Southern Blot and Sequence Analysis of Cloned Recombinant Plasmid

Objective

The objective of this lab unit is to learn Southern blot analysis by confrming the presence of nbs sequence in the cloned recombinant plasmid.

Introduction

To confirm the success of DNA cloning done in the past three weeks, we need to obtain the recombinant plasmid and analyze the cloned DNA. There are ways to analyze DNAs, such as direct DNA sequencing, restriction mapping, and Southern blot analysis, a procedure to identify specific sequence in a DNA sample to reveal information about its identity, size, and abundance (copy number). The major steps of Southern blot analysis include the separation of DNA fragments on an agarose gel and their transfer onto the surface of a membrane followed by probe hybridization, washing and detection.

To confirm the presence of *nbs* sequence in the cloned plasmid, we will have to isolate plasmid DNA from an overnight *E. coli* bacterial culture (unit 2-1). In addition to outsourcing for direct sequencing of the cloned DNA insert in the plasmid, we will also carry out restriction mapping and Southern blot analysis. We first cut the cloned plasmid and a known grape nbs-containing plasmid, pSCA7 (T1-T3-W6)[1,2], with restriction enzymes (Type II), of which DNA fragments are then electrophoresed through an agarose gel to separate them based on sizes (unit 2-2). A restriction map of the recombinant plasmid can be constructed. The DNAs in the gel are then denatured and transferred onto a nitrocellulose or nylon membrane, so called Southern transfer/blot. Meanwhile, a sample of restriction digested plasmid containing a known grape *nbs* sequence is also separated by agarose gel electrophoresis and extracted from the gel to be used to synthesize a Dig-labeled DNA probe (unit 2-3) for Southern hybridization (unit 2-4). After washing and detection (*colorimetric*, chemiluminescence, or autoradiograph), the presence and location of the *nbs* sequence in the plasmid can be identified. With results of DNA sequencing, we will also analyze your cloned *nbs*-containing sequence using free web-based sequence analysis programs (unit 2-5)

[1]Ming-Mei Chang, P. DiGennaro, and A. Macula (2009) PCR cloning partial *nbs* sequences from grape (*Vitis aestivalis* Michx). Biochem & Mol Biol Education 37 (6):355-360.
[2]Nucleotide [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [2010]. Accession No. HM562670, Vitis aestivalis P-loop NTPase, partial, mRNA; [cited 2021 01 20]. Available from: https://www.ncbi.nlm.nih.gov/nucleotide/HM562670.1?report=genbank&log$=nuclalign&blast_rank=1&RID=ZBRXFJV6016

Week 1 Plasmid DNA Isolation and Quantification; Endonuclease Restriction Digestion

Objective

To learn the principle and practice techniques of plasmid DNA isolation and restriction digests for Southern blot analysis and resctriction mapping.

Background Information

Isolating plasmid DNAs from *E. coli* is a common routine in research laboratories. There are two major methods for plasmid DNA isolations, boiling vs. alkaline lysis. We will use a commercially available "mini-prep" kit to perform the commonly-practiced plasmid DNA isolation procedure that involves alkaline lysis of bacterial cells. Alkaline lysis isolates plasmid DNAs by breaking the cells open. Bacteria containing the plasmid of interest are first grown overnight, and then allowed to lyse with an alkaline lysis buffer consisting of a detergent sodium dodecyl sulfate (SDS) and NaOH (a strong base). The alkali ruptures cells and denatures the DNAs and proteins involved in maintaining the structure of the cell membrane while the detergent breaks open the phospholipid bilayer of membrane. A neutralization step is followed, which causes the renature of the plasmid DNA but not the bacterial chromosomal DNA. Through a series of steps of mixing, precipitation, centrifugation, the cellular debris is removed and the plasmid in the supernatant is obtained and purified. The preparation yields fairly clean DNA quickly and ready for DNA sequencing or other downstream manipulation, i.e. restriction digest.

Restriction enzymes, found in bacteria and archaea, are thought to provide a defense mechanism against bacteriophage. Inside a bacterial host, the restriction enzymes selectively cut up foreign DNAs while bacterial DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme's activity (restriction modification system). The restriction enzymes play an important role in constructing recombinant DNA molecules, such as those in gene cloning experiments, and mapping the locations of restriction sites in DNA. The name of each restriction enzyme contains one or more **capital letters** (genus initials) and **two small letters** (species) followed by a Roman numeral (the number of enzymes discovered in the organism. Historically the numeral identified the protein peak in which the enzyme eluted during chromatography.) Additional information may be added as a letter. For ***Eco*** RI, the R indicates the particular strain of *E. coli*.

Restriction enzymes cleave the **phosphodiester bonds** in each strand of double-stranded DNA. There are mainly three types of restriction enzymes. Type II restriction enzymes recognize a specific site of 4, 6 or 8 bp and cleave within this sequence (cleavage site) which is generally palindromic (the same sequence being read on the two strands in reverse directions). The cleavage may produce a "blunt end" or a 3' or 5' overhang of a single strand, "sticky end".

A few buffers are used for most restriction enzymes but **no single buffer** allows activity of every enzyme. Suppliers always provide a reaction buffer (10x concentrate) that works best for a specific enzyme. The 1x buffer usually contains 10-100 mM **Tris** at pH 7.3 - 8.5, **salts** like KCl and NaCl (10 to 150 mM), 10 mM $\mathbf{Mg^{+2}}$, 2 mM beta-mercaptoethanol. Sometimes, 0.01% **Triton-X100** (a detergent) and **BSA** (a stabilizers) or swine skin gelatin that is stable to

autoclaving and costs about 1/15 as much as BSA.Since restriction enzymes can require different buffer conditions, some strategies are used to set up "double digests." The preferred method is to simultaneously digest with both enzymes in a compatible buffer even if one enzyme is not fully active (e.g., 75% active). More of one enzyme can be added (1 U of enzyme A plus 1.33 U enzyme B) for equal cutting efficiency. There are limits to adding excess enzymes due to **increased glycerol in the reaction that can reduce specificity of some enzymes**. Or, we can set up a tube to digest with the "low salt" enzyme then add more buffer and the "high salt" enzyme to complete the digest. In extreme cases, the DNA can be precipitated after one digest and dissolved in the 2nd digest buffer. Most digests are carried out at 37°C unless specified for the enzyme.

For the 1st part of today's lab, you will isolate plasmid DNA from the overnight bacterial culture. After quantification, the purified DNA sample is sent out for sequencing. Restriction mapping is the other way to analyze cloned DNA. For the 2nd part of the lab, we will digest the cloned plasmid and a known nbs-cotaining plasmid with restriction enzymes. Next week, you will separate the digested DNAs by agarose gel electrophoresis, which will then be used for Southern blot analysis. In addition, a DNA band corresponding to the known grape nbs-containing DNAinsert will also be isolated from gel and purified for DIG-DNA labeling by PCR.

Growing Overnight Bacterial Culture the night before the lab

1. Pick one **white colony** from your Amp/X-Gal plate using one sterile pipette tip, twirl it in one 15 ml Falcon tubes containing 2 ml LB/Amp+ medium and label the tube with your group number.
2. Repeat the step 1 with the known nbs containing bacterial colony and label the tube "nbs" and your group number.
3. Next morning, add 1 ml of new culture medium into each culture tube and put it back on the shaker

In order to obtain enough plasmid DNA, each group will perform two isolations of the same clone.

BEFORE STARTING

- All centrifugation steps should be carried out at 16,000 x g (~13,000 RPM).
- Add 4 volumes of ethanol (≥ 95%) to one volume of Plasmid Wash Buffer 2.
- If precipitate formed in Lysis Buffer (B2), incubate at 30–37°C, inverting periodically to dissolve.
- Store Plasmid Neutralization Buffer (B3) at 4°C after opening, as it contains RNase A.

Plasmid DNA Isolation (NEB Monarch Plasmid Miniprep Kit)

1. Pipette 1.2 ml (600 µl twice) (1–5 ml, not to exceed 1.5 OD units, *12-16 hours is ideal*) bacterial culture (one is your bacterial conlony and the other is the known "nbs" containing bacterial colony) into each of two 1.5 ml centrifuge tubes and spin both at the top speed for 30 seconds. Discard supernatant. (**Remember to change tip for the 2nd culture)**

2. Repeat step 1.

3. Suspend each pellet in 200 µl all Plasmid Resuspension Buffer (B1).
   Vortex or pipet to ensure cells are completely resuspended without visible clumps.
4. Add 200 µl all Plasmid Lysis Buffer (B2), gently invert tube 5–6 times, and incubate at room temperature for 1 minute. **Do not vortex**.
   Color should change to dark pink, and the solution will become transparent and viscous.
5. Add 400 µl all of Plasmid Neutralization Buffer (B3), gently invert the tube until neutralized, and incubate at room temperature for 1 minute. **Do not vortex.**
   Sample is neutralized when color is uniformly yellow and precipitate forms.

6. Centrifuge lysate for 2- 5 minutes.
   For culture volumes >1 ml, we recommend a 5 minute spin to ensure efficient RNA removal by RNase A. Pellet should be compact; spin longer if needed.
7. Carefully transfer supernatant to the spin column (This step is critical in determining the purity of your plasmid.) and centrifuge for 1 minute. Discard flow-through.
8. Re-insert column in the collection tube and add 200 µl all of Plasmid Wash Buffer 1. Centrifuge for 1 minute. Discarding the flow-through.
9. Add 400 µl all of Plasmid Wash Buffer 2 and centrifuge for 1 minute.
10. Transfer column to a clean 1.5 ml microfuge tube.
    Use care to ensure that the tip of the column does not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute.
11. Add 40 µl all DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.
    Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated. For larger size DNA, (≥ 10 kb), heating the elution buffer to 50°C prior to use can improve yield.

You need to watch the following two videos before coming to the lab.

- **Monarch Plasmid Miniprep Kit protocol** https://www.youtube.com/watch?v=7Xgy5_i6iOc
- **Tips for Using the Monarch Plasmid Miniprep Kit** https://www.youtube.com/watch?v=Z-hDsUCLM2s

Restriction Endonuclease Digestion of DNA

For today's lab, you need to change pipette tip for each solution/enzyme used.

1. Calculate how many µl of DNA to make up 0.5 µg of your plasmid DNA sample
2. Label **four** 1.5 ml microcentrifuge tubes on ice (Make sure that no ice gets into your tube.) You are going to set up four 20 µl restriction digestion reactions.
3. **Two groups** will also set up one extra tube (tube 5)
4. Set up reactions as shown in Table 1. Remember that enzymes are added last.
5. Add 2 µl the corresponding 10X reaction buffer into each tube.
6. Add 0.5 µg DNA into each of tubes 1- 4 and **1.5 µg *nbs* plasmid DNA (**provided by the instructor) into tube 5.
7. Mix by pipetting up and down **without making bubbles**.
8. Incubate the tubes at 37°C for 1-2 hr.
9. Store at -20°C to be used for agarose gel electrophoresis for a Southern blot (Tubes 1-4), and for isolating restriction digested DNA insert from gel (Tube 5) next week.

Setup for Restriction Digest

Promega Enzymes	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**
10x buffer H	2 µl	2 µl	2 µl	2 µl	2 µl
0.5 µg DNA in H_2O	18 µl	16 µl	16 µl	14 µl	15 µl (2 µg *nbs* plasmid)
Restriction enzyme	0 µl	2 µl *Eco* RI	2 µl *Pst* I	2 µl each	4 µl *Eco* RI
Total	20 µl	20 µl	20 µl	20 µl	20 µl

Restericton enzymes, *EcoR*I and *Pst*I and their recognition sites

***Eco* RI (II)**
Source: *E. coli*

5′-G^A A T T C- 3′
3′-C T T A A^G- 5′

5'—G AATTC-3'
3'-CTTAA G-5'

***Pst I* (II)**
Source: *Providencia stuartii*

5′-C T G C A^G-3′
3′-G^A C G T C-5′

5'—CTGCA G-3'
3′--G ACGTC-5′

Week 2 Agarose Gel Electrophoresis and Southern Transfer

Objective:

To learn the principle and techniques of Agarose Gel Electrophoresis and Southern Transfer

Background Information:

As discussed in the last lab, restriction enzymes play an important role in constructing recombinant DNA molecules, such as those in gene cloning experiments. Another applications of restriction enzymes are to construct a map of restriction sites of a DNA molecule and to locate a specific DNA region (i.e. our cloned *nabs* sequence) in the molecule after Southern blot analysis. A simple form of the Southern blot is a plasmid-to-plasmid hybridization, where plasmid DNA digested with restriction enzymes is subjected to electrophoresis on a gel and blotted onto a membrane. A piece of the plasmid DNA (target) is used as a hybridization probe (i.e. a PCR-amplified Dig-labeled nbs sequence).

Southern blot was originally developed in 1975 by Sir Edwin Southern and earned him a Lasker Award in 2005. It's also one of the most highly cited scientific publications. The goal of Southern blot is **to locate DNA sequence (gene) of interest**, in our case, to locate the *nbs* sequence in the recombinant plasmid, The procedure involves the transfer of electrophoresis-separated DNA fragments onto a membrane and subsequent fragment detection by probe hybridization using a **radioactive/chemical labeled ssDNA/RNA probe** made of the known DNA sequence to be located in a population. The target molecules (heterogeneous population of *denatured* DNA) are fixed on a nylon or nitrocellulose membrane. The s.s. probe (suspended in the hybridization solution) and the denatured target hybridize to each other. The membranes are rinsed to eliminate any non-specific hybridization and retain hybrids which are then detected by exposing the membrane to a film (radioactive or chemiluminescent probe) or revealed by other means, such as colorimetric test on the membrane (in our case).

During agarose gel electrophoresis, intact plasmid DNA may appear in one of the five conformations, which run at different speeds. The different plasmid conformations are listed in the order of electrophoretic mobility (slowest to fastest): (i) **Nicked Open-Circular DNA** which has one strand cut; (ii) **Relaxed Circular DNA** which are ***enzymatically* relaxed** and is fully intact with both strands uncut; (iii) **Linear DNA** which has free ends because both strands have been cut; (iv) **Super-coiled (or Covalently Closed-Circular) DNA** is intact with both strands uncut, and with a twist built in, resulting in a compact form. Sometimes, there is (iv') **super-coiled denatured DNA**, like super coiled DNA but with unpaired regions which make it *slightly less compact* due to the excessive alkalinity during plasmid preparation. Finally, there may also be (v) **single-stranded** super coiled DNA that runs at the front end.

In today's lab, we will separate the uncut and restriction digested DNA fragments via agarose gel electrophoresis and transfer the separated DNA fragments from gel onto a nylon membrane. We will also excise the digested known nbs DNA insert from gel for Dig DNA labeling next week.

I. Agarose Gel Electrophoresis

1. Heat ~ 0.45 g of agarose in 45 ml of 1 X TAE (0.9 ml of 50 X TAE 44.1 ml of H_2O) in 125 ml flasks until agarose is completely melted. Allow to cool to about 60°C with swirling.

2. Add 5 µl GelRed and mix well.
3. Pour each agarose solution into a **pre-sealed gel tray** with the sample comb and allow to set.
4. After gelling, place the gel tray in the electrophoresis box and add ~ 270 ml 1 X TAE to cover the gel.
5. Load 5 µl of DNA molecular weight size marker (instructor) and then your samples.
6. Load sample no 5 into the small gel and load DNA samples no. 1- 4 into the large gel as known below.

Lane	1	2	3	4	5	6	7	8
Sample	Uncut	*Eco*RI	*Pst*I	Both	DNA marker	x	nbs insert	

7. Electrophorese both gels at ~100 V/gel, until the bromophenol blue is at least ~ 3/4 through the gel.
8. While waiting, pre-weigh a 1.5 ml microfuge tube for later use.
9. Visualize DNA fragments under Biospectrum UVP GelDoc-It®**2** Imager and photograph with a ruler.
10. Cut the lower band containing the known nbs DNA insert from **lane 7** from the gel with a clean, sharp scalpel, put it in the preweighed microfuge tube prepared in step 8, weigh it again, and store in the freezer for next week's DNA extraction and Dig-labeling.

II. Southern Transfer

1. Using a razer blad/scaple and ruler to trim off lanes 7 and 8 and **measure the size of the gel (Be precise!)**.
2. Transfer the gel to a small container.
3. Add a proper amount of denaturing solution (1 M NaC1, 0.5 M NaOH) to cover the gel (~70 ml) and shake at low speed for 30 min.
4. While you are waiting, unfold and using a paper cutter to cut a stack of paper towel **(** ≥ 1.5 inches thick after being pressed down**)** that matches the size of the gel obtained from step 1 above.
5. Cut a piece of nylon membrane and two pieces of Whatman paper **EXACTLY** the same size as the gel
6. Place them in a dish of distilled H_2O and then 2XSSC. **(<u>Wear gloves when you handle both!</u>)**
7. Cut a piece of Whatman paper the same length and twice the width as the gel tray
8. Flip your gel tray and place it in a 2nd container, place the Whatman pape from step 7 on top and add a proper amount of denaturing solution (1 M NaC1, 0.5 M NaOH) solution to the paper (~200 ml).
9. Pour off denaturing solution and transfer the gel **upside down** on top of the Whatman paper.
10. Place the nylon membrane from step 5 on top of the gel. (**Carefully lay down the membrane and do NOT move the membrane once it touches the gel**), smooth out any bubbles.
11. Place the pre-wet Whatman papers one by one on top of the membrane and again remove bubbles.
12. Cut pieces of plastic wrap and lay them along the edge of the gel.
13. Place the stack of paper towels from step 4 on top of the Whatman paper.
14. Place a small weight (pipette box) on top of the whole stack and allow the DNA to transfer overnight.
15. **Next morning**, remove the paper towels but ***leave the Whatman paper, membrane and gel together.***
16. Flip the entire stack right side up and **<u>mark the position of each well on the membrane with a pencil</u>**.
17. Remove the gel and filter papers from the membrane. Rince the membrane in 2xSSC.
18. Place the membrane (*DNA face up*) on sheets of KimWipe and cross-linking with the Stratelinker.
19. Air-dry the membrane.
20. Place a few more Kimwipes on top of the membrane and then wrap loosely in aluminum foil.
21. Label **sec. no.** and **group no.** on the outside of the aluminum foil and store it in the refrigerator (4°C).

1X TAE (2 liter)**, Agarose**
1 M NaC1, 0.5 M NaOH (2 liter per section)
1 liter 20X SSC: 175.3 g/l of NaCl; 88.2 g/l of $C_6H_5Na_3O_7.2H_2O$ Adjust pH to 7.0 with NaOH or HCl

Week 3 DNA extration from agarose gel and Digoxigenin DNA labeling by PCR

Objective:

To learn the principle and practice techniques of

(i) DNA extraction from agarose gel

(ii) Digoxigenin DNA labeling by PCR.

Background Information:

Nucleic acid hybridization is a common molecular technique used (i) to screen cDNA or genomic libraries, (ii) to analyze gene expressions in cells (Northern blot analysis), and (iii) to study the organization of specific regions of the genome (Southern blot analysis). In our case, restriction digest was used to make a restriction map of the recombinant plasmid cloned earlier and nucleic acid hybridization will be used to locate the DNA insert in the plasmid. The technique depends heavily on the success of making labelled DNA probes with radioactive or chemically modified nucleotides. Probes can be labeled **internally** or **at the end** of an oligonucleotide. For the **end labeling**, an oligonucleotide is labeled with a single atom, and thus it has a low specific activity. Two enzymes are used to achieve the labeling of each end; **T4 polynucleotide kinase** catalyzes the transfer of radioactive phosphate of [γ-^{32}P]NTP to the **5'-Pi end** and **terminal transferase** is to label its **3'-OH end** with a [α-^{32}P]NTP.

For **internal labeling**, three common labeling methods are , **Nick Translation, Random Primed Labeling,** and **PCR Labeling**. For "**nicked translation**" labeling, DNA to be processed is treated with **DNase I** and **Mg^{+2}** to produce **single-stranded "nicks**." This is followed by strand replacement in nicked sites by **DNA polymerase I** with **5'-3' exonuclease activity** to remove nucleotides "in front," and the **5'-3' polymerase activity** to add nucleotides to the available 3' ends. The "**random primed**" method is based on the hybridization of oligonucleotides of **all possible sequences** to the denatured template DNA to be labeled. The complementary DNA strand is synthesized by a **"Klenow" fragment of DNA Polymerase I**, using the random oligonucleotides as primers. For **PCR labeling**, two primers (degenerate primers in our case since we don't know the cloned nbs sequence), Taq polymerase, a buffer and the DNA template are added into a PCR reaction tube for labeling reaction. By putting a radio- or non-radiolabeled nucleotide for a non-radioactive equivalent in the reaction mixture, the newly synthesized complementary DNA is made radioactive or labeled non-radioactively. There are advantages of using PCR to label probes. a) It requires only a small amount of **template DNA, 10 – 100 pg** and genomic DNA, 1 – 50 ng (ideally, 10 ng). b) Impure templates can be used. c) It requires less optimization than other methods. d) It produces a large quantity of labeled probe. e) It is recommended for very short probes (< 100 bp). f) It produces very sensitive probes.

Today, we will make the Dig-labeled DNA probe (corresponding to the NBS DNA sequence that you cloned) by PCR amplification. Before DNA labeling, you need to purify the DNA insert from the agarose slice using silica gel based membrane. After agarose gel electrophoresis of restriction digested plasmid DNA last week, the desired DNA band was cut from the gel and weighed. Today, we will isolate the DNA from the gel piece by heating it in a salt solution to

melt the agarose, which is ready for binding to the silica gel based membrane. Subsequent low salt washes will remove excess salt followed by eluting from the column. The purified DNA is ready for Dig DNA labeling reaction. Next week, we will hybridize the membrane with the Dig-labeled *nbs* probe to detect the location of the target DNA (the *nbs* insert) in the cloned recombinant plasmid.

DNA Purification from Gel (GeneJET PCR Purificaiton Kit, Thermo Fisher Scientific)

1. Pre-weigh one 1.5 ml Eppendorf tube
2. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel and place it into the tube from step 1.
3. Weigh the gel slice in the tube again and subtract the tube weight (step 1) for gel weight.
   Note: We already did steps 1-3 last week.
4. Add 1:1 volume of Binding Buffer to the gel slice (volume: weight) (e.g., add 100 µL of Binding Buffer for every 100 mg of agarose gel).
   For gels with an agarose content > 2%, add 2:1 volumes of Binding Buffer to the gel slice.
5. Incubate the tube at 50-60 °C for 10 min or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Vortex the gel mixture briefly before loading on the column.
   Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
   For ≤500 bp and >10 kb DNA fragments Optional: use this step only when DNA fragment is ≤500 bp or >10 kb add 1 gel volume of water to the solubilized gel solution (e.g. 100 µL of water should be added to 100 mg gel slice solubilized in 100 µL of Binding Buffer). Mix thoroughly.
6. Transfer up to 800 µL of the solubilized gel solution to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. **Close the bag with GeneJET Purification Columns tightly after each use!**
   If the total volume exceeds 800 µL, the solution can be added to the column in stages. After each application, centrifuge the column for 30-60 s and discard the flow-through after each spin. Repeat until the entire volume has been applied to the column membrane. Do not exceed 1 g of total agarose gel per column.
7. Add 700 µL of Wash Buffer (diluted with ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
8. Centrifuge the empty column for an additional 1 min to completely remove residual buffer.
   This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
9. Transfer the column into a clean 1.5 mL microcentrifuge tube (not included).
10. Add 25 µL of Elution Buffer to the center of the purification column membrane. Incubate column for 1 min at room temperature before centrifuging for 1 min.

For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.

11. Discard the GeneJET purification column, quantify with NanoDrop and store the purified DNA at -20 °C.

DIG PCR Labeling

1. Add 4 µl of your **purified DNA** into 0.2 ml PCR tube
2. Add 10.5 µl of the pre-made PCR mix below to set up the PCR labeling reaction.

Pre-made PCR mix (16 µl)

10.6	µl	Sterile water
2	µl	PCR Buffer with $MgCl_2$ 10x (vial 3)
2	µl	PCR DIG Probe Synthesis Mix (vial 2) 2 mM each of dATP, dCTP, dGTP; 1.3 mM dTTP, 0.7 mM Dig-11-dUTP
2x 0.5	µl	Each of LM638 and LM 637 Primers (25 pmole each)
0.4	µl	Enzyme Mix (vial 1)

3. Place the tube in a thermocycler with heated lid.
   94 ºC for 1 min (1 cycle); 94 ºC for 30 sec; 50 ºC for 30 sec; 72 ºC for 30 sec (35 cycles)
4. Store the tube at -20ºC. (This will be used as a probe for Southern hybridization later.)

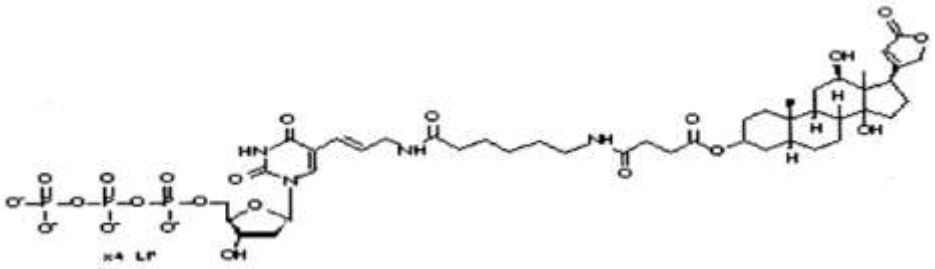

Figure 1. Structure of dUTP (left) and **Alkali-labile Digoxigenin (DIG)-dUTP (right)**
This alkali-labile DIG-dUTP is used for nonradioactive DNA labeling applications.

Week 4 Southern Blot Hybridization and Detection

Objective:

(i) To confirm the presence of NBS sequence cloned in the recombinant plasmid.
(ii) To learn the principle and practice techniques of Southern blot hybridization/detection.

Background Information:

Hybridization relies on the chemical properties of nucleic acids: (1) the complementarity of the DNA bases (A/T, G/C) and RNA (A/U, G/C) and (2) the reversibility of DNA denaturation and reannealing. If the two strands that hybridize contain exactly the same sequences complementary to each other, the hybride will be stable under **high temperature** and **high stringency conditions,** a solution with a **very low salt concentration** similar to water. In contrast, if the two do not have similar sequences, the hybridization will not occur. With partial similarity, the hybrid will be unstable under high stringency conditions but stable under low stringency. The temperature (Tm) at which the two strands separate is specific to their sequence. If it contains high G and C content, the double stranded molecule will be stable as compared to that of the A/T pairs.

There are different techniques for detecting specific nucleic acids in samples. **Southern blot** is used to detect specific DNA in DNA samples. The target DNAs are separated on gels, denatured and transferred onto membrane before hybridizing it with nucleic acid probes. **Northern blot** is used to detect specific RNA molecules among a mixture of RNA isolated from a tissue and then separated by electrophoresis on an agarose gel before hybridization. Northern blot hybridization can be used to estimate the size of the target mRNA as well as its amount in a given tissue at a given time point at different developmental stages of an organism or under different environmental conditions. For **Dot-blot/slot-blot**, the target nucleic acids are NOT separated by electrophoresis. Instead, they are directly deposited on a membrane at a known concentration in the form of a dot or a slot. Equipment adapted to this technique is commercially available. After hybridization, the intensity of the radioactive/fluorescent/colored patch obtained reflects the concentration of the nucleic acid studied. This is a faster method than the Southern and Northern blots. However, the probe must be **highly specific** to the target sequence because of the absence of electrophoresis that prevents the detection of non-specific hybridization.

The DIG Nonradioactive System provides a sensitive method for nucleic acid labeling and detection. Last week, we made a DIG-labeled DNA probe by PCR. This week, we will use it to hybridize and detect the cloned nbs-containing sequence on a nylon membrane (Southern blot analysis).

After confirming the success of Dig DNA labeling through agarose gel electrophoresis, we will use the labeled probe for our Southern membrane. The night before, we have to set up prehybridizaiton and hybridization. Detection of the Dig labeling is caried out the following day in the lab.

Prehybridization

Pre-heat appropriate volume of DIG Easy Hyb solution in 50 ml Falcon tube to 42°C.

1. Calculate the size (cm^2) of your membrane and figure out the volume needed (20 ml/100 cm^2).
2. Place the membrane in a glass tube. The DNA side of the membrane faces INWARD of the tube.
3. Prewet the membrane in 2XSSC slowly and evenly, and then discard the solution.
4. **Add pre-hybridize** membranes with a proper amount (20 ml/100 cm^2 filter or 5 ml) of prewarm DIG Easy Hyb solution (42°C) $\geq$ 30 minutes at appropriate temperature. Solution should move freely. *AVOID BUBBLES trapped between the membrane and glass wall.*
5. While waiting, make 50 µl sterile H_2O containing a proper amount of DIG-labeled DNA probe [2.5 µl/ml of pre-hybridization solution (3.5 ml/100 cm^2) into a 0.2 ml PCR tube]. Too much probe will have VERY high background.
6. Denature the probe at 95°C for 5 minutes in a PCR machine and then rapidly cool on ***slushy ice***.

Hybridization Overnight

1. Discard the initial 5 ml of DIG Easy Hyb solution from the glass tube after $\geq$ 30 min
2. Replace with a proper amount fresh DIG Easy Hyb solution (3.5 ml/100 cm^2 membrane)
3. Add the 50 µL denatured probe into the solution without touching the membrane and pipette up and down for several times but avoid making bubbles that may lead to background.
4. Incubate in HYBAID oven with rotation overnight.

Post-Hybridization Washes: Amount of solution depends on container/ membrane size.

1. Pour off the overnight hybridization with DNA probe solution and save at -20°C for re-use if needed. Probes are typically good for 2-4 uses.
2. Wash membrane 2X 5 minutes each in 2XSSC, 0.1% SDS (50 ml/100 cm^2) at room temp.
3. Wash the membrane with preheated 0.5XSSC, 0.1% SDS twice 15 minutes each at 60°C.
4. Swirl the tube and try to make the membrane move to the top of the tube.d

Detection procedure

1. Pull out the membrane and place it in a square box containing 25 ml of Washing Buffer to wash membranes briefly 5 minutes on a shaker at a low speed.
2. After discarding the solution, add 25 ml of blocking solution into box which is put back on the shaker at room temperature for 30 minutes. Make sure the membrane is covered in solution.
3. While waiting, you should make antibody solution, add 4 µL of antibody (1:5000) to 20 ml of blocking solution. Make sure the membrane is covered.
4. Discard the blocking solution and add the 20 ml Antibody solution and shake for 30 minutes.
5. Wash membranes 2X15 minutes in 30 ml of Washing buffer. Make sure solution covering the membrane.
6. Equilibrate membranes 2-5 minutes in 20 ml of Detection Buffer.
7. Incubate membrane in 10 ml of freshly prepared color substrate solution in the dark. **Do not shake during color development**. It takes about 10 min for the bands to appear.
8. After 15- 20 minutes, stop the reaction by rinsing the membrane with dH_2O.

Preparation of Working Solutions

Solution	Composition / Preparation	Storage/ stability	Use
Washing buffer	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20°C); 0.3% (v/v) Tween 20	15-25°C, stable	Removal of unbound antibody
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5. (20°C)	15-25°C, stable	Dilution of Blocking solution
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)	15-25°C	Adjustment of pH 9.5
Blocking reagent	Dissolve Blocking reagent (bottle 5) in Maleic acid buffer to a final concentration of 10% (w/v) with shaking and heating either on a heating block or in a microwave oven. Autoclave stock solution.	2-8°C or 15-25°C	Preparation of Blocking solution
Blocking solution	Prepare a lx working solution by diluting 10x Blocking reagent 1: 10 with Maleic acid buffer.	prepare fresh	Blocking of unspecific binding sites
Antibody solution	Centrifuge the antibody for 5 min at 10,000 rpm in the original vial prior to each use, and pipet necessary amount carefully from the surface.Dilute anti-digoxigenin-AP 1:5 000 (150 mU/ml) in Blocking solution. (1 µL to 5 ml)	12 hours at 2-8°C	Binding to the DIG-labeled probe
Color substrate solution	Add 40 µl of NBT/BCIP stock solution (vial 5) to 2 ml of Detection buffer. Note: Store protected from light!	prepare fresh	Visualization of anti-body-binding