Introductory Laboratory - Basic Techniques

<u>Theory</u>

- **PCR Amplification**
  - Follows the steps: Denaturation, Primer annealing, and primer extension.
  - **Denaturation ➜** high temperature. Converts dsDNA to ssDNA.
    - Too high = rapid DNA degradation
    - Too low = partial/no denaturation.
  - **Primer annealing ➜** T_a is generally 5 degrees below the lowest T_m.
    - Too high = Lower amplification yield. Primers have a more difficult time binding to their complementary sequences.
    - Too low = non-specific amplification
  - **Primer extension ➜** Done at roughly ~72 degrees.
  - Annealing temperature increases at last few cycles due to more complete binding between primers and strands
- **Agarose gel electrophoresis on DNA**
  - Separation of charged macromolecules via an electric field.
  - DNA samples loaded near the cathode.
  - Electrophoresis buffer contains ions to facilitate the carrying of current.
    - Also contains a buffer to maintain pH.
  - Higher agarose concentration = smaller pores = lower migration distances (more resistance).
  - Ethium bromide (EtBr) is used to visualize the DNA on the gel.
    - In this lab, SYBR safe (invitrogen) was used, as it is relatively safe.
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Lab 1- Amplification

<u>Theory</u>

- **Sense Strand ➜** Coding strand
- **Antisense Strand ➜** Non-coding (template) strand
- **Antiparallel ➜** Side to side but in opposite directions (5'-3' vs 3'-5)
- **Reverse Complement ➜** Complementary and in opposite directions

- **cDNA Development**
  - Conversion of mRNA to DNA with *reverse transcriptase*.
    - Forms antisense strand
    - RNase H removes the mRNA.
    - DNA Polymerase I synthesizes sense strand.

- **Primer design**
  - **Forward Primer**
    - Reads identical to coding (sense) strand
  - **Reverse primer**
    - Reads identical to template (antisense) strand.
  - NOTE: Both primers begin with a ~6 nt 'docking' site, followed by the restriction site for the endonucleases.
- **Subcloning**
  - T7 RNA Polymerase PCR Product is subcloned into the MCS (multiple-cloning-site) of the pTAC-MAT-Tag1 vector.
    - Both the PCR product and the vector are digested with the same restriction enzymes.

Key Steps

- **Step 2:** PCR samples were kept on ice to avoid denaturing of the polymerase enzyme

Lab 2 - Ligation

Theory

- **Restriction Endonucleases (Restriction enzymes) ➜** Cleave (hydrolyze) double-stranded DNA at their recognition sequences.
  - Cleave performed through hydrolysis (breaking a phosphodiester bond with water)
  - They are re-joined by a condensation reaction (splitting a water molecule to reform the phosphodiester bond).
    - Done by ligases.
- **Restriction Sites ➜** Usually palindromic. Cut sites for restriction enzymes.
- **Restriction Cuts ➜** Result in 5' or 3' overhangs, or blunt ends. Blunt ends are less desirable (less efficient to re-ligate).
  - Longer overhang determines the type (e.g if the 5'-P is longer than the 3'-OH, it's a 5' overhang).
- **Ligation**
  - Accomplished with T4 DNA ligase.
  - Typically requires 3:1 insert:vector molar ratio, with 20-75 ng of vector DNA in 40uL.
  - Ligation can't be performed without a 5'- phosphate group on one of the DNA strands.
    - Allows for blocking of ligation through dephosphorylation (using alkaline phosphatase).
- **Directional Ligation ➜** Two different restriction enzymes used.

        - Prevents ligation of inverted DNA strands and re-circularization of the plasmid with no insert.
- **Non-directional Ligation ➜** Only one restriction enzyme used
    - DNA can insert inverted, or plasmid can re-circularize without insert.

- **Reading Frame**
    - Assess the location of insertion of the ATG of the T7 RNA Polymerase insert. Ensure that it is in-frame with the vector's ATG.

- **Control Treatments for ligation**
    - **Positive Control ➜** Plasmid vector digested with one restriction enzyme. Recircularizes.
    - **Negative control ➜** Doubly-digested plasmid with incompatible termini. No ligation occurs. Partial plasmid cuts (only one restriction enzyme cuts) results in compatible termini which can re-ligate.

Key Steps

- **Step 1:** Both the amplicon and the plasmid were digested with the same restriction enzymes
- **Step 4:** Formula for calculation of a 3:1 insert:vector molar ratio:
    - $Mass\ of\ insert\ =\ (\frac{size\ of\ insert}{size\ of\ vector})\ (mass\ of\ vector)(molar\ ratio)$

Lab 3 - Transformation

Theory

- **Bacterial Competency**
    - Made competent via addition of divalent cations such as Ca^{2+}.
        - Ca^{2+} improves transformation efficiency by forming transitory complexes between lipopolysaccharides on the cell membrane, the Ca^{2+} itself, and the phosphate groups of DNA.

- **Plasmids and vectors**
    - **ORI (Origin of Replication)**
        - Allows replication independent to that of the host chromosome.
            - Control of copy numbers (e.g. chromosome must have one replication event per division, but plasmids may replicate at much different ratios).

    - **MCS (Multiple Cloning Site)**
        - Region containing recognition sequences
            - Unique and not found anywhere else on the plasmid.

        - **Selectable Marker**
            - A gene on the vector that indicates successful transformation within a colony.
            - pTAC-MAT-Tag-1 vector contains a gene coding for β-lactamase, which hydrolyzes the β-lactam ring of ampicillin.
- **Sterile Conditions**
    - Disallow growth of other microorganisms on the LB agar plates.
    - Required since ampicillin degrades (half-life of 2-5 days), and since it is metabolized by transformed cells.
        - Contamination would thus allow growth after ampicillin is depleted.
- **KRX® Cell Line**
    - No F^+ plasmid - no conjugation.
    - No $RecA1^-$ - no plasmid-chromosome recombination events.
    - No $EndA^-$ - no plasmid degradation.
    - No ompP and ompT genes, which are outer-membrane proteases which have been shown to fragment the T7 RNA Polymerase enzyme.
- **Transformation Controls**
    - **Positive control ➜** Undigested pTAC-MAT-Tag-1 (contains resistance to ampicillin, colonies will form).
    - **Negative control ➜** Competent cells incubated without plasmid DNA, and thus no ampicillin resistance was transferred, resulting in no colonies forming.
        - Assesses background resistance to ampicillin with no plasmid DNA.

<u>Key Steps</u>

- **Step 5:** RF2 contains higher concentrations of $CaCl_2$, allowing more Ca^{2+} to form transitory complexes between lipopolysaccharides, and incoming DNA strands, facilitating the induction of competence.

Lab 4- Screening and Sequencing

<u>Theory</u>

- **Basic procedural sequence**
    - Lysis ➜ neutralization (renaturing) ➜ purification (cleansing of lysates)
    - The lysis step is performed using NaOH, and sodium dodecyl sulphate (SDS).
    - Neutralization is performed with potassium acetate.
        - Allows for reannealing. The smaller plasmid should anneal quickly, whereas the chromosome, being very large, will likely get caught up in proteins during this process, and precipitate as an insoluble complex.
            - Removed via centrifugation.
    - Plasmid is then precipitated using ethanol.

- **DNA Sequencing**
    - **Step 1 - PCR Amplification**
        - Use dNTPs and ddNTPs. Overall, there should be some occasions where a ddNTP terminates the elongation process. The ddNTPs are distinctively tagged using a dye.
    - **Step 2 - Analysis of amplicon via capillary electrophoresis**
        - Sequences of varying lengths are obtained in this amplification process.
            - Fused-silica capillary is used.
                - Strong electric current (electric field) used to distinguish by sequence length.
    - **Step 3 - Automatic base-calling**
        - Fluorescence signal recorded at capillary output.
            - Signal analyzed by computer to determine sequence (base-calling, where each nucleotide is assigned a fluorescence peak).

<u>Key Steps</u>

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Lab 5- Protein Expression

<u>Theory</u>

- T7 RNA Polymerase expression uses the regulatory process of the *lac* operon.
- An allolactose analog, isopropyl-β-D-thio-galactoside (IPTG).
    - Cannot be hydrolyzed, so its concentration is constant.
    - Behaves similarly to allolactose.

Lab 6- Protein Purification

<u>Theory</u>

- **Immobilized Metal ion Affinity Chromatography (IMAC)**
    - Contains agarose beads that are covalently linked to iminodiacetic acid (IDA).
        - Metal chelator that can immobilize metal ions to allow them to form coordination bonds with other molecules.
    - The metal ion used here is Nickel Ni^{2+}. Nickel forms <u>**three**</u> coordination bonds.
    - Imidazole ring of histidine coordinates with the nickel ion, and other proteins (that do not possess an imidazole/histidine on the outer-surface) are eluted.

        - The histidine-tagged (MAT-tagged) proteins can then be eluted using a high concentration of imidazole.
            - At this point, the eluted proteins are contaminated with imidazole.

- **Polyacrylamide Gel Electrophoresis (PAGE)**
    - **Electrophoresis ➜** The application of a potential difference across two electrodes on opposite sides of a gel, whereby molecules bearing a charge travel along the gradient towards the opposite charge.
    - Proteins are treated with sodium dodecyl sulfate (SDS) and β-mercaptoethanol.
        - B-mercaptoethanol reduces the disulfide bridges between residues, contributing to the unfolding of proteins.
        - SDS immerses the proteins in a negative charge, contributing to unfolding, as well as the application of a size-independent negative charge density on each protein.
            - Allows migration of proteins along gradient to be solely based on molecular weight.

Lab 7/8- Western blot & Enzymatic Assay

<u>Theory</u>

- **SDS-PAGE on gradient gels**
    - A gradient of lower to higher concentrations of polyacrylamide.
    - Issue is that at higher polyacrylamide concentrations, proteins get tightly wrapped, and thus immobile within the smallest gel pores, making protein transfer onto a membrane difficult.
        - Solved by using longer protein-transfer times.
- **Detection of Antigen by Western blotting**
    - Primary antibody targets the MAT epitope.
        - The proteins are denatured to allow full exposure of the MAT epitope.
    - Secondary antibody is conjugated with alkaline phosphatase
        - Catalyzes conversion of colorless substance into a blue insoluble product

- **Enzymatic Activity of the T7 RNA Polymerase**
    - Requires:
        - The polymerase (T7 RNA Polymerase)
        - A DNA template
            - Must contain the T7 promoter motif, as the T7 RNA Polymerase is extremely promoter-specific

            - NTPs (not dNTPs, as the T7 RNA Polymerase forms RNA strands)
            - Pyrophosphatase
                - Removes pyrophosphate
                    - Pyrophosphate inhibits the T7 RNA Polymerase.
            - DFHBI
            - (Recommended) Ribonuclease inhibitors.
                - Prevent degradation of RNA products.
- **Enzymatic Assay using the 5S RNA-dBroccoli template**
    - The DNA template contains 4 domains that are functional when transcribed:
        - T7 Promoter
        - 5S RNA domain
            - Stabilizes the 2° structure of the RNA transcript.
        - dBroccoli domain
            - Functional domain of the transcript
            - Folds into a hairpin loop
            - Forms a structure known as a G-quadruplex
                - DFHBI binds into one of these G-quadruplexes and emits fluorescence.
                - DFHBI does not emit fluorescence in the absence of the G-quadruplexes. Instead, the lack of binding (and thus lack of rotational constriction), allows for the dissipation of light energy signals via rotation of the sigma-bond.
                    - When bound to a G-quadruplex, the rotation is inhibited, and the sole method of dissipation is fluorescence.
        - Rho-independent terminator.