DNA Extraction using Qiagen DNeasy Mini Prep Kit

Protocol: Pretreatment for Gram-Positive Bacteria
This protocol is designed for purification of total DNA from Gram-positive bacteria. The protocol describes the preliminary harvesting of bacteria and incubation with lysozyme to lyse their cell walls before DNA purification.

1. Harvest cells (maximum 2 x 10^9 cells) in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm). Discard supernatant.

2. Resuspend bacterial pellet in 180 μl enzymatic lysis buffer.

3. Incubate for at least 30 min at 37°C.
After incubation, heat the heating block or water bath to 56°C if it is to be used for the incubation in step 5.

   Note: Do not add proteinase K directly to Buffer AL. Ensure that ethanol has not been added to Buffer AL

5. Incubate at 56°C for 30 min.

6. Add 200 μl ethanol (96–100%) to the sample and mix thoroughly by vortexing.
   It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.
   A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

7. Pipette the mixture from step 6 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

8. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μl Buffer AW1, and centrifuge for 1 min at _6000 x g (8000 rpm). Discard flow-through and collection tube.

9. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).
10. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 μl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

11. Hold Eluted DNA on ice

DNA Extraction using “Dirty” Method or Colony Pick Method

**Protocol: Rapid Pretreatment for Gram-Positive Bacteria**
This protocol is designed for the rapid separation of total DNA from Gram-positive bacteria. The protocol describes the treatment of bacterial isolates for further manipulation through PCR methods.

1. **Isolate a pure colony from a bacterial culture from an agar plate.**
   Using a sterile toothpick, remove a portion of a single colony from an agar plate. This should be roughly about a quarter or a third of the colony. Less is more in this instance. Place the portion of culture in a sterile eppendorf tube.

2. **Cell lysis**
   Place the eppendorf tubes containing the sample in a microwave on High for ~3 min. This will lyse the bacterial cells in the sample causing a release of DNA.

3. **Suspension and Hydration of DNA**
   Suspend the sample in 100μL of Nuclease free, sterile water. This can be attained by adding 100μL of the sample and gently pipetting the sample up and down until dissolved in the water.

4. Hold the suspended DNA on ice or 4°C (no long than a week) until further work.
DNA Quantification and Purity

Introduction

DNA quantity and quality are important because they will influence the efficiency of PCR in a number of ways. When we describe DNA quantity, we typically report values as ng/µl or mg/ml. The weight of a nucleotide is 330 Daltons therefore a base pair is 660 Daltons. However, we use spectrophotometry as an indirect measurement to quantify DNA due to our inability to efficiently “weigh” it. Purines (A’s and G’s) contain a pyrimidine ring fused to an imidazole ring which absorb energy at A260 (UV spectrum). The relationship between absorption and DNA concentration is linear; greater absorbance at A260 = higher quantity of DNA. Traditionally, DNA concentrations are determined in quartz cuvettes which require a minimum of 0.5 ml of sample. This is typically not practical when quantifying DNA, especially if the initial source/sample is limited (e.g., mosquito trapped in amber). The new alternative is Nanodrop technology.

How It Works

Pedestal Measurements

The patented sample retention system used by The Thermo Scientific NanoDrop 2000 and NanoDrop™ 2000c allows for the analysis of 0.5 µl - 2.0 µl samples, without the need for cuvettes or capillaries.

With the arm open, a sample is pipetted directly onto the pedestal.

After the arm is closed, a sample column is formed.

The pedestal then moves to automatically adjust for an optimal path length (0.05 mm - 1 mm).

When the measurement is complete, the surfaces are simply wiped with a lint-free lab wipe before going on to the next sample.

- **Sample ID** - field into which a sample ID is entered. The appropriate sample ID should be entered prior to each measurement.
• **Type** - a drop down list from which the user may select the (color-keyed) type of nucleic acid being measured. Options include DNA-50 for dsDNA, RNA-40 for RNA, and ssDNA-33 for single-stranded DNA. Additional options include Oligo DNA and Oligo RNA which utilize the appropriate extinction coefficient based upon user-defined base sequences.

• **Conc** - concentration based on absorbance at 260 nm

• **A260** - displays absorbance at 260 nm normalized to a 10 mm pathlength.

• **A280** - displays absorbance at 280 nm normalized to a 10 mm pathlength.

• **260/280** - ratio of absorbance at 260 nm and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

• **260/230** - ratio of absorbance at 260 nm and 230 nm. This is a secondary measure of nucleic acid purity. Residual ethanol and salts absorb at 230 nm.

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<table>
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<tr>
<th>Sample ID</th>
<th>Conc</th>
<th>A260</th>
<th>A280</th>
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*Adapted from Nanodrop by Thermo Scientific*
Nucleic Acid Calculations
For nucleic acid quantification, the Beer-Lambert equation is modified to use a factor with units of ng-cm/microliter.

The modified equation used for nucleic acid calculations is the following:
\[ c = \frac{A \cdot \varepsilon}{b} \]
c = the nucleic acid concentration in ng/microliter
A = the absorbance in AU
\( \varepsilon \) = the wavelength-dependent extinction coefficient in ng-cm/microliter
b = the pathlength in cm

The generally accepted extinction coefficients for nucleic acids are:
• Double-stranded DNA: 50 ng-cm/μL
• Single-stranded DNA: 33 ng-cm/μL
• RNA: 40 ng-cm/μL

When the pedestal mode is selected, the NanoDrop 2000/2000c spectrophotometer uses short pathlengths between 1.0 mm to 0.05 mm to enable measurement of concentrated samples without dilution.
Agarose gel electrophoresis & Interpretation

Introduction

*You must change tips after every use. No exceptions*

The goal of today’s laboratory is to determine the results of our PCR reactions:

1. *Salmonella* control
2. *Listeria monocytogenes*
3. Negative control
4. Unknown

1. **Load PCR product in to agarose gel**

Load 10µl PCR product in to 1.5% agarose gel

2. **Stain agarose gel in Ethidium Bromide**

   Place gel in 0.0001% EtBr soln. for 30s. Remove.

3. **Destain agarose gel in water for 30 min.**
4. **Visualize PCR product using UV light and image capture system**
5. **Interpret results**

<table>
<thead>
<tr>
<th>PCR template</th>
<th>Amount of template</th>
<th>+/- reaction</th>
<th>Correction band size</th>
<th>Y/N</th>
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Primer Preparation and PCR

Introduction

*You must change tips after every use. No exceptions*

The goal of today’s laboratory is to set up 4 independent PCR reactions using 4 independent templates:

1. Salmonella control
2. Listeria monocytogenes
3. Negative control
4. Unknown

1. PRIMER HYDRATION

The first step is to hydrate your custom primers. Primers arrive as a lyophilized powder which must be reconstituted prior to use in PCR reactions. Primer are hydrated with sufficient 10 mM Tris-HCl pH 7.4 to achieve a 100µM stock concentration prior to further dilution to our working stock concentration of 10 µM. On the detailed primer insert provided by the manufacturer, determine the amount of primer in the tube.

To achieve a 100µM stock concentration of primer, simply move the decimal to the right.

EX. 25.09nMol of primer would be hydrated with 250µl 10 mM Tris-HCl to achieve a 100µM stock concentration.

_____ nMol

_____ volume Tris-HCl added

Our working stock (i.e. the concentration we use in a PCR reaction) is 10µM, which means we need a _____ dilution. This calculation is up to you!
2. PCR REACTION SETUP

Once you have prepared your 10µM working stock concentration of primers, you are ready to assemble your PCR reaction. We are using a commercially available “Master Mix” which contains DNA Taq Polymerase, dNTPs, MgCl₂, and buffer. It DOES NOT contain primers, DNA template, and sufficient water to dilute the buffer to 1X concentration.

GoTaq® Master Mix Protocol

1. Thaw the GoTaq® Green Master Mix at room temperature. Vortex the Master Mix, then spin it briefly in a microcentrifuge to collect the material at the bottom of the tube.
2. Prepare the following reaction in a 0.2 mL PCR Tube
   a. 50 µl reaction volume (per culture)
      i. GoTaq® Green Master Mix, 2x 25 µl
      ii. Forward primer, 10 µM 2 µl
      iii. Reverse primer, 10 µM 2 µl
      iv. DNA Template 1 µl
      v. Nuclease Free Water 20 µl
3. Place the reaction in a thermal cycler.


3. THERMAL CYCLING CONDITIONS
4. SCHEDULE a time to do “PCR clean-up” on Thursday, Dec 9.
PFGE Sample Preparation

Bacterial Sample Preparation

1. Harvest cells.
From an overnight broth culture, place 1mL of culture in a 1.5mL eppendorf tube. Centrifuge sample for 5min at 8,000rpm. Remove the supernatant (liquid) and resuspend the pellet (solid) in 1mL TE buffer.

2. Sample standardization.
Using the nanodrop, adjust the OD_{610} of the sample to 1.0±0.2. Adjust the sample with TE buffer.

3. Lysozyme addition
Transfer 240μL of bacterial sample to a sterile 1.5mL tube. Add 60μL of lysozyme (10mg/mL). Mix by gently pipetting up and down. Do not shake or vortex tube. Place sample at 56°C for 10 min.
Note: Make duplicate samples of for further processing of every target sample.

SSP Solution

1. Ingredients for SSP solution
SSP is the solution used to suspend the bacterial sample in semi-solid state for further lysis and digestion. SSP is made up of 1.2% agarose, 10%Sodium dodecyl sulfate (SDS) and proteinase K (20mg/mL). All three ingredients will be prepared and set aside.

2. Preparation of SSP solution
Add 10% SDS and 1.2% agarose together a sterile 1.5mL tube. Keep mixture at 56°C until ready to process samples. When ready to continue, add proteinase K to the warm SDS/agarose mixture. Mix the sample gently by inverting or gently pipetting as to not create any bubbles. Immediately move on to the plug preparation step at this point.

The following are the volumes of each ingredients to add:
Table 1.
<table>
<thead>
<tr>
<th>Number of samples</th>
<th>10% SDS (μL)</th>
<th>1.2% Agarose (mL)</th>
<th>20mg/mL Proteinase K (μL)</th>
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<tr>
<td>1</td>
<td>30</td>
<td>0.267</td>
<td>3</td>
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<tr>
<td>10</td>
<td>300</td>
<td>2.66</td>
<td>30</td>
</tr>
</tbody>
</table>

Plug Preparation

1. Addition of sample to SSP solution
Take 300μL of the warm SSP solution freshly prepared and mix with 300μL of sample. Mix gently as to not create any bubbles in the mixture. Pipette up and down slowly, do not vortex.

2. Plug formation
Immediately, add the SSP solution/sample mix to the plug molds provided. Allow to cool for >15 min at room temperature.
3. Storage
Place plugs in 1.5mL of TE buffer in a fresh eppendorf tube
PFGE Sample Preparation

Lysis

1. **Cell lysis is to be performed on the previously prepared plugs.**
   Cell lysis buffer consists of 50mM Tris pH 8.0 50mM EDTA pH 8.0, 1% Sarkosyl, and 0.15 mg/mL proteinase K. This buffer is provided to you.

   In individual sterile tubes, combine 4mL of cell lysis buffer and each of the previously prepared plugs.

   Incubate the plugs at 54°C for 2 hr.

2. **Plug washes**
   Carefully, pour off the lysis buffer from the tube. Following removal of lysis buffer, the plugs will be washed with sterile milliQ water and TE buffer successively.

   First, wash the sample with 15mL of sterile milliQ water for 10 min at 54°C. Repeat wash for a total of two water washes.

   Second, wash the sample with 15mL of TE buffer at 54°C for 15 min. Repeat was three more times for a total of four TE buffer washes.

3. **Plug storage**
   Following wash steps, place 15mL of TE buffer in a fresh tube along with each individual plug. Place plugs at 4°C.
PFGE Sample Digestion

Restriction Digestion with Apal

1. Soaking of prepared plug in digestion buffer.
The initial step for restriction digestion involves soaking the plug in the restriction enzyme buffer (RE buffer) without the enzyme first. RE buffer will be provided.

Place 150μL of RE buffer in 1.5mL sterile tubes (one for each plug sample).

Carefully, remove sample plugs from the TE buffer it was stored in. Only a portion of the plug will be used for electrophoresis. With a sterile scalpel, cut a 2mm (height) slice from the plug. Place the rest of the plug in fresh TE buffer for storage (4°C).

Place each cut plug portion into their own tube of RE buffer. Ensure that the entire plug is submerged by gently inverted the tube.

Incubate plug at 37°C for 10 min.

2. Enzyme Digestion
Following incubation, carefully remove all the RE buffer from the tube containing the plug. Ensure not to remove any remnants of the plug with buffer removal.

Add 150μL of RE buffer plus Restriction Enzyme Apal (prepared for you) to the tube containing the plug sample. Incubate sample with Apal at 30°C overnight.
Pulse Field Gel Electrophoresis and Visualization

1. Gel preparation
The gel for PFGE is prepared in advance. It consists of 1% Agarose in 0.5X Tris-Borate EDTA buffer (TBE buffer). This gel is very similar to that which was used for PCR product gel electrophoresis.

2. Loading of enzymatically digested plugs
Remove plugs from incubator and cool to room temperature.

Remove the RE buffer from the sample plug tube carefully without disturbing the plug itself or removing any plug remnants.

Add 200μL of 0.5X TBE buffer to the tube containing the sample plug.

Carefully, remove the plugs from the TBE buffer solution using the blunt end of a spatula and place in the well of the gel.

*Note: Plug standards are used for comparison of band profiles in PFGE. The plug standards will be used as an example by the proctor to demo how to place the plug in the gel.*

Fill in the gaps in the wells with 1% Agarose following sample addition ensuring sample stability in the gel.

3. Pulse Field Gel Electrophoresis
Remove the gel from the gel casting unit by removing the end gates. Trim additional agarose from the corners of the gel.

Place the gel in the gel electrophoresis chamber. The following are the conditions for electrophoresis. The proctor will program these conditions in.

Running Buffer – 0.5XTBE @ 14°C
Select Auto Algorithm on the Chef Mapper keypad
Enter in 30kb for Low MW and 700kb for High MW.
Press ‘Enter’ for default option.
Run Time – 19hr for 14cm by 13cm gel or 21 hr for 21cm by 14cm gel.

4. Gel staining and visualization
Place the gel in Tupperware containing ethidium bromide solution (prepared in advance). Stain gel for 30 min.

Place the gel in water in order to destain it for 60 min, replacing the water every 20 min.

Visualize the gel using UV light and imager.

5. Interpret results
Using predetermined sizes for the standard lanes, compare band sizes and frequencies between lanes on the gel along with further analysis.