Bacterial and Yeast Cell Culture

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Why Use Bacteria and Yeast?

- Bacteria and yeast are among the most widely used organisms in molecular biology labs.
- Unlike mammalian cells, they are inexpensive to grow, require relatively simple media, and can produce large numbers of cells within a short period of time.
- A single bacterial colony can contain millions of cells. After overnight growth, that number can increase to billions.
- For this reason, bacteria and yeast are routinely used for DNA cloning, plasmid maintenance, recombinant protein production, and genetic engineering.
- Many molecular biology workflows begin with a single colony growing on an agar plate.

Strains, Plasmids and Selection Markers

- Before inoculating any culture, confirm both the strain and the plasmid.
- Two cultures may both be *E. coli*, yet behave very differently.
- For example, DH5α is commonly used for cloning because it maintains plasmids efficiently, whereas BL21(DE3) is designed for protein expression.
- The plasmid should also be checked for its selectable marker.
- A plasmid carrying AmpR requires ampicillin selection. A plasmid carrying KanR requires kanamycin selection.
- Using the wrong antibiotic is one of the simplest ways to lose an entire experiment.

Agar Plates and Liquid Broth

- Agar plates and liquid cultures serve different purposes.
- Agar plates are used when individual colonies need to be separated and examined.
- Liquid cultures are used when larger quantities of cells are required for procedures such as minipreps, transformations or protein expression.
- For routine bacterial culture, LB agar and LB broth are widely used.
- For yeast culture, YPD often serves the same role.

Looking at an Agar Plate

- Before picking a colony, examine the entire plate.
- A good plate usually contains colonies that are:
  - well separated
  - similar in appearance
  - growing only where expected
- Look carefully for unusual colonies, spreading growth, fungal contamination, or colonies growing within contamination.
- The quality of the colony chosen here influences every subsequent step.

Streaking for Isolation

- When colonies are too crowded to distinguish clearly, the culture should be restreaked.
- Using a sterile loop, a small number of cells are dragged across the agar surface.
- The loop is then passed into a fresh region of the plate, progressively reducing the number of cells deposited.
- After incubation, the final sectors should contain isolated colonies derived from individual cells.
- A successful streak plate typically shows:
  - dense growth in the first sector
  - lighter growth in later sectors
  - isolated colonies in the final region

Choosing a Single Colony

- Choose a colony that is clearly separated from neighbouring colonies.
- Avoid colonies that:
  - touch adjacent colonies
  - grow within contamination
  - have a markedly different appearance from the rest of the plate
- The colony can be picked using a sterile loop, sterile pipette tip, or sterile toothpick.
- Only a very small amount of material is needed.
- Everything that follows: overnight cultures, minipreps, sequencing and protein expression depends on this colony.

Setting Up an Overnight Culture

- An overnight culture is usually prepared by inoculating a single colony from an agar plate into 3 - 5 mL LB broth containing the appropriate antibiotic.
- The culture is grown in a sterile culture tube or flask.
- For routine *E. coli* cloning work, incubation is typically performed at:
  - 37°C
  - 180 - 250 rpm shaking
- By the following morning, the medium should appear visibly cloudy.
- A culture that remains completely clear usually indicates:
  - incorrect antibiotic
  - failure to transfer a colony
  - incorrect incubation conditions
  - loss of cell viability
- The overnight culture becomes the starting material for glycerol stocks, minipreps and many downstream procedures.

Preparing a Glycerol Stock

- Once a culture has grown successfully, it is usually worth preserving it immediately.
- A common approach is to combine:
  - 850 µL overnight culture
  - 150 µL sterile glycerol
- in a labelled cryovial.
- The final glycerol concentration is approximately 15%.
- The stock is then stored at -80°C.
- Many researchers prepare glycerol stocks before verification is complete. If sequencing later confirms the clone is correct, the stock becomes a permanent backup.
- A good glycerol stock can often be recovered years later.

Why Colonies Must Be Verified

- Growth on a selective plate does not guarantee that the correct clone has been obtained.
- A colony may contain:
  - the wrong insert
  - an incomplete insert
  - a self-ligated vector
  - unwanted mutations
- For this reason, colonies are usually screened before extensive downstream work is performed.
- Verification reduces the risk of investing time and reagents into the wrong clone.

Colony PCR

- Colony PCR provides a rapid method for screening multiple colonies.
- A small amount of material is taken directly from the colony and introduced into a PCR reaction.
- The remaining colony is usually inoculated into liquid medium so that it can be recovered later if the PCR result is positive.
- PCR products are analysed using agarose gel electrophoresis.
- For example, if an insert is expected to be 1.2 kb, colonies producing a band near 1.2 kb become candidates for further analysis.

Plasmid Miniprep

- A plasmid miniprep is used to isolate plasmid DNA from a bacterial culture.
- Most commercial kits begin with 1 - 5 mL of overnight culture. Cells are first pelleted by centrifugation, producing a compact off-white pellet at the bottom of the tube.
- The pellet is resuspended thoroughly before alkaline lysis is performed. During lysis, bacterial membranes are disrupted and cellular contents are released.
- A neutralisation buffer is then added. Plasmid DNA remains in solution, while much of the chromosomal DNA and protein precipitates.
- After centrifugation, the cleared lysate is transferred onto a silica spin column. Under high-salt conditions, plasmid DNA binds to the membrane while contaminants are removed during the wash steps.
- The DNA is finally eluted in approximately 30 - 50 µL of nuclease-free water or elution buffer.
- A successful miniprep should produce DNA suitable for restriction digestion, sequencing and further cloning work.

Restriction Digest Screening

- Restriction digest analysis provides a second level of clone verification after colony PCR.
- A restriction enzyme recognises a specific DNA sequence and cuts the plasmid at that location.
- The aim is to generate DNA fragments of predictable sizes.
- A typical reaction might contain:
  - 200 - 500 ng plasmid DNA
  - restriction enzyme
  - reaction buffer
  - nuclease-free water
- Digests are usually incubated at 37°C for 30 - 60 minutes, although the exact conditions depend on the enzyme being used.
- The resulting fragments are analysed on an agarose gel.
- If a 3 kb vector contains a 1 kb insert and the enzyme cuts on either side of the insert, two bands should be visible at approximately 3 kb and 1 kb.

Why Sequencing Still Matters

- Colony PCR confirms size.
- Restriction digest confirms structure.
- Neither confirms sequence.
- A clone can produce the expected PCR band and the expected restriction digest pattern while still containing mutations.
- These mutations may have been introduced during PCR amplification, DNA synthesis or cloning.
- For important constructs, Sanger sequencing is normally the final verification step.
- Only after sequencing confirms the correct DNA sequence should the construct be considered fully validated.

Why Overnight Cultures Are Not Experimental Cultures

- An overnight culture is useful for expanding a clone, but it is usually a poor starting point for an experiment.
- After 16 - 18 hours of growth, nutrients have been consumed and waste products have accumulated.
- The cells have generally entered stationary phase and are no longer dividing rapidly.
- Cell physiology in stationary phase differs substantially from log-phase growth.
- For this reason, fresh cultures are normally prepared before expression experiments, growth studies or physiological analyses.

Subculturing into Fresh Medium

- Subculturing is the process of transferring a small volume of an existing culture into fresh medium.
- Typical inoculations range from 1:50 to 1:100.
- For example:
  - 100 µL overnight culture into 10 mL fresh LB
  - 1 mL overnight culture into 100 mL fresh LB
- The fresh medium provides new nutrients and allows the cells to re-enter active growth.
- The culture is then incubated under the same temperature and shaking conditions used previously.
- Growth is monitored until the desired cell density is reached.

Bacterial Growth Phases

- Bacterial cultures follow a predictable growth pattern.
- Immediately after inoculation, cells enter lag phase.
- During this period, cells adapt to the new environment and prepare for division.
- This is followed by log phase, where cell numbers increase exponentially.
- Eventually nutrients become limiting and growth slows as the culture enters stationary phase.
- Many molecular biology procedures are performed during log phase because cell growth and metabolism are most consistent during this stage.

Measuring Growth with OD600

- As bacterial numbers increase, the culture becomes progressively more turbid.
- Rather than counting cells manually, growth is usually monitored using optical density measurements at 600 nm (OD600).
- A small volume of culture is transferred into a cuvette and analysed using a spectrophotometer.
- Typical observations:
  - OD600 0.1 - 0.3 → early growth
  - OD600 0.4 - 0.8 → mid-log phase
  - OD600 >1.0 → dense culture
- When OD600 exceeds approximately 1.0, samples are often diluted before measurement because accuracy decreases at high cell densities.
- OD600 measurements allow cultures to be compared objectively rather than relying on visual estimates.

Choosing the Correct Growth Phase

- The success of many experiments depends on using cells at the correct stage of growth.
- For example, protein expression is often induced during mid-log phase when cells are metabolically active and dividing rapidly.
- Transformation protocols also frequently specify a target OD600 range.
- Two cultures incubated for the same amount of time may be at very different growth stages if inoculation density or aeration differed.
- For this reason, OD600 is generally a more reliable guide than incubation time alone.

Competent Cells

- Under normal conditions, bacterial cells do not readily take up plasmid DNA.
- Competent cells are prepared using specialised methods (eg: chemical transformation or electroporation) that temporarily increase membrane permeability.
- Commercial competent cells are usually supplied frozen and should be thawed on ice immediately before use.
- Typical transformation efficiencies range from:
  - 10^6 - 10^8 CFU/µg DNA for routine competent cells
  - 10^9 CFU/µg DNA for high-efficiency competent cells
- Repeated freeze-thaw cycles reduce transformation efficiency and should be avoided.
- Competent cells should remain cold until the transformation procedure begins.

Bacterial Transformation

- In a standard heat-shock transformation, 1 - 5 µL plasmid DNA is mixed gently with competent cells.
- The mixture is incubated on ice for approximately 20 - 30 minutes.
- Cells are then exposed to 42°C for 30 - 45 seconds before being returned to ice for approximately 2 minutes.
- Although the exact mechanism remains unclear, this treatment promotes DNA uptake.
- Only a small fraction of cells successfully acquire the plasmid.
- The remainder remain untransformed.

Recovery After Transformation

- Immediately after heat shock, transformed cells are transferred into fresh SOC medium.
- SOC is a rich recovery medium that helps cells recover from the transformation process.
- Cultures are typically incubated for 30 - 60 minutes at 37°C with gentle shaking.
- During this period, transformed cells begin expressing the antibiotic resistance gene carried by the plasmid.
- Skipping the recovery step often reduces the number of colonies obtained after plating.

Reading a Transformation Plate

- Transformation plates are usually examined after overnight incubation.
- Successful transformations produce discrete colonies on the selective plate.
- The number of colonies varies depending on:
  - DNA quality
  - competent cell quality
  - plasmid size
  - transformation efficiency
- A plate containing no colonies suggests that the transformation failed or that selection conditions were incorrect.

Picking Transformants

- Several colonies are usually selected from a successful transformation plate.
- Choosing multiple colonies increases the likelihood of identifying a correct clone.
- Each colony is inoculated into a separate overnight culture and analysed independently.
- At this stage, colony PCR, restriction digest analysis or sequencing may be used to identify the desired construct.

Cloning Strains vs Expression Strains

- A strain that performs well for cloning may perform poorly for protein expression.
- Cloning strains such as DH5α and TOP10 are designed to maintain plasmids efficiently and minimise unwanted DNA rearrangements.
- Expression strains such as BL21(DE3) are designed to produce recombinant proteins. BL21(DE3) carries a chromosomal copy of T7 RNA polymerase, which allows genes downstream of a T7 promoter to be expressed at high levels.
- For this reason, plasmids are often constructed and verified in a cloning strain before being transferred into an expression strain.

Induction of Protein Expression

- Once the plasmid has been transferred into an expression strain such as BL21(DE3), the culture is grown in fresh medium until it reaches the desired cell density.
- Many laboratories monitor growth using OD600 and induce expression at approximately 0.4 - 0.8, when cells are actively dividing.
- Expression is commonly induced using IPTG, which activates transcription from lac-based promoters. Initial experiments often use 0.1 - 1 mM IPTG
- Following induction, cultures may be incubated:
  - 2 - 4 hours at 37°C for rapid expression
  - overnight at 18 - 25°C when protein solubility is important
- The optimal conditions depend on the protein being expressed. A condition that works well for one construct may perform poorly for another.

Common Expression Problems

- A successful transformation does not guarantee successful protein production.
- One of the most common outcomes is that little or no detectable protein is produced after induction. This may result from poor plasmid stability, incorrect induction conditions, or low expression levels.
- Another common problem is the formation of inclusion bodies. In this situation the protein is produced, but accumulates as insoluble aggregates rather than remaining soluble in the cytoplasm.
- Reducing the induction temperature from 37°C to 18 - 25°C often improves protein folding and solubility.
- Some recombinant proteins are toxic to the host cell. Cultures may stop growing shortly after induction, or produce substantially lower biomass than expected.
- When troubleshooting expression experiments, it is useful to vary one parameter at a time, such as:
    - IPTG concentration
    - induction temperature
    - induction duration
    - host strain
- Small changes in culture conditions can sometimes produce dramatic improvements in protein yield.

How Yeast Culture Differs from Bacteria

- Although yeast is grown using many of the same laboratory techniques as bacteria, it is biologically very different.
- Yeast cells are larger, grow more slowly and possess membrane-bound organelles.
- A bacterial colony often appears overnight.
- A yeast colony may require 48 - 72 hours before it becomes clearly visible.
- Because yeast is eukaryotic, it can perform many cellular processes that bacteria cannot, making it useful for studying protein localisation, gene regulation and eukaryotic cell biology.

YPD and Selective Media

- YPD is one of the most commonly used rich media for yeast culture.
- It contains:
  - yeast extract
  - peptone
  - dextrose
- When genetic selection is required, defined media are often used instead.
- Unlike bacterial cloning, where antibiotics are commonly used, yeast selection frequently relies on nutritional markers.
- For example, a strain unable to synthesise uracil can be selected using medium lacking uracil.
- Only transformed cells carrying the appropriate marker will grow.

Yeast Transformation

- One of the most widely used yeast transformation methods is the lithium acetate / PEG protocol.
- Cells are first grown to mid-log phase and harvested by centrifugation.
- The transformation mixture typically contains:
  - competent yeast cells
  - plasmid DNA
  - carrier DNA
  - lithium acetate
  - polyethylene glycol (PEG)
- PEG helps bring DNA into close contact with the cell surface, while lithium acetate increases transformation efficiency.
- The mixture is incubated before a heat-shock step is performed.
- Although the procedure takes longer than bacterial transformation, it is robust and widely used.

Verifying Yeast Transformants

- Growth on selective medium indicates that selection has occurred, but verification is still required.
- The exact verification strategy depends on the experiment.
- Common approaches include:
  - colony PCR
  - phenotype analysis
  - fluorescence microscopy
  - sequencing
- For example, a GFP-tagged construct may be verified by observing fluorescence in the expected cellular compartment.

Preserving a Verified Yeast Strain

- Verified strains should be preserved before downstream experiments begin.
- Long-term storage is commonly performed using glycerol stocks stored at -80°C.
- A typical stock contains:
  - yeast culture
  - sterile glycerol

Reviving Frozen Stocks

- Frozen stocks should be treated as long-term archives.
- Remove only a small amount of material using a sterile loop or pipette tip.
- Return the stock to the freezer immediately after sampling.
- Avoid repeated freeze-thaw cycles.
- A common workflow is:
  - frozen stock
  - agar plate
  - single colony
  - liquid culture
- This helps preserve stock quality and reduce contamination risk.

Avoiding Contamination

- Most contamination events originate during handling rather than media preparation.
- Good aseptic technique includes:
  - using sterile consumables
  - minimising exposure of open vessels
  - working in a clean area
  - avoiding contact between sterile and non-sterile surfaces
- Contamination may appear as:
  - unexpected colonies
  - fungal growth
  - unusual turbidity
  - altered colony morphology

Labelling Cultures Properly

- Every tube, plate and flask should be labelled before use.
- Labels should normally include:
  - strain
  - plasmid or construct
  - antibiotic or selection marker
  - date
  - initials
- Agar plates should be labelled on the base rather than the lid.
- Clear labelling becomes increasingly important as experiments become larger.
- Poor labelling is a common cause of sample mix-ups.

Common Laboratory Mistakes

- Many culture problems arise from simple procedural errors.
- Eg: using the wrong antibiotic, selecting the wrong colony, mislabelling cultures, or incubating cells under incorrect conditions.
- These mistakes are often easy to make and sometimes difficult to detect immediately.
- Careful attention to detail is one of the most valuable lab skills a researcher can develop.

From Colony to Experimental Data

- A typical molecular biology workflow begins with a single colony.
- That colony is expanded into an overnight culture, preserved as a glycerol stock, screened, verified and eventually used in downstream experiments.
- Along the way, cultures are grown, DNA is purified, clones are validated and proteins may be expressed.
- Each stage depends on the quality of the previous stage.
- Good microbiology rarely depends on one clever technique. More often, it depends on performing a series of simple techniques carefully and consistently.