HiPer® Plasmid DNA Extraction Teaching Kit (Solution Based)

Product Code: HTBM003

Number of experiments that can be performed: 20

Duration of Experiment: 3 days

Day 1: Revival of Host
Day 2: Inoculation of culture
Day 3: Plasmid DNA Extraction and Agarose Gel Electrophoresis

Storage Instructions:

- The kit is stable for 12 months from the date of manufacture
- Store Control DNA and Ampicillin at -20°C
- Store 6X Gel Loading Buffer, *E.coli* cells (with plasmid) and Resuspension Solution at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)

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WHO GMP CERTIFIED
ISO 13485-2016 CERTIFIED

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Aim:

Isolation and purification of plasmid DNA (using solution based method).

Introduction:

Plasmid is an extra-chromosomal DNA molecule different from the chromosomal DNA which is capable of independent replication. The term "Plasmid" was first introduced by the American molecular biologist Joshua Lederberg in 1952. Plasmid size varies from 1 to over 1000 kilo base pairs (kbps). Plasmids are mostly circular and double-stranded. Plasmids are found in a wide variety of bacterial species; but are sometimes found in eukaryotic organisms e.g., in *Saccharomyces cerevisiae.*

Fig1: A bacterium showing that the plasmids are not part of the chromosomal DNA

Plasmids typically have two important elements:

- An origin of replication
- A selectable marker gene (e.g. resistance to ampicillin)

Conformations of plasmid: Plasmid DNA may appear in one of the five conformations which are as follows:

- **"Nicked Open-Circular"** DNA has one strand cut.
- **"Relaxed Circular"** DNA is fully intact with both strands uncut, but has been enzymatically "relaxed" (supercoils removed).
- **"Linear"** DNA has free ends, either because both strands have been cut, or because the DNA was linear *in vivo*.
- **"Supercoiled"** (or "Covalently Closed-Circular") DNA is fully intact with both strands uncut, and with a twist built in, resulting in a compact form.
- **"Supercoiled Denatured"** DNA is like supercoiled DNA, but has unpaired regions that make it slightly less compact.

The conformations listed above are in order of electrophoretic mobility from slowest to fastest; and for a given size, run at different speeds in the gel during electrophoresis.

Plasmid DNA Extraction Teaching Kit (Solution Based) provides a fast and easy method of purification of plasmid DNA for reliable applications in PCR, library screening and sequencing, etc.

The DNA purification using precipitation method comprises following steps.

- Resuspension of bacterial cell pellet
- Lysis of cells
- Precipitation of Genomic DNA

- Precipitation of Plasmid DNA
- Wash to remove residual contamination
- Elution of Plasmid DNA

Principle:

The alkaline lysis method for isolation of plasmid DNA from *E.coli* has been used for more than 20 years. This method takes advantage of the physical difference between linear, closed and supercoiled DNA. The bacterial suspension is first exposed to a strong anionic detergent (i.e. SDS) at high pH which helps to rupture the cell wall by hyperlytic osmosis releasing the DNA (chromosomal and plasmid), proteins and other contents which are denatured. The strands of the closed circular plasmid DNA are not completely ruptured in this process as the plasmid has a highly supercoiled confirmation. On addition of neutralization solution, the proteins, polysaccharides and genomic DNA are precipitated, whereas the plasmid DNA remains in the solution. The plasmid DNA is then precipitated by addition of isopropanol. Subsequently, other contaminants are removed by addition of Wash Solution I and II. The pure plasmid is then eluted in Elution Buffer.

Kit Contents:

This kit can be used to perform plasmid DNA extraction from *E.coli* strain.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			20 expts	
1	TKC005	Control DNA	0.22 ml	-20^{O}C
2	TKC006	*E. coli* cells (with plasmid)	1 No.	2-8^{O}C
3	DS0020	Resuspension solution (HP1) with RNase A	6 ml	2-8^{O}C
4	DS0021	Lysis solution (HP2)	6 ml	R T
5	DS0022	Neutralization solution (HN3)	8 ml	R T
6	MB063	Isopropanol	22 ml	R T
7	DS0032	Wash solution (HPB)	12 ml	R T
8	DS0024	Wash solution II	16 ml	R T
9	DS0040	Elution Buffer (ET) [10mM Tris-Cl, pH 8.5]	1.2 ml	R T
10	MB002	Agarose	9.6 g	R T
11	M1245	Luria Bertani (LB) Broth	10 g	R T
12	MB053	Agar Powder, Bacteriological	2 g	RT
13	MB104	Ampicillin	2x 0.025 g	-20^{O}C
14	PW1139	Collection Tubes, Polypropylene (2.0 ml)	40 Nos.	R T
15	ML016	50X TAE	240 ml	R T
16	ML015	6X Gel Loading Buffer	0.1 ml	2-8^{O}C

Materials Required But Not Provided:

Glasswares: Conical flask, Measuring cylinder, Beaker
Reagents: Distilled Water, Ethidium bromide (10 mg/ml)
Other requirements: UV Spectrophotometer, Tabletop microcentrifuge (with rotor for 2.0 ml tubes), Electrophoresis apparatus, Incubator, UV Transilluminator, Micropipettes, Vortex Mixer, Shaker, Tips, Adhesive tape, Microwave/ Hotplate/ Bunsen, sterile loop

Storage:

HiPer® Plasmid DNA Extraction Teaching Kit (Solution Based) is stable for 12 months from the date of manufacture without showing any reduction in performance. On receipt, store the Control DNA and Ampicillin at -20°C. Store *E. coli* cells (with plasmid), 6X Gel Loading Buffer and Resuspension solution at 2-8°C. Other kit contents can be stored at room temperature (15-25°C).

Important Instructions:

Read important instructions before starting the experiment.

1. Read the entire procedure carefully before starting the experiment.
2. Thaw all refrigerated samples before use.
3. Thoroughly mix the reagents. Examine the solutions for any kind of precipitation. If any solution (except Resuspension Solution) forms a precipitate warm at 55-65°C until the precipitate dissolves completely, allow it to cool to room temperature before use.
4. Ensure that clean & dry eppendorf tubes and tips are used for the procedure.
5. **Ampicillin Preparation**: Dissolve 25 mg of the Ampicillin antibiotic in 500 µl of sterile distilled water to prepare a stock concentration of 25 mg/500 µl. Store at -20°C.
6. **Preparation of Luria Bertani broth (10ml):** Dissolve 0.25 g of Luria Bertani broth in 10 ml of distilled water and autoclave.
7. **Preparation of LB (Luria Bertani) agar plates with ampicillin (50 ml):** Dissolve 1.25 g of LB media and 0.75 g of agar in 50 ml of distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45 °C. Add 50 µl of ampicillin into it and pour on sterile petriplates.

Procedure:

Day 1: Revival of Host

1. Open the vial containing culture and resuspend the cells with 0.25 ml of LB broth.
2. Pick up a loopful of culture and streak onto LB agar plate with ampicillin
3. Incubate overnight at 37°C.

Day 2: Inoculation of culture

1. Pick up a single colony from LB agar plate and inoculate in 10 ml of LB broth containing 10 µl ampicillin.
2. Incubate the test tube overnight at 37°C.

Day 3: Plasmid Extraction:

1. Harvest Cells

Take 1.5 ml of the overnight grown culture into a micro centrifuge tube and centrifuge the cells at 13,000 rpm for 3 minutes. Discard the supernatant culture medium.

NOTE: For good plasmid yields, the $O.D_{600}$ of the culture should be around 3.0×10^6 cells/ml.

2. Resuspend Cells

Resuspend the cell pellet in 250 µl of Resuspension Solution and mix well by gentle vortexing till no cell clumps are visible.

3. Lyse Cells

Add 250 µl of Lysis Solution to lyse the cells. Mix thoroughly by gently inverting the tube 4-6 times.

NOTE: Do not vortex the tubes as it may result in the shearing of genomic DNA which may contaminate the plasmid DNA. Do not allow this lysis reaction to exceed for more than 5 minutes.

4. Neutralize

Add 350 µl of Neutralization Solution and immediately mix thoroughly by inverting the tube gently 4-6 times.

NOTE: On addition of Neutralization Solution, genomic DNA will precipitate out. The mixture should become cloudy and the precipitation should be homogeneous.

5. Centrifuge the sample at 13,000 rpm for 10 minutes to obtain a compact white pellet.

NOTE: A compact white pellet of genomic DNA will form. If the supernatant is not clear, transfer the supernatant to a fresh tube and spin for an additional one minute at 13,000 rpm to remove the interfering salts/precipitates completely.

6. Precipitation of Plasmid DNA

Carefully transfer the supernatant containing plasmid DNA to a new collection tube. Add 1 ml of isopropanol to precipitate plasmid DNA and mix by gentle inversion for 5 minutes.

7. Centrifuge at 13,000 rpm for 15 minutes. White pellet of plasmid DNA will be seen, sticking to the sides of the tube. Discard the supernatant and invert the vial on blotting paper to drain out left over supernatant.

8. First Wash

Resuspend the pellet by adding 500 µl of Wash Solution I (HPB) and centrifuge at 13,000 rpm for 3 minutes.

9. Second Wash

Discard the supernatant and add 700 µl Wash Solution II. Centrifuge at 13,000 rpm for 3 minutes. Discard the supernatant and air dry the pellet for 10-15 minutes at room temperature.

11. DNA Elution

Resuspend the pellet in 50 µl of Elution Buffer. Centrifuge at 13,000 rpm for 5 minutes to remove insoluble material and transfer the supernatant containing pure plasmid DNA into a new collection tube.

Storage of the eluate with purified DNA: The eluate contains pure plasmid DNA. For short term storage (24-48 hours) of the DNA, 2-8°C is recommended. For long-term storage, -20°C or lower temperature (-80°C) is recommended. Avoid repeated freezing and thawing of the sample which may cause denaturation of DNA. The Elution Buffer will help to stabilize the DNA at these temperatures.

Agarose Gel Electrophoresis:

Preparation of 1X TAE: To prepare 500 ml of 1X TAE buffer, add 10 ml of 50X TAE Buffer to 490 ml of sterile distilled water*. Mix well before use.

Preparation of agarose gel: To prepare 50 ml of 0.8% agarose gel, add 0.4 g agarose to 50 ml 1X TAE buffer in a glass beaker or flask. Heat the mixture on a microwave or hot plate or burner by swirling the glass beaker/flask occasionally, until agarose dissolves completely (Ensure that the lid of the flask is loose to avoid buildup of pressure). Allow the solution to cool to about 55-60°C. Add 0.5 µl Ethidium bromide, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.

NOTE: Ethidium bromide is a powerful mutagen and is very toxic. Appropriate safety precautions should be taken by wearing latex gloves; however, use of nitrile gloves is recommended.

Loading of the DNA samples: To prepare sample for electrophoresis, add 2 µl of 6X gel loading buffer to 10 µl of DNA sample. Mix well by pipetting and load the sample into the well. Load the Control DNA after extracting the DNA sample.

Electrophoresis: Connect power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100-120 volts and 90 mA until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

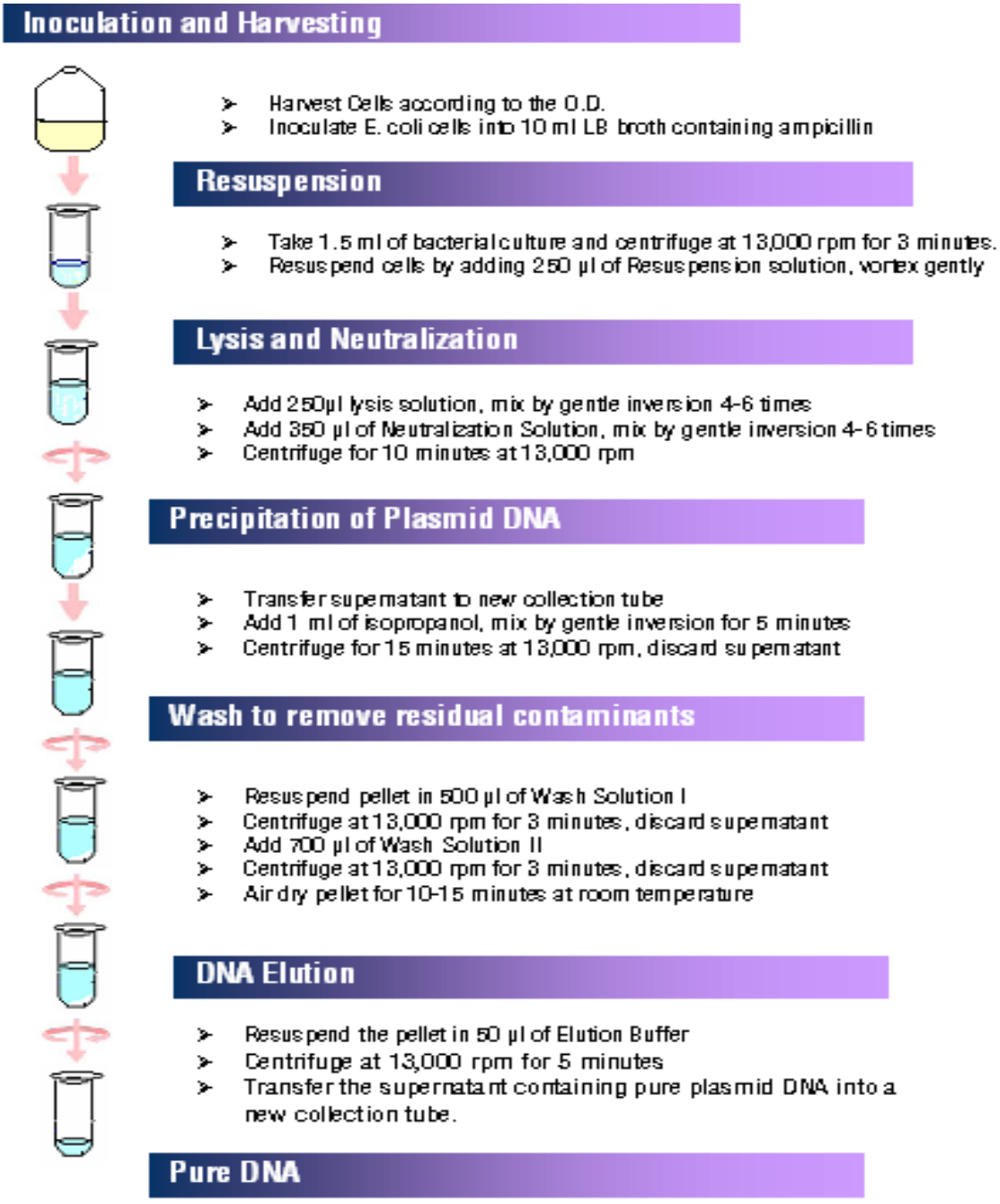
* Molecular biology grade water is recommended (Product code: ML024).

Quantitation of DNA:

Spectrophotometric analysis and agarose gel electrophoresis will reveal the concentration and the purity of the genomic DNA. Use Elution Buffer to dilute samples and to calibrate the spectrophotometer, measure the absorbance at 260 nm, 280 nm, and 320 nm using a quartz microcuvette. Absorbance readings at 260 nm should fall between 0.1 and 1.0. The 320 nm absorbance is used to correct background absorbance. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. The concentration of DNA is calculated by the following formula:

Concentration of DNA sample (µg/ml) = 50 x A_{260} x dilution factor

Flowchart:


Inoculation and Harvesting
Harvest Cells according to the O.D.
Inoculate E. coli cells into 10 ml LB broth containing ampicillin
Resuspension
Take 1.5 ml of bacterial culture and centrifuge at 13,000 rpm for 3 minutes.
Resuspend cells by adding 250 µl of Resuspension solution, vortex gently
Lysis and Neutralization
Add 250µl lysis solution, mix by gentle inversion 4-6 times
Add 350 µl of Neutralization Solution, mix by gentle inversion 4-6 times
Centrifuge for 10 minutes at 13,000 rpm
Precipitation of Plasmid DNA
Transfer supernatant to new collection tube
Add 1 ml of isopropanol, mix by gentle inversion for 5 minutes
Centrifuge for 15 minutes at 13,000 rpm, discard supernatant
Wash to remove residual contaminants
Resuspend pellet in 500 µl of Wash Solution I
Centrifuge at 13,000 rpm for 3 minutes, discard supernatant
Add 700 µl of Wash Solution II
Centrifuge at 13,000 rpm for 3 minutes, discard supernatant
Air dry pellet for 10-15 minutes at room temperature
DNA Elution
Resuspend the pellet in 50 µl of Elution Buffer
Centrifuge at 13,000 rpm for 5 minutes
Transfer the supernatant containing pure plasmid DNA into a new collection tube.
Pure DNA

Observation and Result:

Perform Agarose Gel Electrophoresis. Visualize the DNA bands using UV Transilluminator and calculate the yield and purity using UV Spectrophotometer.

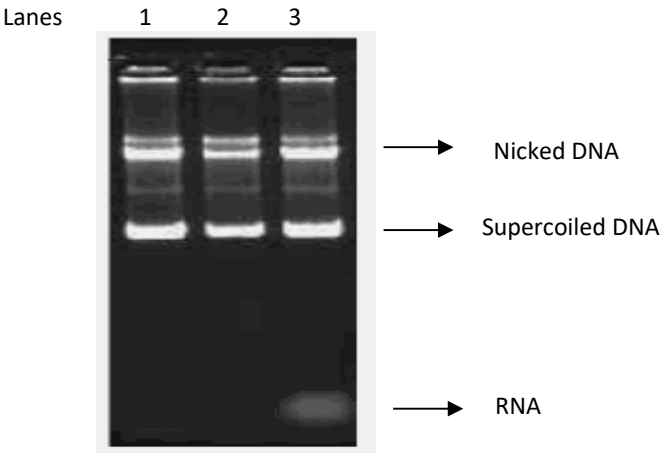


Fig 2: Gel picture of isolated plasmid DNA

Lane 1: Control DNA
Lane 2: Extracted plasmid DNA
Lane 3: Plasmid DNA with RNA contamination

Table 2: Absorbance of the extracted plasmid DNA at 260 nm and 280 nm

Sample	Dilution Factor	A_{260}	A_{280}	A_{260}/A_{280}	Concentration (µg/ml)
1					
2					
3					

Calculate the concentration of isolated DNA using following formula:

Concentration of DNA sample (µg/ml) = 50 x A_{260} x dilution factor

Interpretation:

On analyzing plasmid DNA after electrophoresis, two bands were observed.
1) Nicked DNA
2) Supercoiled DNA
The supercoiled being more compact runs faster than the nicked form. The nicked form runs slowly since their open structure experiences more resistance while passing through the gel matrix. These are seen as bands above the supercoiled form.

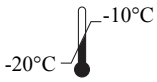
The data in Lanes 1 and 2 demonstrate that highly purified plasmid DNA has been obtained with no visible RNA contamination when electrophoresed on agarose gel. If RNA contamination is present, one would see a faint and smeary RNA band below the plasmid DNA as shown in Lane 3. RNA being of lower molecular weight runs faster than the plasmid DNA. RNA contamination is observed when the RNase treatment has not been carried out properly. An absorbance of 1.0 at 260 nm corresponds to approximately 50 µg/ml of DNA. If the A_{260}/A_{280} ratio is 1.6-1.9, then the isolated DNA sample is considered to be pure. If higher A_{260}/A_{280} ratio is observed it indicates the possibility of RNA contamination.

Troubleshooting Guide:

Sr. No.	Problem	Possible Cause	Solution
1	Poor or low plasmid DNA recovery	Alkaline lysis is done for longer time	The lysis time should not exceed more than 5 minutes
		Plasmid DNA is contaminated with chromosomal DNA	Do not use cultures that have grown for more than 24 hours or if cells are in the death phase. Do not vortex or vigorously shake the cells during the lysis reaction or neutralization procedure
2	Additional band seen ahead of supercoiled plasmid during gel electrophoresis	A portion of the plasmid DNA is permanently denatured	Do not allow the lysis reaction to exceed 5 minutes NOTE: The nicked or covalently open double-stranded plasmid DNA runs slower than the super coiled DNA during electrophoresis
3	Poor performance in downstream enzymatic applications	Purification is incomplete	Salts in one or more of the solutions may have precipitated. Examine the solutions for any kind of precipitation, if any solution forms a precipitate warm at 55-65°C until the precipitate dissolves completely; allow it to cool down to room temperature before use

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com



Storage temperature

Do not use if package is damaged

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03/2026

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