MOLECULAR CLONING

<u>Cloning process summary</u>

1. Transformation
    a. Competent cells of E.coli treated with CaCl2 solution
        i. Ca2+ cation **neutralizes** repulsive negative charges of **phosphate backbone** of the competent cell DNA and **phospholipids** of cell membrane
        ii. When subjected to heat shock → increase permeability of cell membrane to DNA
        iii. Competent E. coli cells take up plasmids
2. Recovery
    a. Cells "rest" after being subjected to stressful conditions
        i. LB nutrient broth added
        ii. helps cells grow and **express ampicillin-resistant gene B-lactamase**
        iii. So that competent cells can survive on am-selection plates
3. Selection
    a. First selection: Select for transformants (containing vectors)
        i. Selection medium: **antibiotic e.g. ampicillin**
        ii. Cells containing transformants are conferred **ampicillin-resistance** due to **expression of B-lactamase**
        iii. Transformants selected for (others die)
    b. Second selection: Select for recombinant plasmids
        i. LacZ gene present
            1. Selection medium: **antibiotic + X-gal**
            2. In recombinant plasmids: insert cloned into lacZ gene, lacZ gene disrupted, B-galactosidase **NOT FUNCTIONAL**
            3. X-gal **NOT BROKEN DOWN**
            4. **WHITE COLONIES** (negative: blue colonies)
        ii. Second antibiotic resistance e.g. tetracycline
            1. Selection medium: **antibiotic + 2nd antibiotic**
            2. Conferred by insert probably
            3. Cells with recombinant plasmids are conferred **tetracycline-resistance** due to expression of **gene on insert**
        iii. Non-expression of Barnase gene (kills bacteria)
            1. Selection medium: **antibiotic**
            2. In recombinant plasmids: Barnase gene **REPLACED** by insert → not expressed → not killed
        iv. GFP gene expression
            1. Under UV light → GFP gene expressed → colonies fluoresce **bright green**
            2. Some (e.g. pGLO) contain **arabinose** which regulates synthesis of GFP
4. Confirmation

Molecular cloning questions

1. Perform Polymerase Chain Reaction (PCR)
    a. **Two primers** each binding to a DNA strand are used to **bracket the target region** at the start and the end to be **amplified**
    b. Gene of interest (insert) is **obtained**
2. **Restriction digestion** of **insert** of GFP gene and **Plasmid Z** with the **restriction enzyme HindIII**
3. **Ligation of** HindIII-digested insert to **linearized** Plasmid Z with **enzyme DNA ligase**, to obtain a **recombinant plasmid**
4. Transform ligation mix **containing putative recombinant plasmids** into **Ca2+-treated, competent E. Coli cells** by **heat shock**
    a. Competent host cells used are **tetracycline-sensitive**
5. Select the **transformants with recombinant plasmids** on (LB agar) selection medium containing: **tetracycline + (__)**
    a. Transformant cells will survive and grow as they have become **tetracycline-resistant** due to **expression of Tcr gene** on **Plasmid Z**
    b. Transformants **with recombinant plasmids** will **fluoresce** a brilliant green **under UV light** due to **expression of GFP gene** on **DNA Insert**
6. Confirmation using Restriction digestion: Digest **putative recombinant plasmids** using **Ehe1**. Following agarose gel electrophoresis of the reaction products,
    a. Positive recombinant plasmids give an **insert** band (__bp) and a **vector** band (___bp)
    b. Negative recombinant plasmids give one vector band (__bp)
7. OR Confirmation using Polymerase Chain Reaction
    a. **Primers bracket** the insert sequence/ MCS region
    b. Electrophoresing of the reaction products on agaorse gel to identify cells containing recombinant plasmids
        i. Non-recombinant plasmids will yield a small product corresponding to **size of MCS region** between primer binding sites
        ii. Recombinant plasmids will yield a larger product consisting of **insert** and **residual MCS sequences**
        iii. *Foreign DNA/**truncated inserts** will yield a band that **does not correspond** to the size of insert/MCS
    c. *PCR product: 0.5kb band = **0.4(insert) + 0.1(MCS!)**

[IMPT!]
If insert REPLACES gene e.g. Barnase gene
During confirmation by RD
Vector band = original size (e.g. 3455bp) MINUS BARNASE GENE (300bp) = 3155bp!!