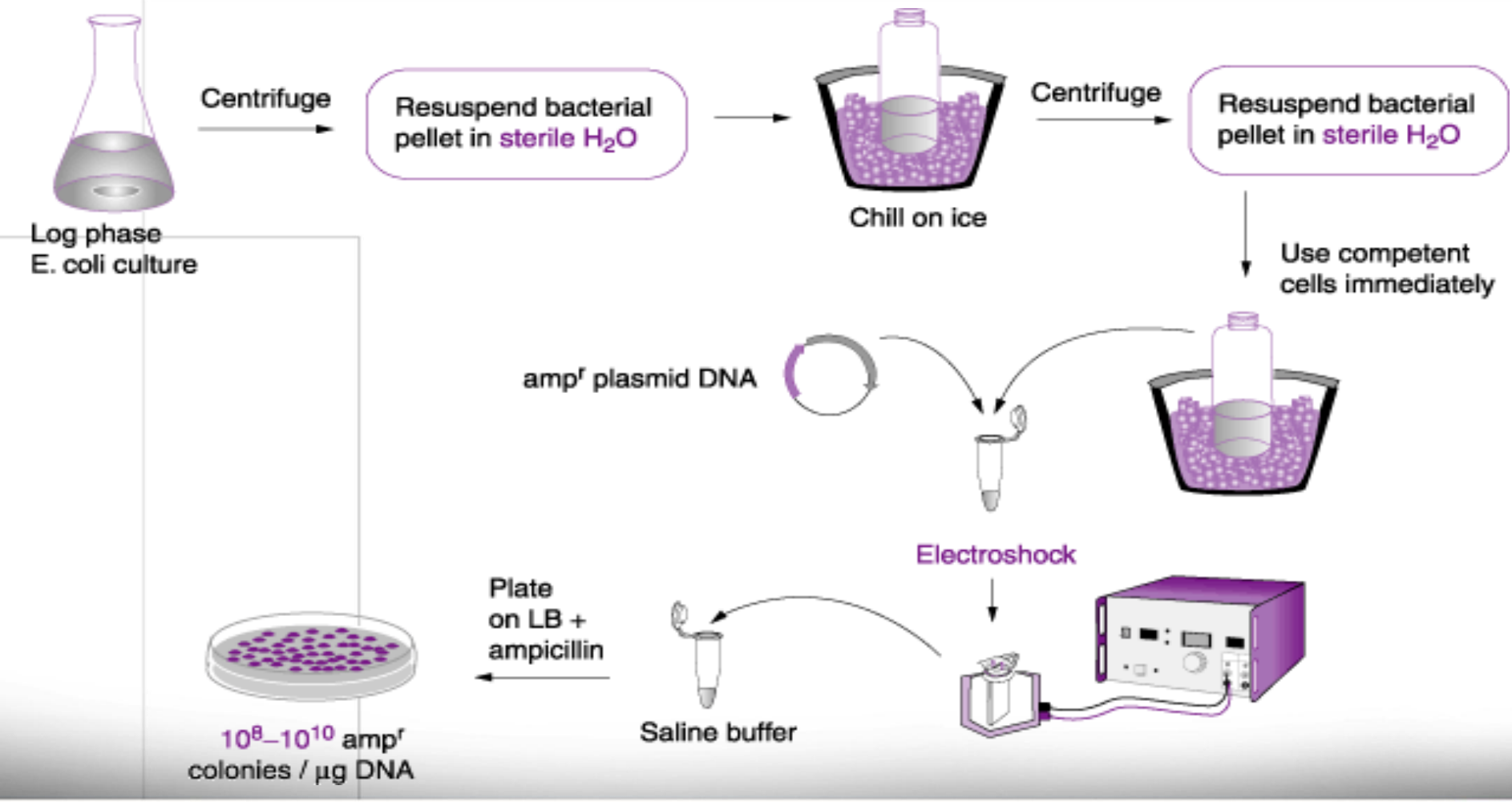
Transformation: Electro method

Electroporation involves the application of an electric current across the cell, which is thought to create momentary “pores”in the cell membranes and force the negatively charged DNA into the cells by an electrophoresis-type effect.

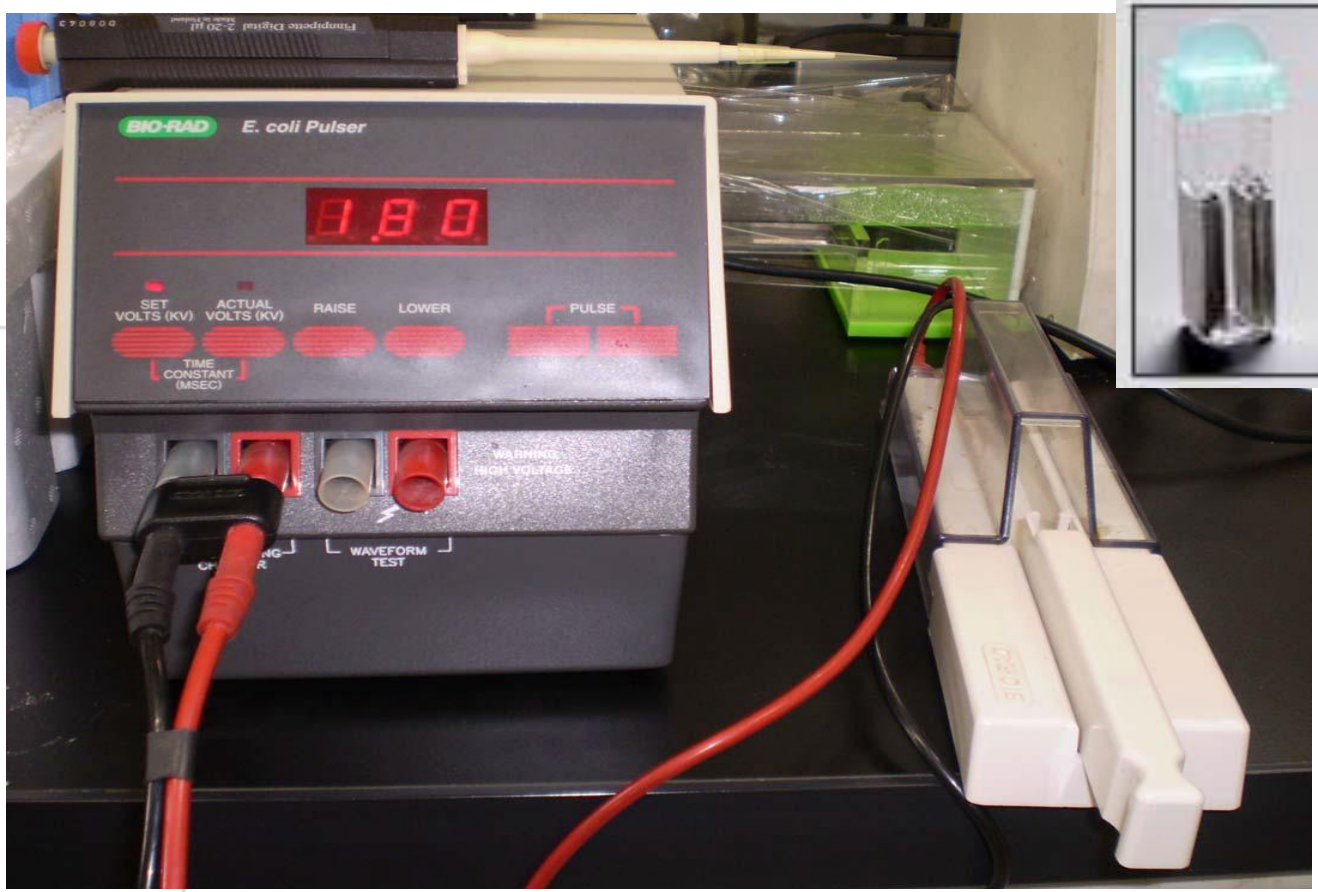
CHEMICAL TRANSFORMATION WITH CALCIUM CHLORIDE



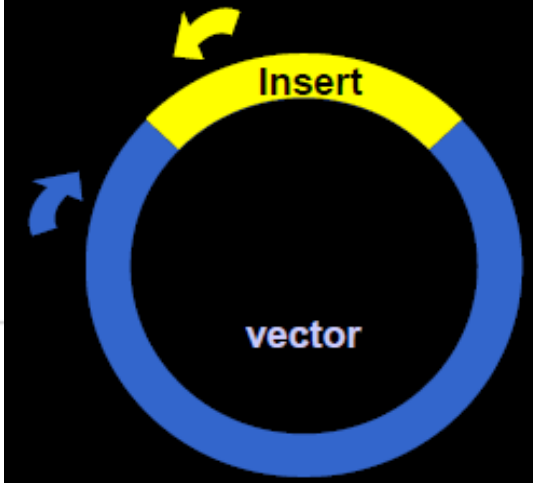
TRANSFORMATION BY ELECTROPORATION



ELECTROPORATOR

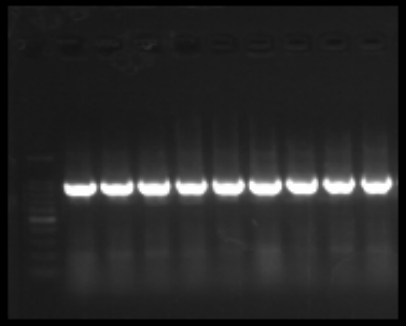


Colony PCR: selecting the true-transformed colonies

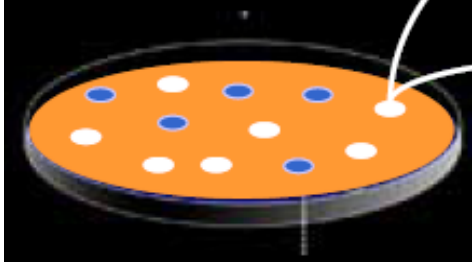


PCR using vector-insert primer pair allows the identification of insert in the vector

PCR



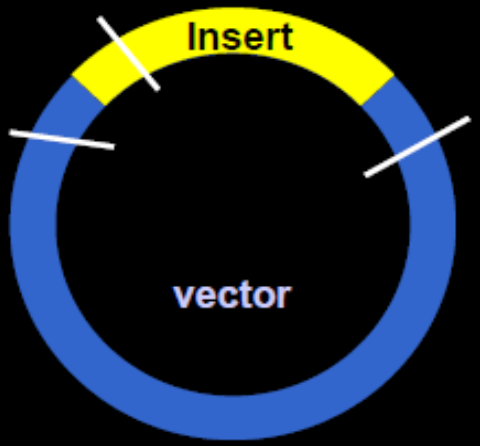
Contained PCR mix



Contained LB-medium

Over night culture in LB + antibiotics at 37°C (shaking)

Boil-method: selecting the true-transformed colonies



Cutting the plasmid by certain restriction enzyme allows the identification of insert in the vector

Over night culture in LB + antibiotics at 37°C (shaking)

Collect cells, add STET and lysozyme (on ice)

Vortex

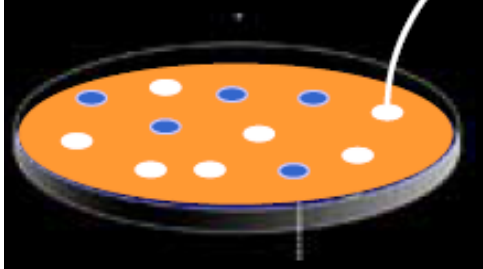
Boil in 100°C for 40"

Centrifuge 15 000 rpm at 4°C

Discard pellet (i.e. by toothpick)

Isopropanol precipitation

Plasmid



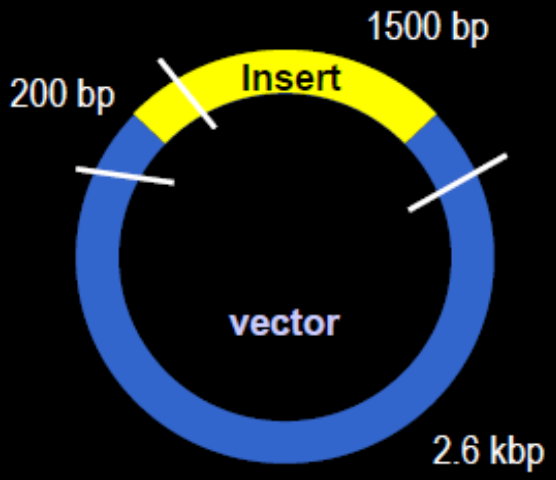
Boil-method: selecting the true-transformed colonies

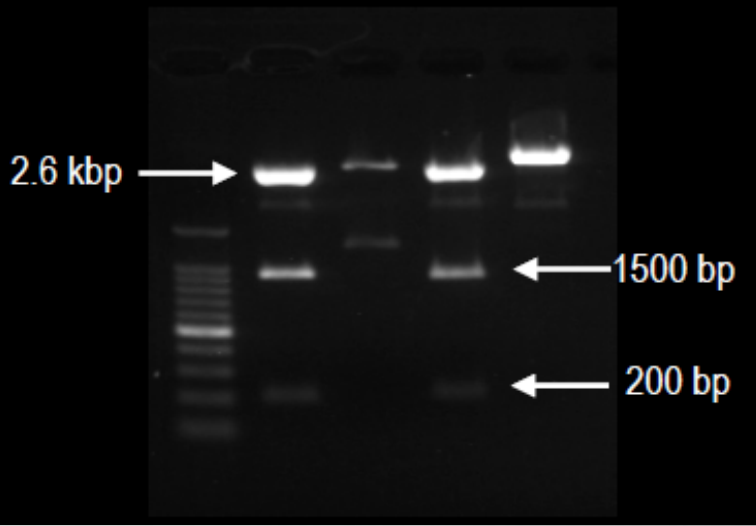
Plasmid digestion by restriction enzyme:

Components	Final concentration
10x Buffer	1x
Enzyme	1 unit/ µg DNA
Plasmid	> 1 µg
Water	

→ Incubate at reaction temperature (i.e. 37°C for Hind III)

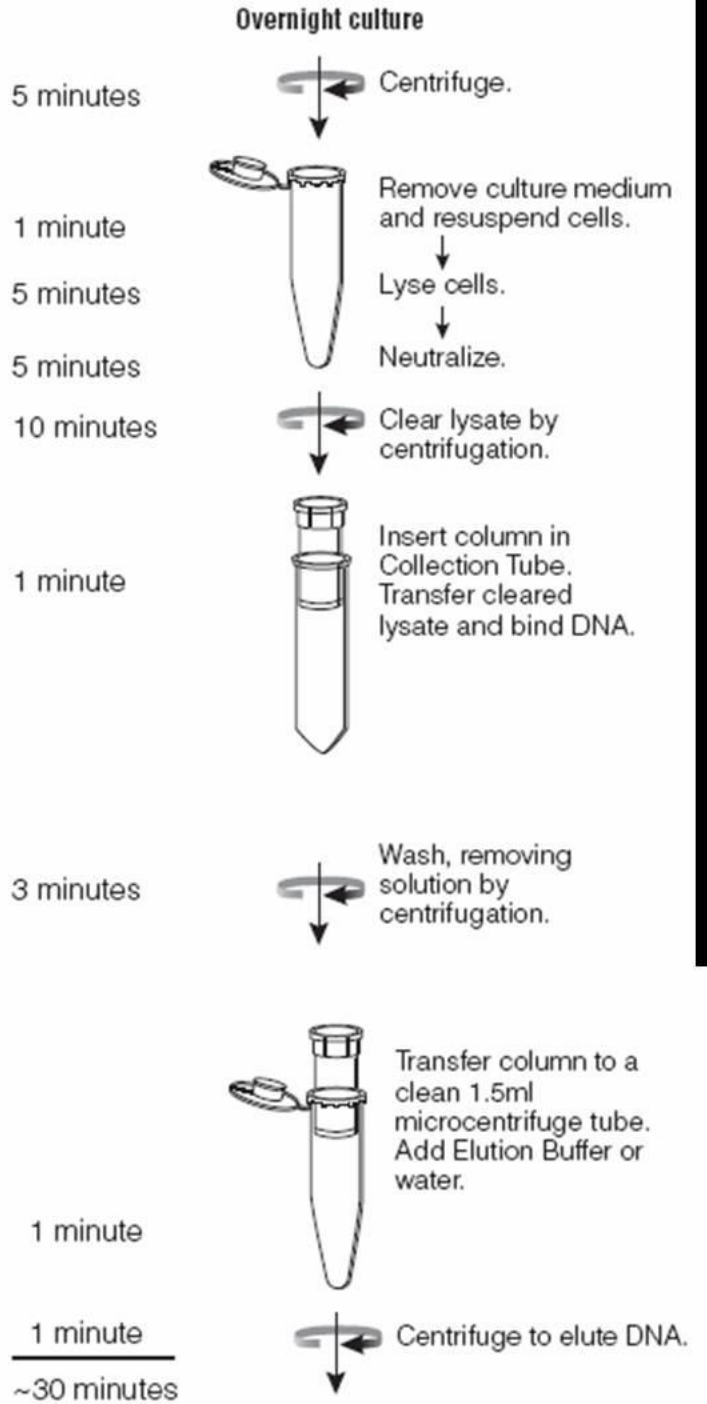
→ Electrophoresis in TAE gel





Plasmid Purification

- Lysis: Disruption of most cells is done by chaotropicsalts, detergents or alkaline denaturation
- Neutralization: neutralize the alkaline pH to prevent plasmid degradation.
- DNA binding: binding plasmid DNA (i.e. by silica in the binding column)
- Washing: removing the remaining salts with an alcohol based wash
- Elution: elute DNA by water or TE buffer




THANK
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