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Educational Handbook of Practical Workshop

Molecular Biology Workshop 4

Genetic Engineering

Department: Second Cycle

Level: Second Year

Option: Molecular Biology

Field: Biological Sciences

Domain: Natural and Life Sciences

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Molecular Biology Workshop 4: Genetic Engineering

Molecular cloning and heterologous expression: from in silico experiment design to recombinant protein purification

Preamble

Genetic engineering emerged in the early 1970s with the first recombinant-DNA experiments, Paul Berg's work and the Cohen Boyer demonstration of plasmid cloning in *Escherichia coli*. Since then, it has transformed the life sciences by enabling the rational design, assembly, and expression of DNA constructs in living cells. Today, the field underpins research and applications across biotechnology, medicine, agriculture, and industry. This workshop introduces genetic engineering through a balanced combination of concise theoretical instruction and guided practical training. The curriculum focuses on essential concepts, techniques, and applications, encouraging students to engage actively in experimental planning, data interpretation, and responsible practice.

Objectives

- Learn foundational genetic-engineering techniques.
- Prepare for advanced study and project work in cloning and heterologous expression.

Target Audience

- Second-year, second-cycle (Master's level) students specializing in molecular biology.
- Learners with established theoretical knowledge who wish to consolidate it through supervised, practice-oriented training.

Prerequisites

- Prior coursework in molecular biology, genetics, and biochemistry.
- Familiarity with core laboratory techniques and consistent adherence to biosafety procedures.

Workshop Modules (overview)

- Solutions and buffer preparation.
- Primer design for cloning (in silico).
- Enzymatic digestion and molecular cloning.
- Bacterial transformation and selection of recombinant clones.
- Heterologous expression.
- Purification and analysis of recombinant proteins.

Contents

List of Abbreviations

YT: Yeast Extract–Tryptone medium
A: Absorbance
Amp: Ampicillin
Bp: Base pair
BSL-1: Biosafety Level 1
°C: Degree Celsius
Ca^{2+}: Calcium ion
$CaCl_2$: Calcium chloride
CFU/µg: Colony Forming Units per microgram
CO_2: Carbon dioxide
Conc: Concentration
dNTP: Deoxynucleotide Triphosphate
DNA: Deoxyribonucleic Acid
dsDNA: Double-stranded Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
EcoRI: E. coli RI
EtBr: Ethidium bromide
g: Relative centrifugal force (gravity)
GFP: Green Fluorescent Protein
high-MW: High Molecular Weight
His-tag: Polyhistidine tag
IDT: Integrated DNA Technologies
IMAC: Immobilized Metal Affinity Chromatography
IPTG: Isopropyl β-D-1-thiogalactopyranoside
kb: Kilobase
kDa: Kilodalton
L: Liter
LB: Luria-Bertani medium
mM: Millimolar
mm: Millimeter
Mg^{2+}: Magnesium ion
MW: Molecular Weight
NaAc: Sodium Acetate
NaOH: Sodium Hydroxide
nm: Nanometer
NTC: No-Template Control
OD_{600}: Optical Density at 600 nm
ORF: Open Reading Frame
pH: Hydrogen potential (measure of acidity/alkalinity)
PCR: Polymerase Chain Reaction
PPE: Personal Protective Equipment
QC: Quality Control
RA Cloning: Restriction/Assembly Cloning
RBS: Ribosome binding site
Ref: Reference
RNase: Ribonuclease (RNA-degrading enzyme)
rpm: Revolutions per minute
rDNA: Recombinant DNA
SDS: Sodium Dodecyl Sulfate
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOC: Super Optimal broth with Catabolite repression
SOP / SOPs: Standard Operating Procedure(s)
T_m: Melting temperature
TAE: Tris-Acetate-EDTA
TB: Terrific Broth
Taq: Thermus aquaticus DNA polymerase
TBE: Tris-Borate-EDTA
TE: Tris-EDTA
Tris: Tris(hydroxymethyl)aminomethane
U: Enzyme activity unit
UV: Ultraviolet
V/cm: Volts per centimeter (electrophoresis field strength)
v/v: Volume per volume
w/v: Weight per volume
WHO: World Health Organization
XhoI: Restriction enzyme (Xanthomonas holcicola I)
µg/mL: Microgram per milliliter
µL: Microliter

Introduction

This hands-on workshop establishes both the practical and conceptual foundations of modern genetic engineering. Students will use recombinant DNA (rDNA) techniques to clone a bacterial gene (illustratively, *Thermus aquaticus* DNA polymerase, "Taq") and express it heterologously in *Escherichia coli*.

The workshop emphasizes design logic, reproducibility and biosafety, while highlighting factors relevant to research and industry (yield and cost).

Activities are aligned with internationally recognized biosafety frameworks that require protocol-driven risk assessment, containment practices and waste management (NIH SOP, 2024; Organization, 2020).

We adopt a widely used *E. coli* workflow: DH5α as a high-efficiency cloningstrain and BL21(DE3) as a T7-based expression host carrying the λDE3 prophage (T7 RNA polymerase under lacUV5 control) and reduced proteolysis background (Lon/OmpT-deficient).

Plasmids are pET-family vectors (e.g., pET-21a(+), pET-28a(+)) with T7/T7lac expression cassettes and optional His_6-tags for analysis and purification. We also introduce auto-induction media to illustrate induction strategies and trade-offs (glucose to lactose shift). *In-silico* design (e.g., SnapGene/Benchling and our locally made software **G-synth**) supports primer design and restriction-based cloning to de-risk wet-lab steps.

Across all sessions, students' progress from buffer/media preparation and primer design to cloning strategy, transformation and clone screening. Expression trials are run under varied conditions, followed by His-tag IMAC purification, SDS-PAGE analysis, Western blot confirmation, and a basic enzyme activity assay, completing the path from construct design to functional verification. Participants will learn the essentials from molecular cloning to the production of a functional recombinant protein under rigorous standards of reproducibility and biosafety.

Session 1 (8h): Preparations and Strategies for Cloning

Objective

The aim of this session is to introduce students to a genetic engineering workshop, beginning with the preparation of buffers and solutions with refreshers on key solution-chemistry concepts (molarity, pH). It then reviews culture media with refreshers in fundamental microbiology (aseptic technique, growth). The second part introduces cloning and provides guided practice in cloning primer design using bioinformatics tools.

Solutions & Culture Media

Buffer chemistry (fundamentals)

Henderson–Hasselbalch equation

Use this equation: pH = pK_a + log10([base]/[acid]), to reason about solution pH and buffer behavior. A buffer's effective range is roughly ±1 pH unit around its pK_a, and buffer capacity increases with total concentration.

Practical design

Choose a conjugate acid/base pair with a pK_a near the target pH. Remember that temperature and ionic strength can shift a buffer's apparent pK_a, so always calibrate and verify pH at the working temperature.

Dilutions

Keep concentration and volume work symbolic using the dilution equation ($C_1V_1 = C_2V_2$) when preparing stock solutions and diluting to working concentrations.

Buffers across the workflow

Buffers are indispensable throughout molecular biology protocols. For example, DNA extraction and purification steps use carefully formulated buffers (for cell lysis, neutralization, washes) to maintain pH and protect nucleic acids. PCR and restriction enzyme reactions rely on specific buffers (with defined pH, salt, and cofactor conditions) to ensure optimal enzyme activity. Cell handling and transformation also depend on proper solutions (e.g., $CaCl_2$ for competent cells) and rich recovery media (SOC) to maximize viability. Even protein analysis techniques such as SDS-

PAGE and Western blotting use specialized buffer systems (running buffers, transfer buffers, blocking solutions) to achieve correct protein separation and detection. Mastery of buffer chemistry and solution preparation thus underpins the success of every step in the cloning and expression pipeline.

Acceptance criteria

- When preparing buffers, document that the pH meter was calibrated and ensure the final pH is within ±0.05 of the target (at room temperature).
- Label each buffer with its name, concentration, pH at specified temperature, date, and preparer's initials. If the buffer is sterilized (e.g., autoclaved), re-check and record the pH after sterilization as well (since pH can drift upon cooling) to confirm it remains in range.
- For thermolabile components, do not autoclave, sterilize by membrane filtration under aseptic conditions (e.g., using a sterile 0.22 µm syringe filter).

Culture media selection (cloning and expression)

Defined / complex media

Defined media have a fully known, specific chemical composition, whereas complex media include nutrient-rich digests/extracts (e.g. yeast extract, tryptone) with undefined components that support robust growth.

Selective / differential media

Selective media contain agents that inhibit certain organisms (allowing only the desired strain to grow), while differential media contain indicators that visibly distinguish between organisms based on metabolic traits (e.g. color changes on agar).

Example application

For cloning and plasmid propagation (e.g. using *E. coli* DH5α), use Luria–Bertani (LB) medium (tryptone, yeast extract, NaCl) is well matched to *E. coli* physiology (Baev et al., 2006; Tuttle et al., 2021), providing sufficient nutrients and osmotic balance for routine cloning and plasmid propagation. supplemented with the appropriate antibiotic for selection.

After transformation, include a recovery step in SOC medium (a nutrient-rich, non-selective broth) to allow cells to express antibiotic resistance before plating.

For expression cultures (e.g. *E. coli* BL21(DE3) carrying an expression plasmid), use LB or a high-density medium like Terrific Broth (which contains glycerol and phosphate for buffering) to support greater cell yields. Auto-induction media can also be used for expression strains, as they permit lactose/glucose-regulated induction of T7-driven gene expression while achieving high cell density without manual IPTG addition.

Biosafety note

Conduct a risk assessment for all work with cultures and recombinant DNA. Use appropriate containment measures (BSL-1/2 practices as required), and handle wastes properly (e.g., decontaminate culture waste with disinfectant or autoclave and dispose in the correct biohazard stream). Adhere to institutional and international biosafety guidelines (NIH SOP, 2024; Series, 2021; WHO, 2020, p. 4) when preparing and sterilizing solutions and media.

Cloning Primer Design

A cloning primer is a synthetic DNA oligonucleotide used in PCR to amplify a target fragment and add sequence features needed for insertion into a vector such as 5′ restriction sites or overlap sequences (for Gibson/HiFi), start/stop codons, tags, or regulatory elements while maintaining the correct reading frame (Scharf, 1990; Shaker & Buniya, 2025).

Insert construction

Before designing primers, confirm the open reading frame of your target gene. Ensure you know the exact start codon (ATG) and stop codon positions and that the coding sequence translates to the expected protein with no frameshifts. This may involve using ORF-finding tools or aligning the gene sequence with a known protein sequence.

Ribosome binding site (RBS) context

Understand whether your cloning strategy needs an RBS. For pET-type expression vectors, the plasmid typically provides a Shine-Dalgarno sequence upstream of the insert site, so you do not need to include an RBS in the insert (X. Li et al., 2023). If you do need to design an RBS (for custom plasmids or special cases), recall that efficient translation in *E. coli* generally requires a Shine-Dalgarno sequence about 5–9 nucleotides upstream of the start codon, with minimal secondary structure in that region (Shilling et al., 2020; Spindler et al., 2025).

Core primer properties

- **Length and GC%:** aim for primers 18–30 nucleotides in length with a GC content around 40–60%. This range balances specificity (long enough to bind uniquely) with efficiency (not so long that T_m is too high or binding kinetics are slow).
- **Melting temperature (T_m):** calculate T_m using nearest-neighbor thermodynamics (SantaLucia model) with appropriate corrections for Mg^{2+} and salt concentration (e.g., Owczarzy formula). For a given primer pair, ensure their T_m values are within 2–5 °C of each other to enable a single annealing temperature in PCR (Zhou et al., 2025).
- **3′ end stability:** avoid primers with problematic 3′ features. Do not end a primer with runs of identical nucleotides (especially avoid 3 or more G/C in a row at the 3′ end) as this can promote nonspecific binding or primer-dimer formation. A mild "*GC clamp*" (1–2 G/C in the last 5 bases) can stabilize binding, but the very 3′ terminus should ideally not be G or C if it creates strong self-complementarity.
- **5′ additions for cloning:** when designing primers for cloning, you often need to add extra sequences at the 5′ ends that are not part of the template. For traditional restriction/ligation cloning, append the restriction enzyme recognition site to the 5′ end of each primer. Additionally, include ~4–6 extra nucleotides *upstream* of the restriction site as a "clamp or a stuffer" to ensure the enzyme can bind and cut at the end of the PCR product, many enzymes won't cut efficiently if their site is too close to the DNA end (New England Biolabs, 2021).

Reading frame and tags

Maintain the correct reading frame between your insert and the vector. If your plasmid adds an N-terminal tag (like a His-tag or GFP), you may need to omit the native start codon of the insert so that translation initiates at the vector's start (upstream of the tag) and continues seamlessly into your gene.

Conversely, if adding a C-terminal tag from the vector, design the reverse primer to exclude the gene's stop codon, allowing the tag to be translated in-frame. If no C-terminal tag is used and the native protein should terminate, ensure the stop codon is included in the reverse primer.

In summary, double-check that any fusion junctions (insert-tag or insert-vector junction) do not introduce a frameshift or unwanted amino acids.

Specificity and documentation

Primer-BLAST verification

- Once primer sequences have been designed, their specificity should be validated using NCBI Primer-BLAST.
- The search should be restricted to the relevant organism or taxonomic group (e.g., set the "Organism" field to *Escherichia coli* when amplifying an *E. coli* gene) to minimize cross-species matches.
- The output should confirm the intended target as the primary amplicon, with no additional high-confidence products, and with the expected size.
- Any off-target alignments must be recorded, if multiple significant off-targets are observed, the primer design should be refined (e.g., by adjusting length, binding position, or specificity parameters). All Primer-BLAST parameters (database, organism, allowed mismatches, product-size range, etc.) and results should be documented for reproducibility.

Design record-keeping

Bioinformatic tools

Tools like **G-synth** (*G-Synth is an internal platform developed by Dr. Merzoug Mohamed*), SnapGene, or Benchling.

The G-Synth platform, for example, can input your entire ORF sequence and automatically design primer pairs with the desired cloning method in mind (restriction sites or overlaps, inclusion or removal of stop codon, etc.). G-Synth will output primer sequences, calculate their T_m and GC%, and flag potential issues (like internal restriction sites that might interfere, or if the added overhangs create frameshifts). Use such tools to double-check your manual design.

Finally, use sequence visualization software (SnapGene or Benchling) to map your primers onto the vector and insert: this helps verify that the product of your PCR will indeed clone correctly into the plasmid (e.g., check that restriction sites are in the expected positions, and the ORF remains in-frame with any tags). Export or screenshot these designs as needed. Keeping an

organized record ensures you can justify your design choices later and easily share the rationale with others.

Biosafety and waste

Disinfect benchtops and the work area before and after handling biological materials or chemicals. Use separate waste containers for chemical waste (e.g., acidic or basic solutions) and for biohazardous materials (e.g., culture waste, agar plates, DNA gels). Inactivate biological waste by autoclaving or with appropriate disinfectants, then dispose of it according to your institution's guidelines. Follow local regulations and applicable biosafety guidance (e.g. Laboratory Biosafety Manual (WHO, 2020)) to protect personnel and the environment.

Mini-timeline (8 h)

0:00–0:20 – Orientation and Quick Refresher

Henderson–Hasselbalch and Dilution Demo: instructor demonstrates a quick pH calculation using the H–H equation and a dilution calculation ($C_1V_1 = C_2V_2$) to set the stage. Media Classification Poll: a rapid-fire class poll on examples of defined vs. complex media and selective vs. differential agents. (Keep theory to ~20 minutes total.)

0:20–1:10 – Practical Block 1 — Buffers

Teams design a buffer: Each group chooses a target pH and appropriate buffer system, then writes a symbolic recipe (no numbers, just formulas) for making, say, 1 L of that buffer at a given molarity. They outline how they would adjust pH (with HCl or NaOH) and record the expected pH at room temperature.

1:10–2:00 – Practical Block 2 — Culture media

Groups draft symbolic recipes for common media (LB, SOC), listing ingredients conceptually (no need for grams, just what and why). They then discuss and document the rationale for using each medium in cloning and expression. For example, why might SOC be preferable for plasmid preparation. Each group also specifies the appropriate antibiotic for selection in cloning, and notes if the medium is selective or not.

2:00–2:20 – Primer Design Essentials (Mini-Lecture)

Instructor presents a concise overview of primer design best practices: T_m calculations and the importance of GC%, designing clean 3′ ends (avoiding dimers and hairpins), and adding 5′ extensions for cloning. Introduce the software tools that will be used (Primer-BLAST for specificity checks, and design tools like G-Synth and SnapGene/Benchling for planning and visualization). *(~20 minutes theory)*

2:20–3:20 – Practical Block 3 — Primer Drafting

Students are given a target gene sequence (ORF) and must design a forward and reverse primer pair to amplify it and clone it into a provided vector. In this block, they work on paper or in a notebook to:

- Identify the ORF boundaries (start/stop) and determine if the gene includes a stop codon or if one should be omitted for tagging.
- Decide on appropriate restriction sites or overlap sequences for cloning into the vector (ensuring the sites do not cut within the ORF and that reading frame will be correct).
- Draft the primer sequences (5′ overhang and gene-specific region) and calculate approximate length, %GC, and T_m for each (using given tables or an online calculator).
- Check the 3′ ends of the primers for potential self-complementarity or secondary structures.

3:20–4:00 – Simulation 1 — G-Synth

Students now use the G-Synth software (or a similar primer design aide) to validate and refine their primer designs:

- Input the full ORF sequence and specify the cloning strategy (e.g., which restriction sites and stuffers).
- Generate suggested primer pairs and compare them to the student's own designs. G-Synth will provide T_m and GC% for each primer and highlight any potential problems (like if the primers introduce a frameshift or if a chosen restriction site is incompatible).
- Students can adjust their designs as needed based on the software feedback.

4:00–5:00 – Main Break (1 h)

5:00–5:40 – Simulation 2 — Primer-BLAST

Back from break, students use NCBI Primer-BLAST (or a similar tool) to check the specificity of their primer pair in silico:

- Set up Primer-BLAST with the appropriate organism or database
- Enter the primer sequences and desired product size range.
- Run the search and interpret the output: confirm that the intended target is found at the correct size, and note if any off-target hits appear.
- Students should record the settings used and the results (even if no off-targets, note that).

5:40–6:20 – Simulation 3 — SnapGene/Benchling

Students import the vector sequence (provided by instructor) into a DNA editing tool like SnapGene or Benchling, along with their newly designed primer sequences:

- Stimulate the cloning virtually: for restriction/ligation, perform an *in-silico* digest of the vector and see where the insert would ligate, for Gibson or overlap assembly, create an alignment of the PCR product with the vector.
- Check that the insert is in the correct orientation and frame, and that any tags (His-tag, etc.) will be translated properly. Also verify the presence or absence of stop codon as intended. The software can also be used to generate an annotated plasmid map showing the insert, vector features (promoter, tag, origin, antibiotic resistance), and the positions of the primers.

6:20–6:50 – Safety Mini-Brief

Instructor leads a short discussion on laboratory safety specific to the day's tasks: proper handling of acids/bases when adjusting pH, precautions with UV if mentioned (for future gel work), and handling of bacterial cultures and antibiotics. Emphasize labeling of all reagents and waste containers, and the importance of personal PPE.

Deliverable: Each student (or group) signs off on a safety checklist confirming they understand the procedures for spill cleanup, waste disposal, and emergency protocols.

6:50–7:30 – Paper-Only Build (Integration)

In this capstone activity for the session, students integrate everything they have learned into a cohesive cloning plan on paper:

They outline a step-by-step plan to go from gene to cloned product, including which buffer they would prepare (and why), which growth medium to use at each stage (e.g., transformation recovery and overnight culture), their primer sequences with annotations, and how they would confirm success in silico and in vitro.

Essentially, they produce a mini project proposal for cloning the gene, referencing the designs and decisions made throughout the day. This can be done in a worksheet format provided by the instructor, prompting them to fill in each component.

7:30–8:00 – Summary and reflection

Students write a brief summary (maximum 300 words) articulating their cloning design validation. A summary handed in or uploaded, will be reviewed to assess each student's understanding of the session's learning outcomes.

Troubleshooting

- **pH drifts after sterilization:** Let solutions cool to room temperature before final pH measurement, adjust pH if necessary, and always record the pH at the working temperature.
- **Primers yield multiple bands in silico (Primer-BLAST shows many hits):** Restrict the Primer-BLAST taxon or database to the target organism, increase stringency (e.g., require near-exact matches, particularly at the 3′ end), and, if necessary, redesign primers by increasing length or repositioning them to a unique genomic region (avoiding primers derived from repetitive elements).
- **High primer dimer or hairpin scores :** primers have 3′ complementarity to each other or internal sequences that form stable hairpins (especially if rich in GC). Redesign primers focusing on the 3′ end, remove any 3′ self-complementary sequences or runs of G/C that foster dimers. You might shorten the primer or alter some bases (without compromising binding) to reduce complementarity. Re-check the new designs with an oligo analysis tool before ordering.

Evaluation

Please answer the following questions to demonstrate your understanding of the concepts behind the practical work.

1. **Buffer capacity**

   Why is the effective buffering range typically about ±1 pH unit around a buffer's pK_a?

2. **Growth media**

   Give one example of a *defined* medium and one example of a *complex* medium. At which point in this workshop would you use each one, and for what purpose?

3. **Primer design**

   If your two primers have melting temperatures (T_m) that differ by 7 °C (calculated using the SantaLucia/Owczarzy method), what strategies could you use to bring their T_m values closer together?

4. **Restriction sites in primers**

   Why do we add extra nucleotides at the 5′ end of a primer when we include a restriction site? What might happen if you omit these extra bases before a restriction site in a PCR primer?

Session 2 (6h): Template Preparation for Cloning

Objective

In this session, students will learn to extract genomic DNA (gDNA) from bacterial cells, design and perform a cloning PCR, verify the construct in silico, and analyze results using agarose gel electrophoresis. The emphasis is on following Standard Operating Procedures while understanding the underlying scientific principles, including rationale, quality control measures, and data interpretation. All activities are conducted in alignment with best practices in molecular biology and genetic engineering.

Bacterial genomic DNA Extraction

- **Cell Envelope & Lysis Strategy:** Gram-positive bacteria (e.g. *Bacillus*, *Enterococcus*) have thick peptidoglycan walls that require enzymatic weakening (e.g. lysozyme) combined with detergents or mechanical disruption for efficient cell lysis. Gram-negative bacteria (e.g. *E. coli*) have a thinner peptidoglycan layer and are generally easier to lyse with detergents or mild mechanical methods. Choosing an appropriate lysis strategy is critical: over-aggressive mechanical lysis can shear DNA, whereas enzymatic methods help preserve high-molecular-weight DNA (Chassy & Giuffrida, 1980; Trigodet et al., 2022).

- **Impact on DNA Quality:** The extraction method influences DNA yield and fragment size. Harsh physical methods (sonication, bead-beating) may shred genomic DNA, while gentle enzymatic lysis helps retain long DNA strands. In addition, inclusion of reagents like EDTA (to chelate Mg^{2+} and inhibit nucleases) and proteinase K or SDS (to denature proteins) ensures proteins and nucleases are inactivated, improving DNA purity. *RNase* is often added to remove contaminating RNA, which would otherwise inflate A_{260} readings and artificially increase the A_{260}/A_{280} ratio (Pariseau et al., 2024).

Procedure

Genomic DNA Extraction (bacterial culture):

Sample Harvest: Transfer 1.5~3mL of a fresh overnight bacterial culture (≈10^8–10^9 CFU/mL) into a sterile 1.5 mL microcentrifuge tube. Pellet the cells by centrifugation at ~10,000 × *g* for 5 min. Carefully decant or pipette off the supernatant. *(You may need to spin multiple tubes if using >1.5 mL; collect pellets together.)*

Enzymatic Lysis: Thoroughly resuspend the cell pellet in 100 µL of resuspension buffer (e.g. 25 mM Tris·Cl, 10 mM EDTA, pH 8.0) containing lysozyme at 20 mg/mL. *(Lysozyme helps digest the cell wall; EDTA protects DNA by chelating Mg^{2+}.)* If not added in the buffer, also add RNase A to ~20 µg/mL to degrade RNA. Mix gently. Incubate the suspension at 37 °C for 30-60 min. The mixture may become slightly viscous as cells begin to lyse.

Chemical Lysis: Add 700 µL of lysis buffer to the tube. (A typical lysis buffer for genomic DNA contains a chaotropic salt like guanidine hydrochloride and a detergent like SDS. If using a kit, use the provided buffer.) Vortex briefly (5–10 seconds) to mix, the solution should become clear and viscous as cell lysis completes. Incubate at 70 °C for 15 min.

This high-temperature step further inactivates proteins (including nucleases) and helps dissolve membrane components. After incubation, centrifuge the tube at 14,000 × *g* for 10 min to pellet any insoluble debris. Transfer the clear supernatant (contains the DNA) to a new tube, being careful not to carry over the pellet.

DNA Binding: Add 400 µL of binding buffer (high-salt solution, often with alcohol) to the supernatant and mix. This creates conditions for DNA to bind to a silica column (if using a spin-column approach). Apply the mixture to a silica spin column placed in a collection tube. Centrifuge at 14,000 × *g* for ~1 min so that the DNA binds to the column matrix. Discard the flow-through. *(If not using a kit/column, DNA can be precipitated by adding isopropanol and spooled or pelleted; however, the column method is used here)*.

Wash: Add 500–700 µL of wash buffer (usually an ethanol-based buffer) to the column. Centrifuge 1 min and discard flow-through. Repeat with a second wash buffer if provided or use the same wash

again. After the final wash, spin the column for an additional 1-minute dry spin to remove residual ethanol (important: leftover ethanol can inhibit downstream enzymatic reactions).

Elution: Place the column in a clean 1.5 mL tube. Add 50–100 µL of elution buffer (10 mM Tris·Cl or nuclease-free water, preheated to ~65–70 °C for better yield) to the center of the column matrix. Let it sit for 2 minutes at room temperature. Centrifuge at maximum speed (~14,000 × *g*) for 1–2 minutes to elute the DNA into the tube. This is your purified genomic DNA. Label the tube and store on at –20 °C.

DNA Quantification and Quality Control

Spectrophotometric quantification

Theoretical background

Nucleic acids absorb UV light maximally at 260 nm due to their aromatic bases. According to the Beer–Lambert law, an absorbance of 1.0 at 260 nm ($A_{260} = 1$) corresponds to ~50 µg/mL of double-stranded DNA (pathlength 1 cm). Most lab spectrophotometers (including microvolume instruments) use this principle with a built-in factor to directly report DNA concentration. Ensure measurements are in the linear range of the spectrophotometer; dilute the sample if A_{260} is above ~1.5 to avoid saturation (Voet et al., 1963).

The A_{260}/A_{280} ratio assesses protein contamination: ~1.8 is "pure" DNA; significantly lower (<1.7) indicates protein or phenolic contaminants, whereas higher (>2.0) suggests RNA contamination since RNA absorbs strongly at 260 nm. The A_{260}/A_{230} ratio (>2.0 for pure DNA) is sensitive to contaminants like residual guanidine, ethanol, or other organic compounds, values <2.0 (especially <1.5) imply significant salt/solvent presence. Such contaminants can interfere with enzymatic reactions (e.g. PCR, restriction digest).

Procedure

General preparation and instrument QC

- Warm-up & blank: Turn on instrument; allow stabilization. Blank with the exact diluent used for samples (water or Tris buffer).

- Path length: Standard cuvette 1 cm; microvolume instruments employ short path lengths with automatic normalization to 1 cm—ensure the reading is within the instrument's linear range (avoid $A260 > 2.0$ after normalization).
- Turbidity correction (recommended): Record A320–A340 and subtract it from A260/A280/A230 to correct for light scatter (when available on the instrument).

Cuvette method

- Dilution: Prepare a 1:50–1:100 dilution (e.g., 10 µL sample + 490–990 µL diluent). Mix gently, avoid vortexing genomic DNA.
- Rinse the cuvette with a small aliquot of diluted sample; then fill.
- Record A260, A280, A230 (and A320 if available).
- Calculate concentration (apply dilution factor):
    - dsDNA (µg/mL) = $A260 \times 50 \times$ dilution
    - RNA (µg/mL) = $A260 \times 40 \times$ dilution
- Interpret purity.

Microvolume UV–Vis

- Clean pedestal surfaces, blank with 1–2 µL of diluent.
- Load 1–2 µL of undiluted sample, when possible, if absorbance is out of range, dilute and repeat.
- Record A260/A280/A230 (and A320 if available), compute concentration with built-in factors.

Agarose gel electrophoresis (integrity & size assessment)

Gel and buffer selection

- Agarose % by target size: (Sambrook & Russell, 2001)

0.8% (2–10 kb), 1.0% (0.5–10 kb; general use), 1.5% (200–3,000 bp), 2.0–3.0% (50–1,000 bp).

- Buffer:
    - TAE (Tris–acetate–EDTA) typically used for larger fragments and when DNA recovery from gel is planned.

- TBE (Tris–borate–EDTA) provides sharper resolution of small fragments and better buffering during longer runs.

Casting and staining

- Prepare 1% agarose in 1× TAE or 1× TBE (**Figure 1**).
- Heat to dissolve, cool to ~60 °C.
- Add intercalating dye per manufacturer (pre-cast) *or* plan to post-stain.
- Pour into casting tray with comb, allow to set (~20–30 min). Place gel in tank, cover with 1× running buffer.

Sample preparation and loading

- Mix nucleic acid with 6× loading dye to 1× final.
- Typical loads:
  - Genomic DNA: 50–200 ng per lane (avoid shearing).
  - PCR products: 5–50 ng.

  Load DNA ladder appropriate to the expected size range.

Electrophoresis and visualization

- Run at 5–8 V/cm (distance between electrodes), commonly 80–120 V for mini-gels, until the leading dye migrates 60–70% of gel length.
- Visualize on a blue-light transilluminator (preferred for preparative DNA to minimize UV-induced damage to fragments intended for cloning), or on UV for analytical gels.

Agarose Gel Interpretation

- Genomic DNA (gDNA): a high-molecular-weight band with minimal smearing indicates intact DNA; smear toward low MW suggests shearing or nuclease damage.
- RNA (non-denaturing gel): two distinct rRNA bands (28S and 18S in eukaryotes, with ~2:1 intensity ratio) and low background smear indicate good integrity.
- For high-precision integrity metrics (e.g., RIN, DV200), use microfluidic electrophoresis; report according to MIQE 2.0 when RNA supports qPCR (Schroeder et al., 2006).

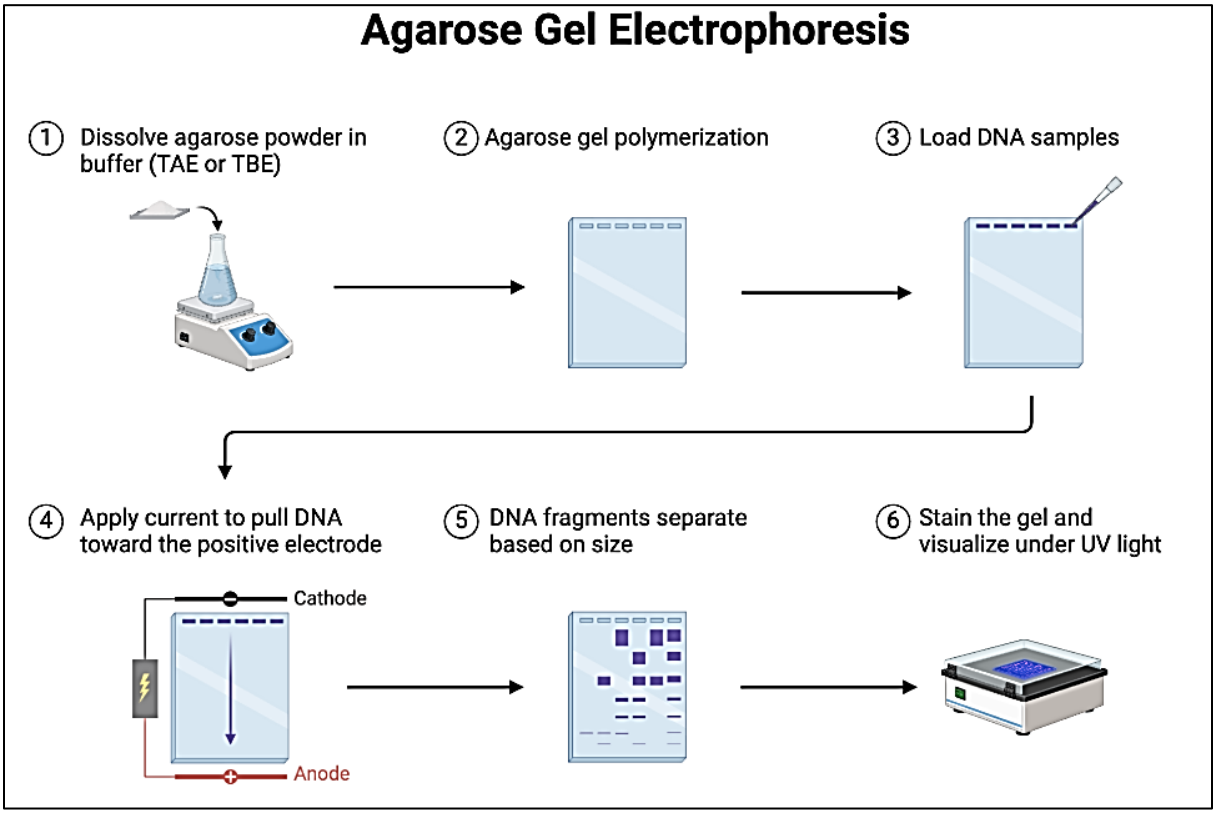

Figure 1: Agarose Gel Electrophoresis for the Separation of DNA Fragments (Lee et al., 2012).

Cloning PCR

Theoretical background

- For cloning applications, a high-fidelity DNA polymerase is strongly recommended. Taq polymerase lacks proofreading and has an error rate ~1×10^{-5}, which could introduce mutations in your clone. Proofreading polymerases (e.g. Phusion, Q5, Pfu) have 50-100× lower error rates and thus greatly reduce the chance of base errors in the PCR product (Liu et al., 2025; McInerney et al., 2014). They also tend to yield blunt-ended products (no 3′ A-overhang), which is important to note if you were planning TA cloning (in our case we use restriction/assembly, so blunt is fine).
- PCR Controls: Always run appropriate controls in parallel with your sample PCR. A no-template control (NTC) contains all reagents except DNA template, it should yield no bands, if it does, your reagents or lab bench may be contaminated with DNA. A positive control PCR

uses a template and primer set known to work (for example, amplify a housekeeping gene from the same gDNA, or a plasmid control), this ensures the PCR setup and cycler are working properly.

Procedure

Set up the PCR reactions on ice. For each sample (including your experimental sample, NTC, and positive control), prepare a 50 µL reaction mix. A typical master mix for one 50 µL PCR might include:

- Nuclease-free water: to bring total volume to 50 µL.
- 10× PCR buffer: 5 µL (provides optimal ionic environment, pH ~8.5). *(If using a polymerase with 5× buffer, use 10 µL of that instead and adjust water accordingly).*
- $MgCl_2$: (if not already in buffer) add to 1.5–2.5 mM final (e.g. 1.5 µL of 50 mM stock for 1.5 mM final). Many high-fidelity buffers include Mg^{2+}, so this step may be unnecessary if already in the 10× buffer.
- dNTP mix (10 mM each): 1 µL for 200 µM final each dNTP.
- Forward primer (e.g. 10 µM stock): 2.5 µL for 0.5 µM final.
- Reverse primer (10 µM): 2.5 µL for 0.5 µM final.
- High-fidelity DNA polymerase: e.g. 0.5–1 µL (usually 1–2.5 U, follow supplier's unit definition). For example, Q5 polymerase might use 0.5 µL. Taq would often use 1 µL (5 U/µL stock).
- Template DNA: add gDNA such that ~50–100 ng of genomic DNA is in the reaction (for a single-copy gene ~1 kb, 50 ng genomic ~10^6 copies of target, which is plenty). If your gDNA is ~100 ng/µL, 0.5–1 µL is sufficient. For positive control, add an appropriate template (e.g. 1 µL of control DNA plasmid). For NTC, add water instead of template.

Gently mix the reaction (pipette up/down or flick tube), spin briefly, and keep on ice until placing in thermocycler.

Thermocycler Program: Perform PCR in a thermal cycler with a heated lid (to prevent condensation). Use cycling conditions appropriate for your primers/polymerase. If using a high-fidelity enzyme (with a high denaturation temp), a general program might be: Initial denaturation: 98 °C for 30 s (to fully denature template DNA). Cycling (30 cycles): Denature at 98 °C for 10 s, Anneal at [~5 °C below your primers' Tm] for 20 s (e.g. 55–65 °C, depending on primer Tm, if uncertain, 60 °C is a reasonable starting point for many ~55–70 °C Tm primers). Extend at 72 °C for time per kb (e.g. 30 s/kb, so if expecting ~1.5 kb, use ~45 s). Final extension: 72 °C for 5 min (to finish any incomplete products and add A-overhangs if using Taq).

Note: proofreading enzymes don't add A-overhangs, but final hold ensures all strands are full-length.

Post-PCR Analysis:

When cycling is complete, retrieve the tubes and add 5–10 µL of each PCR reaction to the prepared agarose gel (with loading dye) for electrophoresis. Run the gel to verify if the PCR was successful. A band of the expected size in the sample and not in the NTC indicates a successful specific amplification. No bands or unexpected bands will require troubleshooting.

Depending on success, the PCR product can be purified (e.g. using a PCR cleanup kit or gel extraction if needed) in the next session before proceeding to cloning (ligation or assembly into the vector will be the next step in the workshop).

Interpreting PCR Results:

After running the PCR samples on an agarose gel, interpret the outcome: A single clear band at the expected size indicates success; you have amplified your target amplicon and can proceed to purification/clone insertion. No band (and no primer-dimer smear) in the sample lane suggests the amplification failed, possible reasons include an issue with primer annealing (check primer sequences in silico for mismatches or hairpins), insufficient template quantity or quality (did the gDNA degrade? inhibitors present?), or simply suboptimal cycling conditions (Lorenz, 2012).

In-silico Cloning Simulation

- Define the objective: assembly method (restriction–ligation or Gibson/Golden Gate), reading frame, tags, and diagnostic sites.

- Gather inputs: target gene sequence (FASTA/GenBank), annotated vector map, primers (with overhangs/sites).
- Import sequences (SnapGene or Benchling) and verify key annotations (ORI, selection marker, MCS, promoter/tag).
- Annotate primers on the template; check Tm, %GC, unique binding, and in-frame overhangs.
- Simulate PCR: generate the amplicon, confirm size and specificity, export the amplicon (preserving overhangs).
- Assemble into the vector:

- ***Restriction–ligation*:** virtual digests of insert/vector with chosen enzymes, (dephosphorylate vector if applicable), *in-silico* ligation.
- ***Gibson/related*:** design 20–40 bp overlaps and assemble via the assembly wizard.
  - Inspect the construct: insert orientation, junction sequences, frame continuity, absence of unintended restriction sites.
  - Verify translation: continuous ORF, correct tag positioning, no premature stop codons.
  - Plan diagnostics: virtual digests of the final plasmid and a table of expected fragment sizes; optional virtual gel.
  - Export deliverables: annotated plasmid map (GenBank + PDF/PNG), amplicon file, digest images/tables, brief report.
  - Final acceptance: single expected PCR product, in-frame complete construct, documented verification plan.

Mini-timeline (7 h) – Overview of session activities and approximate timing:

0:00–0:15 – Orientation and Concept Refresher: Instructor discusses cell envelope differences in Gram (+) vs Gram (−) bacteria and how these inform lysis methods. Overview of session tasks is given (gDNA extraction, PCR, *in-silico* planning, gel analysis). *(~15 min theory/discussion)*.

0:15–2:30 – Practical Block 1: Genomic DNA Extraction and QC – Students perform gDNA extraction from bacterial culture. Steps include cell harvest, enzymatic and chemical lysis, and DNA purification. During the 37 °C enzymatic lysis incubation (~1 h), the instructor leads a brief PCR design primer (covering primer selection and polymerase choice for cloning). After lysis, students

complete DNA binding, washing, and elution. Immediately following extraction, each group measures DNA concentration and purity using a spectrophotometer.

Deliverable: record A_{260}/A_{280}, A_{260}/A_{230} values in a gDNA QC table and note any purity concerns.

2:30–2:45 – *Break* (students can clean up the extraction area and prepare for PCR).

2:45–3:15 – Practical Block 2: Cloning PCR Setup – Using the extracted gDNA as template, students set up a 50 µL cloning PCR with appropriate reagents (buffer, Mg^{2+}, dNTPs, primers with restriction-site overhangs, high-fidelity DNA polymerase). Include a no-template control and a positive control. The instructor reviews thermocycler programming (initial denaturation, cycling temps, etc.) and reinforces primer design logic (e.g. Tm, GC%, clamp bases for restriction sites). PCR reactions are placed in the thermal cycler (run time ~1.5-2 h).

3:15–4:30 – Practical Block 3: *In-Silico* Simulation – While PCR runs, students use SnapGene or Benchling to verify their PCR design and plan the cloning strategy in silico. They will import the target gene sequence and vector sequence, annotate primer binding sites, simulate the PCR amplification to confirm the expected product length, and check that the chosen restriction sites (or assembly overlaps) are correctly positioned and will produce a compatible insert and vector.

If using restriction cloning, simulate a virtual digest-ligation, if using Gibson/overlap, simulate the seamless assembly. Each group exports an annotated plasmid map showing the insert and any relevant features (e.g. open reading frame, tags) and optionally a predicted gel image of the PCR product for their records.

4:30–5:45 – Practical Block 4: Agarose Gel Electrophoresis: students prepare a 0.8% and 1% agarose gel in TAE buffer (suitable for high-MW gDNA and moderate-size PCR products). Once the PCR run is finished (~3:30), samples are mixed with loading buffer and loaded alongside a DNA ladder. If required, genomic DNA samples (from extraction) and control PCRs are also loaded on the gel for quality check. The gel is run at ~4–5 V/cm (e.g. ~100 V for a 20 cm tank) until adequate separation is achieved. DNA is visualized using a safe stain (e.g. SYBR Green in-gel or post-stain) and a gel documentation system.

5:45–7:00 – Analysis & Wrap-Up: Groups consolidate their findings. In a short debrief, discuss whether the gDNA quality was sufficient (yield, purity) and how that impacted the PCR. Emphasize

troubleshooting steps if outcomes were unexpected. Finally, students submit their session artifacts: the gDNA QC data with interpretation, the annotated gel figure with legend and a written primer design & cloning rationale with *in-silico* verification evidence. *(If time permits, an open Q&A or quiz can reinforce key concepts.)*

Table 1. Troubleshooting

Issue	Possible Cause	Solution
A_{260}/A_{280} ratio 1.3–1.6 (low)	Protein carryover or phenol contamination from extraction; or very low DNA concentration (spectral noise)	Re-purify the DNA using cleanup protocol to remove proteins/phenol. Ensure thorough removal of cell debris in lysis step. Re-measure ratios in a buffered solution (Shin, 2013).
Good purity ratios, but no PCR band	Possible primer issue (design or degradation); or template DNA is too fragmented orcontained PCR inhibitors (e.g. ethanol carryover)	Re-check primer sequences *in silico* for specificity (e.g., align in BLAST to ensure they match the target and nothing else)(*Design PCR Primers and Check Them for Specificity*, n.d.). Verify the gDNA integrity on a gel – if smeared/degraded, that could cause PCR failure. If inhibitors are suspected (high A_{260}/A_{230} issues), ethanol-precipitate or re-purify the DNA and retry PCR.
Multiple bands in PCR product	Non-specific primer binding or degraded template causing random priming; too low annealing temperature; or too many PCR cycles amplifying off-targets	Optimize PCR conditions: increase annealing temperature or shorten extension time to reduce non-specific amplification. Redesign primers with better specificity if off-target binding is suspected. If the correct band is clearly one of the products, you can excise that band from the gel and purify it for cloning, but ideally refine the PCR to get a cleaner product (Kayama et al., 2021).

Evaluation

By the end of this session, students will be able to:

- Cell Lysis and gDNA Integrity: explain how bacterial cell envelope structure (Gram-positive / Gram-negative) influences the lysis strategy and affects DNA yield/quality.
- Cloning PCR Design: justify key design choices for a cloning PCR

- *In-Silico* Verification: use molecular biology software (e.g., SnapGene or Benchling) to simulate PCR and cloning in silico, verifying primer binding, predicting amplicon size, checking restriction site compatibility and visualizing the cloned insert in a vector.

Session 3 (7h): PCR Cleanup and Restriction Cloning Setup

Objective

In this session, students will purify PCR products, perform restriction digests on both the insert and the vector, and verify the construct *in silico*. The DNA fragments will be analyzed via agarose gel electrophoresis, excised, and purified for downstream use. Emphasis is on ensuring DNA quality for cloning and confirming that the planned construct is correct prior to ligation and transformation (the focus of Session 4).

Theoretical Background

- PCR products must be cleaned to remove residual polymerase, primers/primer-dimers, dNTPs and salts that can inhibit subsequent reactions like restriction digestion or ligation. These contaminants, if not removed, may reduce enzyme efficiency or introduce background noise in cloning (Brown, 2025).
- High-quality PCR DNA has an A_{620}/A_{280} ratio ~1.8 (for double-stranded DNA) and minimal absorbance at 230 nm (indicating low salt/organic carryover). A clean PCR product should appear as a single band of expected size on a gel, with no smearing. Verifying DNA integrity on an agarose gel is a useful step before proceeding (Sankar et al., 2025).
- For directional cloning, select a pair of restriction endonucleases that cut outside the insert sequence (i.e. sites present in the vector's multiple cloning sites flanking the insert region) and produce incompatible ends. Have optimal activity in the same buffer and temperature, to allow a simultaneous digest.

  (Example: If using a pET-21a(+) or pET-28a(+) vector, one might choose enzymes like NdeI and XhoI, which cut in the polylinker and produce non-compatible sticky ends. These sites would be added to the primers in Session 1 and should not occur in the insert ORF).
- The plasmid can re-circularize. Using two different enzymes helps prevent this, but as an extra precaution, consider dephosphorylating the cut vector ends with alkaline phosphatase (this removes 5′ phosphates, preventing ligase from joining the cut ends).

Procedure

PCR Product Cleanup & Quantification

- Purify the PCR product with a silica spin-column kit (bind, wash, elute per manufacturer). Elute in 30–50 µL nuclease-free water or elution buffer.
- Measure DNA on a spectrophotometer blanked with the eluent. Record concentration (ng/µL), A260/A280 (~1.8 target), and A260/A230 (>2.0 desirable).
- Log sample ID, elution volume, concentration, and ratios. If yield is low, plan to use a larger volume in the digest or reconcentrate.

Double Restriction Digest — Insert and Vector (separate tubes)

- Prepare two reactions (typical 20 µL each): DNA (≈0.5–1 µg), 10× buffer (2 µL), Enzyme 1, Enzyme 2 (5–10 U each), nuclease-free water to volume. Use a common buffer if validated, otherwise perform sequential digests.
- Mix gently, quick-spin, and incubate at 37 °C for ≥60 min (or per supplier). Heat-inactivate only if recommended and compatible with downstream steps.
- (Optional, vector only) Dephosphorylate with alkaline phosphatase (per supplier, typically 10–15 min at 37 °C) to reduce self-ligation.

Digest Verification and Gel Extraction

- Run an agarose gel (≈1% in TAE/TBE) with a suitable ladder. Load aliquots of digested insert and digested vector, optionally include undigested vector as control.
- Identify bands at the expected sizes (linearized vector; insert). If non-specific bands are present, select the band matching the design.
- Excise the target bands with minimal UV exposure (prefer blue light). Place each gel slice into a labeled tube.
- Purify DNA from gel slices using a gel extraction kit
- Quantify gel-purified insert and vector; record concentration and A260/A280 (≈1.6–1.8 acceptable post-gel). Store at −20 °C until ligation.

In-Silico Sequencing simulation

This step can be performed with provided example data

- Import ABI .ab1 chromatogram files into SnapGene or Benchling.
- Align each read to the reference plasmid sequence containing the intended insert.
- Verify that all bases across the insert match the reference and that coverage spans every critical region.
- Assess read quality and trim low-quality bases at the read ends.
- Identify any regions that lack high-quality coverage or contain ambiguities.
- Design internal sequencing primers approximately 200 bp upstream of each unclear region using Primer3 or NCBI Primer-BLAST.
- Confirm primer uniqueness with Primer-BLAST and screen for problematic secondary structures.
- Acquire additional Sanger reads with the new primers and repeat alignment until high-quality, continuous coverage is obtained.
- Confirm that the assembled consensus sequence matches the expected insert exactly.

Mini-timeline (7 h)

0:00–0:15 – Introduction and Safety Brief: the instructor reviews Session 2 outcomes (successful PCR amplification) and outlines Session 3 objectives.

0:15–1:00 – PCR Cleanup and Quantification (Lab): students purify their PCR products using silica spin columns. After purification, they measure DNA concentration and purity (A_{260}/A_{280}) using a spectrophotometer. During incubation/spin steps, the instructor discusses why purity matters for digestion and how to interpret A_{260}/A_{280} and A_{260}/A_{230} ratios.

1:00–1:30 – Digest Planning: students plan a double digestion for their insert and vector. They consult restriction maps (from SnapGene/Benchling) and confirm enzyme choices, buffer requirements, and fragment size predictions. Students set up the restriction digest reactions in parallel: one tube with purified PCR product (insert) + two enzymes, another with plasmid DNA + the same enzymes (and possibly a no-enzyme control for the vector). Tubes are placed at 37 °C to incubate 1 hour.

1:30–3:30 – Agarose Gel Electrophoresis: while digests continue to incubate, students prepare an agarose gel (0.8–1% agarose in TAE buffer) with an appropriate DNA ladder. After incubation, they mix each digest products with loading dye and load the gel. The gel runs for ~30–40 minutes. During this time, the instructor reviews gel extraction techniques and highlights safe practices for working with DNA stains and UV light.

3:30–4:00 – Gel Visualization & Band Excision: students visualize the gel results using Gel documentation system. They examine the banding: the vector should be linearized (single band at expected linear size, plus possibly a faint band of undigested supercoiled form if digestion wasn't 100%), and the insert should appear as a band of the expected length (e.g. ~1 kb).

4:00–4:45 – Gel Extraction & Cleanup: students use a gel purification kit to extract DNA from the gel slices. Gel slices are weighed and dissolved in buffer (at ~50 °C), then spun through silica columns. The DNA is washed and eluted. Each group then measures the concentration of the purified insert and vector DNA. The instructor may need to assist if yields are low (e.g., pooling replicate extractions or discussing DNA recovery rates). This purified DNA will be used for the ligation in the next session, so students verify that they have sufficient quantity (at least ~50–100 ng/µL of each).

4:45–5:15 – Break (30 min) – Cleanup and preparation for the next activity.

5:15–6:00 – In-Silico Sequence Verification: the instructor provides example Sanger sequencing chromatograms (for instance, forward and reverse reads of a previously cloned insert in the same vector). Students practice using SnapGene (or a similar DNA analysis tool) to align these reads to the reference sequence. The instructor demonstrates how to interpret chromatogram peaks (clear versus ambiguous base calls) and how to trim low-quality ends. Students learn to spot common sequencing issues, such as a few ambiguous bases at the insert-vector junctions, and discuss how they would resolve them (e.g., designing a new primer and using NCBI Primer-BLAST to ensure it will bind uniquely to the target region).

6:00–6:30 –Interpretation: the class reconvenes to summarize results. Each group reports their DNA yields and gel results, noting any problems encountered and how they solved them (e.g., one group might share that they saw an unexpected extra band and suspect partial digest).

The instructor highlights how these results feed into Session 4: if purified inserts/vectors are ready and verified, the next step is the ligation into the cloning vector and transformation into *E. coli* DH5α. Proper storage of the purified DNA (at –20 °C) is discussed. Finally, students are reminded of the importance of today's steps in ensuring a successful cloning outcome and are given a preview of the upcoming competent cells preparation and ligation session.

Table 2. Troubleshooting

Issue	Possible Cause	Solution
Variable DNA recovery after cleanup or gel extraction	Kit's size-dependent recovery limitations, DNA damage during purification	Use a purification method better matched to your fragment size (check kit specs for optimal fragment length). Minimize UV exposure during gel excision to prevent DNA damage. If yields are low, consider concentrating the sample (ethanol precipitation) or pooling multiple elutions.
Digest shows unexpected bands on gel	Partial digestion; star activity; extra restriction sites (planned or unplanned)	Confirm that the digest conditions were correct: use fresh enzyme, ensure buffer compatibility, and sufficient incubation. If star activity is suspected, try a different enzyme or shorten the incubation. Including an uncut control can help distinguish partial digests (uncut supercoiled plasmid runs lower than linear).

Evaluation

A written plan describing the restriction digest setup (enzymes chosen and why, DNA quantities, buffer, incubation time) and the expected fragment sizes. Include an annotated gel image: label each lane (ladder, undigested control, digested vector, digested insert) and mark fragment sizes.

Provide a brief interpretation confirming that the digestion was successful (e.g., "vector linearized to 5.4 kb band, insert at 1.0 kb; no undigested plasmid visible, indicating complete digest"). If unexpected bands were observed, note potential explanations. This deliverable demonstrates your understanding of the digest results and that you correctly obtained the DNA fragments of interest.

Session 4 (4 h): Ligation of the gene with the plasmid vector

Objective

The aim of this session is to ligate the pre-prepared insert into a T7 expression vector pET-21a(+) or pET-28a(+), building on products prepared in earlier sessions. This is the key step enabling recombinant production of Taq DNA polymerase in the next sessions. Students will set up directional ligations with appropriate insert:vector molar ratios and essential controls, apply dephosphorylation and/or dual-enzyme cloning to prevent self-ligation, and verify that junctions preserve the reading frame (compatible with the C-terminal His-tag in pET-21a(+) or the N-terminal His-tag in pET-28a(+)). Success is defined by correct orientation and in-frame insertion, ready for transformation and heterologous expression.

Theoretical Background

- In traditional cloning, using directional cloning with two different restriction enzymes reduces undesirable background colonies. The two enzymes are chosen such that they do not cut within the insert, have compatible reaction conditions, and create mismatched sticky ends that cannot ligate to each other (Wang et al., 2014). This way, the vector ends cannot re-circularize without an insert, and the insert will only ligate in the correct orientation. If only one enzyme or blunt ends must be used, additional measures (like dephosphorylation of the vector ends) are taken to prevent vector self-ligation (Green & Sambrook, 2012).
- Calculate the insert:vector molar ratio based on fragment sizes, ~3:1 insert:vector molar ratio is a common starting point, meaning you add roughly a 3-fold molar excess of insert relative to vector (Tan et al., 2018). This increases the probability that a vector will meet an insert before self-ligating. If the insert is much smaller or larger than the vector, adjust the mass amounts accordingly (molar ratio accounts for size differences). Using too high an insert ratio, however, can lead to the formation of multiple inserts in one vector, so there is a balance. After calculating, assemble the ligation by mixing vector and insert DNAs with the T4 DNA ligase and its buffer (which contains ATP and Mg^{2+} required by the enzyme) .
- T4 DNA ligase forms phosphodiester bonds between the 3′-OH of one DNA fragment and the 5′-phosphate of another (Rossi et al., 1997). It can ligate both sticky ends and blunt ends, but

sticky-end ligation is far more efficient because complementary overhangs bring the DNA ends together transiently, increasing the effective concentration and correct alignment for ligase action. Blunt-end ligations lack this base-pairing assistance, so they often require higher concentrations of DNA and ligase, and still yield fewer colonies. When designing the cloning, sticky-end cloning (with two different overhangs for directionality) is preferred whenever possible (Del Prete et al., 2023).

Procedure

- Calculate insert:vector molar ratio (start with 3:1). Set up 10–20 µL ligation: linear vector (50–100 ng), insert (per ratio), 10× ligase buffer (ATP-containing), T4 DNA ligase (per supplier), nuclease-free water to volume.
- Incubate per enzyme guidelines (e.g., 10–30 min at room temperature for quick ligase, or overnight at 16 °C for standard ligase).
- Include controls: vector-only (assess background) and, if useful, insert-only (checks contamination).

Mini-timeline (4 h)

An overview of Session 4 activities and their sequencing:

0:00–0:30 – Introduction & Setup: Orientation to the day's objectives and safety briefing. If not done previously, inoculate a 5 mL overnight culture of DH5α in LB (no antibiotic) to ensure fresh cells for competence. Begin pre-chilling necessary reagents (e.g. $CaCl_2$ solution, glycerol) and equipment (centrifuge rotors, tubes) for competent cell preparation.

0:30–1:30 – Competence Theory & Planning: Brief mini-lecture on the biology of competence and the roles of DH5α vs. BL21(DE3). Students calculate how to measure transformation efficiency and discuss the importance of keeping cells cold. Plan the ligation strategy: choose restriction enzymes and calculate insert: vector molar ratios for the cloning of the target gene.

1:30–3:00 – Ligation Setup: set up the ligation reaction: combine vector and insert DNA at the calculated 3:1 molar ratio with T4 DNA ligase and buffer. Incubate the ligation (overnight at 16 °C, or at least 1–2 h at room temperature if time-constrained). Also prepare small-volume control ligation tubes (vector-only, no-ligase) to transform as negative controls in the next session.

3:00–4:00 – Wrap-up & Storage: Properly label and store the ligation tubes at 16 °C (or on ice for transfer to a 16 °C fridge) to continue ligation overnight.

Evaluation

By the end of Session 4, you will be able to:

- **Design a ligation strategy that minimizes background colonies:** Choose appropriate restriction enzymes for directional cloning such that they do not cut the insert, work in compatible buffers, and produce non-complementary sticky ends to prevent vector self-ligation. Devise control reactions (vector-only, no-insert, no-ligase) to troubleshoot ligation outcomes.
- **Plan a DH5α transformation and predict outcomes:** Outline a transformation experiment for the ligation product, selecting suitable control plates and anticipating colony patterns. Interpret possible results (e.g. colonies on control plates vs. ligation plate) to infer if ligation was successful, without relying purely on protocol steps.
- **Understand biosafety and oversight in rDNA work:** Adhere to institutional biosafety guidelines for recombinant DNA experiments (NIH SOP, 2024) and proper waste management. Recognize the importance of these regulations for safe handling and disposal of cultures, antibiotics, and DNA materials.

Session 5 (6h): Preparation of Competent Cells and Transformation of *E. coli* DH5α

Objective

In Session 5, students will prepare competent cells and transform *E. coli* DH5α with the ligation product. Both the $CaCl_2$/heat-shock and electroporation methods will be employed to enable a comparison of efficiency and practical considerations.

Transformation mixtures will be plated on selective agar, and students will define expectations and criteria for colony screening in the subsequent session.

Theoretical background

Preparing Chemically Competent Cells is induced by treating cells in early log phase with cold temperature and divalent cations (e.g. Ca^{2+}), followed by a brief heat shock. This process makes the bacterial membrane temporarily permeable to plasmid DNA.

The efficiency of transformation depends on factors like the strain of *E. coli*, the method used and careful handling to avoid stress to the cells (Dagert & Ehrlich, 1979). DH5α is a cloning strain optimized for high plasmid yield and stable DNA propagation, it carries mutations such as *recA1* and *endA1* that improve insert stability and plasmid quality (Lennen & Herrgård, 2014).

In contrast, BL21(DE3) is an expression strain used later for protein production; it contains a *lac*UV5-controlled T7 RNA polymerase gene and lacks certain proteases to enhance target protein stability.

DH5α and BL21(DE3) are used at different stages: DH5α for constructing and amplifying recombinant plasmids, and BL21(DE3) for expressing the protein after the plasmid is verified(Lennen & Herrgård, 2014; N. Li et al., 2025).

Procedure

Bacterial competence

- Preculture for 16 hours.
- Measure the cell concentration using a spectrophotometer (λ = 600nm).
- Inoculate 30 ml of LB culture medium with X ml of preculture. Utilize the formula Ci X Vi = Cf *Vf, where the cell concentration should equate to an OD of 0.1.
- Incubate at 37°C for 1 hour until reaching an OD of 0.45~0.55.
- Centrifuge at 4500 rpm for 5 min at 4°C.
- Discard the supernatant and add 10 ml of cold CaCl2.
- Incubate for 45 minutes on ice.
- Centrifuge at 4500 rpm for 5 min at 4°C.
- Discard the supernatant and add 2 ml of cold CaCl2. The cells are now ready for immediate use.
- For long-term storage, aliquot 100 µl of cells with 50 µl of sterile glycerol and store at -80°C.

Transformation

- Prepare LB/ampicillin or kanamycin plates.
- Briefly centrifuge the ligation reactions. Add 2 µl of each ligation reaction to a sterile 1.5 ml tubeon ice. Prepare a control tube with 0.1 ng of uncut plasmid.
- Place the DH5α Competent Cells in an ice bath until just thawed (5 min). Mix cells by gently flicking the tube.
- Carefully transfer 100µl of cells to the ligation reaction tubes from Step 2. Use 150µl of cells for the uncut DNA control tube. Gently flick the tubes and incubate on ice for 20 min.
- Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. DO NOT SHAKE. Immediately return the tubes to ice for 5 min (**Figure 2**).
- Add 700µl room temperature SOC medium (or LB medium) to the ligation reaction transformations and 600µl to the uncut DNA control tube. Incubate for 1 hour at 37°C with shaking (~150rpm).

- Plate 100µl of each transformation culture onto duplicate LB/ampicillin plates. For the uncut DNA control, a 1:10 dilution with SOC is recommended.
- Incubate plates overnight at 37°C.

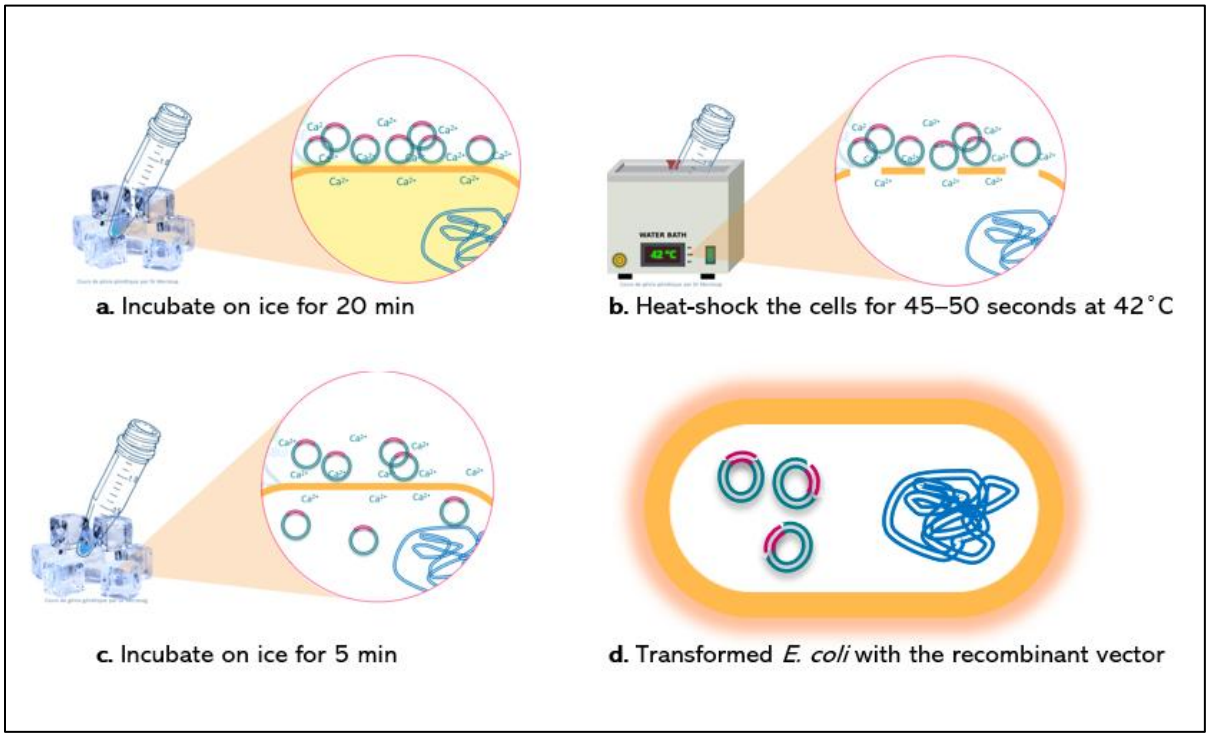

Figure 2. General Schematic of *Escherichia coli* Transformation by Heat Shock ($CaCl_2$ Method).

Mini-timeline (6 h):

0:00–0:20 – Preparation for Transformation: thaw the frozen competent cell aliquots prepared in Session 4 on ice. Retrieve the ligation reaction tubes from the 16 °C incubation (or ice). Set up the workspace for aseptic technique: pre-warm selective agar plates to 37 °C, set a 42 °C water bath (for heat shock). Review the transformation protocol with the team.

0:20–1:00 – Execute Transformations: perform the heat-shock transformation first: gently mix $CaCl_2$-competent DH5α cells with the ligation DNA on ice, incubate 20-30 min, then heat shock at 42 °C for 45 s. Immediately return the tube to ice for 5 min, then add 700 µL of SOC medium. Also transform the control plasmid into 50 µL of competent cells to serve as a positive control for efficiency (Ao et al., 2018).

1:00–4:30 – Outgrowth Recovery: incubate all transformation cultures at 37 °C with shaking (~200 rpm) for about 1 hour. This recovery period allows the bacteria to express the antibiotic resistance gene before exposure to antibiotics. During this time, discuss as a group the expected outcomes and any observations.

4:30–5:30 – Plating on Selective Media: plate aliquots of each transformation onto the appropriately labeled LB–agar -antibiotic plates. For the ligation and control transformations, typically plate 100 µL of the recovered culture directly. For the negative controls (which should have very low colony counts if any), you may plate the entire 500 µL by first pelleting the cells gently and resuspending in ~50 µL, then spreading that on a plate (to concentrate any potential colonies). Flame the spreader (or use sterile glass beads) to distribute the cells evenly. Let the plates absorb the liquid (few minutes with lids ajar), then invert them. Incubate all plates upside-down at 37 °C. Note: These plates will grow overnight; results will be analyzed in Session 6.

5:30–6:00 – Cleanup & Preview of Next Steps: properly dispose of or store any remaining ligation mix and competent. Summarize the experiment: students note in their lab notebook which plates were set up and any deviations or issues. The instructor provides a brief preview of Session 6, where colonies will be counted and verified (colony PCR).

Evaluation

By the end of Session 5, you will be able to:

- Perform transformation via both heat-shock and electroporation: Execute a plasmid transformation using $CaCl_2$-competent cells with a 42 °C heat shoc.
- Outline next steps for clone verification: Plan what to do after colonies grow. This includes picking colonies for colony PCR or mini-preps in Session 6, and understanding that only confirmed clones should be carried forward to an expression host.
- Explain how a colony PCR will verify the insert and how results (band sizes) will inform which colony is a true positive clone.

Session 6 (8 h): Clone Screening and BL21(DE3) Transformation

Objective

This session focuses on screening *E. coli* DH5α transformants for the presence and correct orientation of inserts using colony PCR. Positive clones will be verified through plasmid extraction (miniprep) and quality control checks before being transferred into the expression host *E. coli* BL21(DE3). A short lecture on recombinant DNA technology is integrated to reinforce theoretical principles of rDNA, host–vector systems, and expression strategies in modern applications.

Theoretical background

- After transformation, the ligation mixture is plated on selective media (e.g. LB agar + ampicillin for pET vector clones). Include control plates for comparison: a vector-only control and a no-ligase control (and a known positive control if available). If colonies appear on the vector-only or no-ligase controls, it indicates background growth from undigested or self-ligated vector; a successful ligation should show colonies predominantly on the ligation plate and none (or very few) on the negative controls.
- On ampicillin selection plates, large β-lactamase-producing colonies may allow tiny satellite colonies to grow nearby once the antibiotic is locally degraded. These small satellites do not contain the plasmid and should be ignored (do not pick them). To reduce satellites, some protocols substitute carbenicillin (a more stable analog of ampicillin) in the agar (Stroik, 2023). Always verify that colonies you pick are true transformants (typically larger, well-isolated colonies) rather than satellites.
- To quickly screen for inserts, perform colony PCR using primers that flank the insertion site. For example, in pET-derived constructs, use vector-specific primers such as the T7 promoter and T7 terminator primers, which amplify across the cloning site. This yields a PCR product whose size will increase by the length of the insert, allowing confirmation of insert presence and an indication of its size (Using one vector primer and one insert-specific primer is another strategy to check orientation if needed.). *In-silico tip:* Use SnapGene or Benchling to map primer binding sites and predict the expected PCR product length for your insert; this helps confirm that your primer design and insert are compatible.

- A successful colony PCR will show a single DNA band of the expected size (corresponding to vector + insert) for a true positive clone. If no band is observed (despite the colony growing on the plate), it could mean the insert is absent or the primers did not bind (e.g. the colony might be a false positive or a satellite). Double-check that you picked a proper colony and that the PCR mix and primers were correct. You may re-run PCR or pick a different colony.

  - If multiple bands or a smear appear, it suggests non-specific amplification or a mixed colony. In this case, re-purify the colony (streak it on a fresh plate to isolate a single clone) and repeat the PCR, or proceed with plasmid extraction and verification by restriction digest or sequencing for clarity (Kenkel, 2016; *Troubleshooting Your Plasmid Cloning Experiment*, 2019).

Procedure

1- Colony PCR

Before proceeding with the transformation of *E. coli* strain BL21(DE3), a verification of the presence of the insert in the pET-21a plasmid should be conducted using colony PCR with universal primers of the T7 promoter and terminator:

- Transformed clones were randomly chosen, picked using a sterile Pasteur pipette/stick from the antibiotic-supplemented selection dishes, and resuspended in 10 µl of ultra-pure water.
- 5 µl of each prepared bacterial suspension is individually inoculated into tubes containing 2 ml of LB medium (supplemented with 100 µg/ml ampicillin) and incubated with agitation at 37°C for 24 hours.
- For bacterial lysis, the remaining bacterial suspension (5 µl) is heated at 95°C for 5 minutes, followed by PCR using PCR master mix (2X) with universal primers. A 1Kb size marker is used for determining the molecular weight of PCR products.

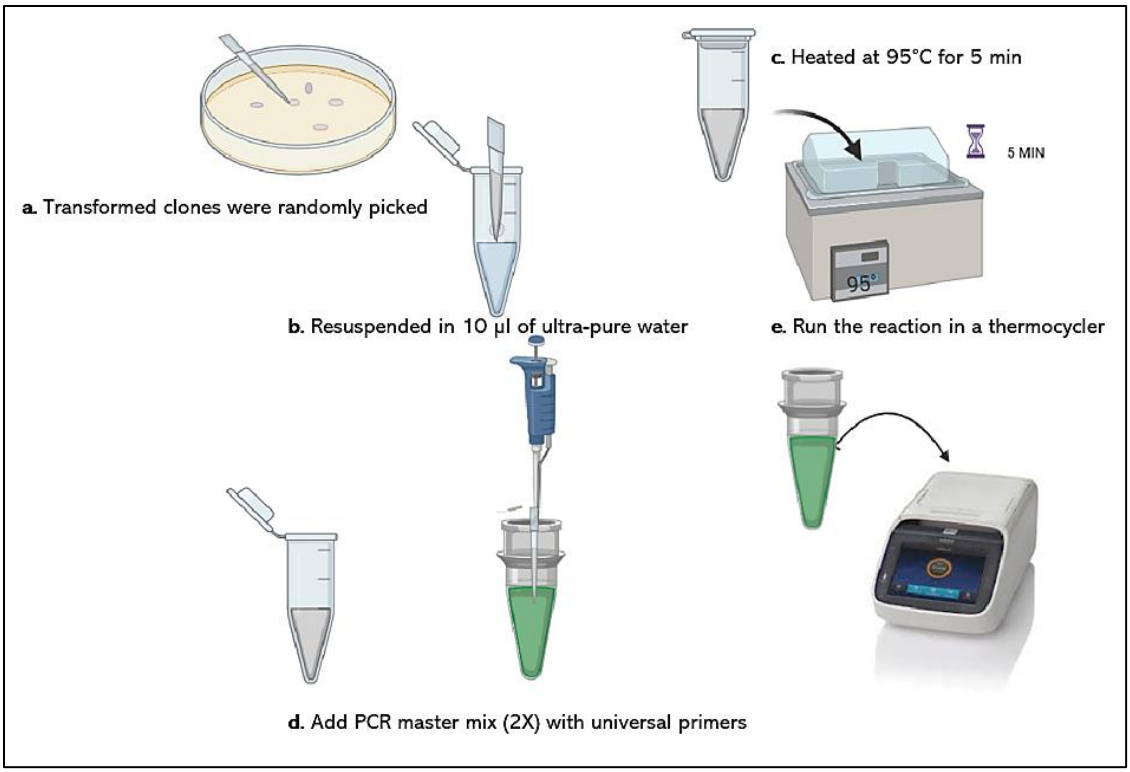

Figure 3: General Schematic of the Colony PCR Method.

2- Plasmid DNA extraction:

In order to transform *E. coli* strain BL21 (DE3), plasmid DNA (recombinant pET21a vector) extraction is performed using a commercial plasmid Miniprep Kit following the provided user manual (Thermo Fisher Scientific, 2025).

3- Transformation of *E. coli* BL21 (DE3) Strain and Selection of Transformed Clones:

The *E. coli* BL21 (DE3) strain was transformed with the recombinant pET-21a plasmid extracted from the *E. coli* DH5α strain. It's noted that the same transformation protocol for the *E. coli* DH5α strain, as described previously, was used again.

Before proceeding with the heterologous expression, selected clones were first analyzed to ensure the presence of the recombinant plasmid by colony PCR.

Table 3. Troubleshooting

Issue	Possible Cause	Solution
Unexpected colonies on control plates (vector-only / no-insert plates)	Undigested or self-ligated vector background	Re-assess the restriction digestion strategy. Confirm that the vector was completely cut (and dephosphorylated if necessary) before ligation. Repeat the ligation with improved controls (*Troubleshooting Your Plasmid Cloning Experiment*, 2019).
No insert band in colony PCR (colony grows but PCR shows no product)	Primer mis-binding, or picked a satellite/non-recombinant colony	Double-check that the primers target the correct sites flanking the insert. Ensure the colony picked was truly recombinant (not a satellite).If the issue persists, pick a different colony and/or redesign primers (Kenkel, 2016; Stroik, 2023).

Evaluation

By the end of this session, you will be able to:

1. Design and interpret colony PCR screens: Select appropriate primers (e.g. T7 promoter/T7 terminator primers for pET vectors) to verify insert presence and orientation in clones before moving to expression hosts. Interpret PCR results to distinguish positive inserts from false positives or negatives.
2. Explain selection on antibiotic plates: Analyze colony patterns on selective plates and recognize background issues such as unintended growth on control plates or “satellite” colonies on ampicillin agar. Justify which colonies to pick for screening based on controls and colony morphology.
3. Justify transfer to BL21(DE3) after verification: Explain the rationale for moving a confirmed construct into BL21(DE3) for T7-expression, and why verifying insert integrity/orientation beforehand is critical to prevent leaky expression or toxicity.

Session 7/8 (8 h): Heterologous Expression Tests: Culture Setup Under Varied Conditions

Objective

Establish a rigorous, comparable framework for heterologous expression of a recombinant enzyme in *Escherichia coli* BL21(DE3), using Taq DNA polymerase as the illustrative target (a thermostable DNA polymerase from *Thermus aquaticus*, expressed from a T7 promoter). Students design and execute a factorial induction matrix (inducer level × temperature × induction duration) in aerated conical shake flasks, biomass-normalize samples, and prepare standardized aliquots for Session 8 SDS-PAGE.

Note: *Taq* is an example target; the same design logic applies to other enzymes (keep plasmid features/reading frame/tag annotations updated).

Theoretical background

- IPTG (isopropyl β-D-1-thiogalactopyranoside) is a non-metabolizable lactose analog that binds the LacI repressor, derepressing lac/lacUV5 promoters. In BL21(DE3), this activates T7 RNA polymerase expression and, in turn, strong transcription from T7 promoters controlling the target gene. Because IPTG is not consumed, its effective concentration remains stable throughout induction.
- Higher IPTG generally increases transcription and protein output but also raises aggregation and metabolic burden (practical starting range is 0.1–0.5 mM).
- Expression temperature modulates translation kinetics and folding. At 37 °C, crude yield is often high while solubility decreases, 30 °C offers a balanced compromise; 18–28 °C improves folding and solubility (overnight incubation).

Procedure

- Prepare a 5 ml pre-culture for each clone using LB medium supplemented with 100 µg/ml ampicillin.
- Inoculate 50 ml of LB medium with 1% of the pre-culture in the presence of ampicillin, and

grow to an OD600nm of 0.6 to 0.8.

- Induce with either 0.3 mM or 1 mM IPTG. Prior to induction, take 1 ml of culture, centrifuge at 14000 rpm for 10 minutes, and store the pellet at -20°C as a negative control.
- Post-induction, incubate for 18 hours at 25°C or 3 hours at 37°C. Then, centrifuge 1 ml of each culture to recover the pellet.
- Resuspend the pellet in 75 µl of distilled water and 25 µl of SDS-PAGE Blue sample buffer (1.5 mL Tris (pH 6.8), 0.03 g Bromophenol Blue, 3 mL Glycerol, 0.35 mL β-mercaptoethanol, 0.6 g SDS, distilled water to a final volume of 7.5 mL).
- Heat at 96°C for 10 min after complete homogenization.
- Post-centrifugation, recover approximately 40 µl from the top of the mixture.
- Analyze all samples and their controls using SDS-PAGE. Load 5 µl of the control (pre-induction) and 2 µl of the sample (post-induction) into the gel wells.
- Use 12-15% SDS-PAGE gels to resolve the recombinant protein effectively.
- Determine successful overexpression by identifying a pronounced band on the gel under optimized conditions.
- Result analysis.

Mini-timeline (≈ 8 h)

0:00–0:20 Briefing & design: confirm construct/antibiotic.

0:20–2:30 Culture setup and growth to induction: inoculate flasks, verify aeration (low fill ratio, baffled flasks), monitor growth toward mid-log, pre-set incubation temperatures.

2:30–3:00 Induction start: initiate per matrix, log condition metadata (time, growth state, temperature band, inducer band).

3:00–8:00 Incubate the first expression assay for 3 hours in a shaking incubator at 150 rpm, 37 °C. During incubation, prepare the SDS-PAGE gel and the required reagents (e.g., loading buffer). At the end of incubation, collect the cell pellet and analyze it by SDS-PAGE.

Troubleshooting

No apparent over-expression: plasmid instability or wrong clone. Shift to cooler temperature band and/or lower inducer band, confirm antibiotic selection, verify clone identity, extend duration only under cooler conditions.

Target mostly insoluble/inclusion bodies: translation outpaces folding. Use cooler temperature band, reduce inducer band, shorten induction, consider chaperone strategies or auto-induction designs in advanced modules.

Evaluation

In this session, you will be evaluated not only on the results of your experiment but also on how you plan, execute, and interpret your work. Your grade will be based on four areas:

1. Preparation & Experimental Design (20%)

We will look at how clearly you set up your induction matrix (IPTG level × temperature × induction time), how carefully you handle your cultures with the correct antibiotic, and how completely you record your metadata (OD_{600}, time points, growth stage, conditions).

2. Technical Execution (30%)

You are expected to use aseptic technique during inoculation and induction, collect pre- and post-induction samples at the right time, and prepare your pellets and buffers properly for SDS-PAGE.

3. Data Acquisition & Analysis (30%)

Your SDS-PAGE gel should show clear, interpretable bands without major artifacts. You should be able to point out the band that corresponds to your recombinant protein and compare expression across the different induction conditions you tested.

4. Scientific Reasoning & Troubleshooting (20%)

Finally, we want to hear your reasoning: can you explain how IPTG concentration and temperature affect expression and solubility? Can you suggest logical changes if your protein is not expressed or forms inclusion bodies?

What you will hand in / present:

- Your lab notebook with complete notes, observations, and an annotated gel image.
- A short written or oral discussion of which condition worked best, what problems you faced, and what you would try next.

Session 9 (8 h): Nickel affinity chromatography for recombinant protein purification

Objective

This session provides a practical application of previously covered theory. Students will purify a His-tagged recombinant protein by immobilized-metal affinity chromatography (IMAC) and conduct SDS-PAGE–based quality assessment. The laboratory exercise is designed to reinforce theoretical understanding and develop operational proficiency in these techniques.

His-Trap affinity purification

Theoretical background

In nickel IMAC, the polyhistidine tag (typically 6×His) binds to a Ni2+–chelate resin (Ni2+–NTA or Ni2+–IDA) through metal ligand bonds:

The imidazole nitrogens (Nδ1 and/or Nε2) of histidine donate lone pairs to the Ni2+ center, yielding multidentate chelation by adjacent histidines (high avidity) (Iyer et al., 2018). Selectivity is tuned with moderate ionic strength (≈300 mM NaCl) and low imidazole (≈5–30 mM) in the binding/wash buffer; imidazole acts as a competitive ligand to suppress nonspecific adsorption. Elution is obtained by high imidazole ≈150–500 mM (**Figure 4**), which occupies the Ni2+ sites left available by the chelator and displaces the His-tag (Islam et al., 2025).

Binding is pH dependent: near pH 7.5–8.0 the imidazole is largely unprotonated (side-chain pKa ≈6.0) and donates efficiently; acidification protonates imidazole and disrupts chelation. Strong chelators (e.g., EDTA) remove Ni2+ from the resin and are reserved for resin regeneration (Crowe et al., 1994).

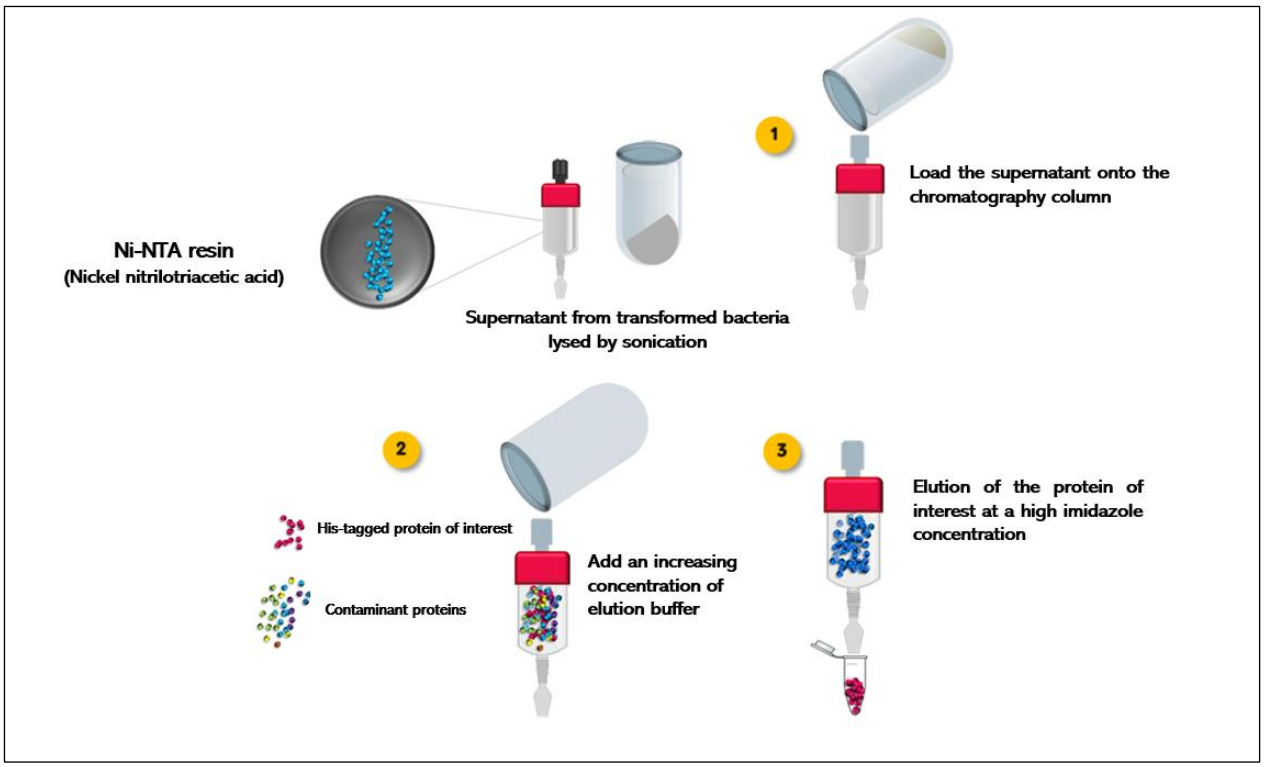

Figure 4: General Schematic of Manual Ni-NTA IMAC Purification with Imidazole Elution.

Procedure

1. Prepare the sample. Clarify the lysate at ≥10,000 × g for 10–20 min at 4 °C. Filter the supernatant through a 0.22 µm membrane. Adjust to the binding buffer (50 mM sodium phosphate, 300 mM NaCl, 10–20 mM imidazole, pH 7.5).
2. Condition the column. Rinse the resin with 5× volume of sterile distilled water. Equilibrate with 10× volume of binding buffer (50 mM sodium phosphate, 300 mM NaCl, 10–20 mM imidazole, pH 7.5).
3. Load the lysate. Apply by gravity or gentle syringe pressure at a flow not exceeding 1 volume per 5–10 min. Collect and label the flow-through.
4. Wash the column. Wash with 10× volume of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20–40 mM imidazole, pH 7.5). Collect sequential wash fractions.

5. Elute the target. Elute with 8× volume of elution buffer (50 mM sodium phosphate, 300 mM NaCl, 200–500 mM imidazole, pH 7.5) using a manual gradient elution (e.g., 200/300/400/500 mM imidazole). Collect small, sequential fractions.
6. Measure absorbance. Measure A_{280} for all fractions using the corresponding buffer as blank. Record volumes and A_{280} values.Select and pool fractions. Identify eluates meeting predefined purity and yield criteria.
7. Perform SDS-PAGE quality control.
8. Aliquot the product and store in storage buffer (20 mM Tris-HCl, 150 mM NaCl, 10 % glycerol, 1 mM DTT, pH 7.5) at 4 °C for short term or −80 °C for long term.

Mini-Timeline (8 h)

0:00–0:30 Briefing, confirm buffers and acceptance criteria.

0:30–1:30 Lysate clarification (or retrieve pre-clarified lysate).

1:30–3:30 IMAC capture: column equilibration, load, wash, elute, $A_{(280)}$ tracing and fraction collection.

3:30–5:30 SDS-PAGE sample preparation and gel run (ladder + key fractions).

5:30–6:00 Gel staining/visualization and documentation; preliminary densitometry.

6:00–7:15 Data review: pooling decisions; record yield and purity.

7:15–8:00 Cleanup, waste routing, notebook finalization, instructor debrief.

Table 4. Troubleshooting

Problem	Probable Cause	Possible Solution
No recombinant protein recovered after elution	Nothing bounds due to protein folding (6×His epitope masked)	Switch to denaturing conditions (urea/GuHCl) to expose the tag.
	Expression levels too low	Optimize expression (strain, induction, temperature, IPTG, time) per your expression guide.
	Protein lost during overly stringent wash	Raise wash pH in high-stringency step and/or reduce wash time/number.
	Not enough sample loaded	Increase sample/lysate load onto the column.

Problem	Probable Cause	Possible Solution
Good recovery but contaminated with non-recombinant proteins	Wash not stringent enough	Lower pH during high-stringency wash and wash more extensively.
	Other His-rich proteins present	Add an extra high-stringency wash at lower pH (pH 6→4) before elution. Repurify eluate: dialyze into binding buffer and reload on a fresh Ni-NTA column.
Low recovery and contamination by non-recombinant proteins	Protein not binding tightly to resin	Use denaturing conditions.

Evaluation

By the end of this session, students will be able to:

- Explain IMAC physicochemistry: describe metal–imidazole synchronization, competitive elution mechanisms, and the influence of pH and buffer composition on binding and elution.
- Define acceptance criteria: set criteria for binding efficiency, wash stringency and elution purity.
- Document purification runs: complete traceable fraction logs, gel annotations, and justify pooling decisions based on QC data.

Session 10 (9h): Recombinant Protein Validation

Objective

This session focuses on confirming the identity of the recombinant enzyme through Western blot analysis and evaluating its functional activity using a systematic enzyme assay. Students will integrate immunodetection and functional analysis to validate the success of their expression and purification workflow. Emphasis is placed on proper experimental design, controls, data interpretation, and documentation practices that are essential for protein characterization in research and industry.

Western Blot

Theoretical Background

Western blotting is viewed as the gold standard for protein detection in molecular biology research. It is used to identify proteins within a cell or tissue lysate. Antibodies against your protein(s) of interest, bind to specific epitopes to identify the target protein within a lysate. Due to the high specificity of the binding, multiple target proteins can be identified on one membrane. Secondary antibodies then bind to your primary antibodies and when exposed to a substrate react, allowing for the visualization of the corresponding protein band (**Figure 5**).

Western Blot Essentials

- ***Transfer & Buffer Systems:*** Western blotting transfers denatured proteins from SDS–PAGE gels to membranes while preserving epitopes. Buffer choice governs efficiency and retention: Tris–glycine often benefits from methanol (especially for hydrophobic proteins), whereas Bis–Tris systems provide more uniform transfer across a wide MW range.
- ***Membrane Selection (Nitrocellulose vs PVDF)****:* nitrocellulose offers strong binding with low background for most applications. PVDF provides superior mechanical strength and supports multiple reprobes but requires methanol activation and may show higher background. Selection depends on target abundance, reprobing needs, and sensitivity requirements (Sule et al., 2023; Xiang et al., 2021).

- ***Detection Strategies:*** chemiluminescence delivers picogram-level sensitivity and a broad linear range ideal for low-abundance targets and semi-quantitative work. Colorimetric detection is robust and equipment-light but less sensitive. Fluorescence enables multiplexing and excellent quantitation, at the cost of specialized imaging hardware (Moela, 2025).
- ***Antibody Selection & Optimization:*** for His-tagged proteins, validated anti-His primaries are reliable with low cross-reactivity. Monoclonals maximize specificity and lot-to-lot consistency, polyclonals can increase sensitivity via multi-epitope recognition. Start with vendor dilutions and optimize empirically to balance signal and background (Lu et al., 2025).
- ***Controls:*** include a positive control (known target), a no-primary control (background check), and a loading control (housekeeping protein or total-protein stain). For recombinant targets, compare induced vs uninduced samples.

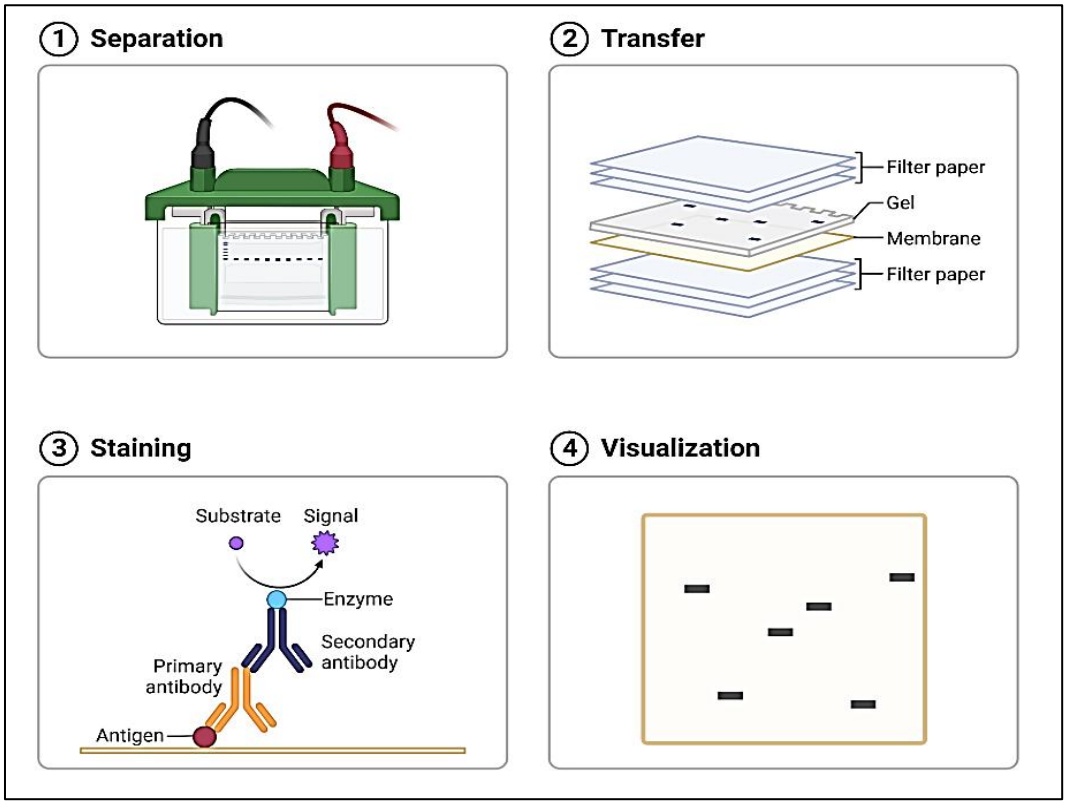

Figure 5: Generalized Workflow for Western Blot Analysis.

Procedure

1. ***Sample Preparation:*** choose key fractions from Session 8 purification, determine loading strategy based on objectives qual volumes for qualitative assessment or equal protein amounts (by Bradford assay) for quantitative comparison. Typical loading: 15-20 µg total protein per lane. Mix samples with 4× SDS-PAGE loading buffer containing reducing agent (β-mercaptoethanol or DTT) and boil for 5 min to ensure complete denaturation. Cool on ice before loading.
2. ***Run SDS-PAGE gel:*** run samples on appropriate percentage polyacrylamide gel (typically 12% for proteins 20-80 kDa). Run until bromophenol blue dye front reaches bottom of gel.
3. ***Prepare transfer:*** choose transfer method based on available equipment and protein size. Prepare transfer buffer according to system requirements, typically Tris-glycine with 20% methanol for standard proteins. If using PVDF, activate in methanol for 15 s, then equilibrate in transfer buffer. Nitrocellulose can be used directly after brief buffer equilibration.
4. ***Assemble transfer stack:*** layer gel, membrane, and filter papers according to manufacturer instructions, ensuring no air bubbles. Proper assembly is critical for uniform transfer.
5. ***Electrophoretic Transfer:*** transfer at appropriate voltage and time for system and protein size, typically 100V for 1-2 hours for standard proteins. Monitor buffer temperature and add cooling if necessary. After transfer, briefly stain membrane with pre-stained molecular weight markers to confirm successful protein transfer.
6. ***Block membrane:*** incubate membrane in blocking buffer (typically 5% non-fat dry milk or 3% BSA in TBS-T) for 1 hour at room temperature with gentle agitation to occupy non-specific binding sites.
7. ***Primary antibody incubation:*** dilute primary antibody (anti-His tag or target-specific) in blocking buffer according to optimization results, typically 1:1000 to 1:5000 for anti-His antibodies. Incubate overnight at 4°C or 2 hours at room temperature.
8. ***Wash membrane:*** remove primary antibody and wash membrane 3× 10 min in TBS-T to remove unbound antibody while preserving specific binding.

9. ***Secondary antibody incubation:*** apply appropriate secondary antibody (anti-mouse or anti-rabbit) conjugated to HRP or AP at optimized dilution (typically 1:5000 to 1:10000). Incubate 1 hour at room temperature.
10. ***Final washes:*** wash membrane 3× 10 min in TBS-T, followed by brief rinse in TBS to remove Tween-20 that may interfere with substrate development.
11. ***Prepare detection substrate:*** mix chemiluminescent substrate components immediately before use. Apply evenly to membrane and incubate according to manufacturer instructions (typically 1-5 min).
12. ***Image acquisition:*** capture images using appropriate system (film, CCD camera, or direct imaging). Take multiple exposures (10 s to 10 min) to ensure optimal signal capture within linear range.

Enzyme Activity Assay

Polymerase Activity Principles

DNA polymerase activity can be measured through various approaches: incorporation assays (monitoring nucleotide incorporation), extension assays (primer extension products), or functional PCR assays. For Taq polymerase, thermostability is a key characteristic that can be evaluated through heat treatment controls (Zweitzig et al., 2012).

Procedure

1. Prepare the controls. Use a positive control with commercial Taq, a negative control without enzyme, and a heat-inactivated control using the test enzyme preheated.
2. Keep all reagents on ice and label tubes or wells in triplicate for each condition and control.
3. Prepare a master mix without enzyme. Include reaction buffer, Mg^{2+}, dNTPs, primers, template, and nuclease-free water.
4. Prepare a serial dilution of the test enzyme. Use a series such as 1×, 1/2×, 1/4×, and 1/8× in enzyme dilution buffer or in 1× reaction buffer.

5. Dispense the master mix into the labeled tubes or wells. Add the enzyme last for each condition. Add water for the negative control, commercial Taq for the positive control, and the preheated enzyme for the heat-inactivated control.
6. Run a short PCR program appropriate for the polymerase. Use an initial denaturation at 95 °C followed by 10 to 20 cycles with denaturation at 95 °C, annealing near primer Tm minus three degrees, and extension at the manufacturer's recommended temperature and time per kilobase. Finish with a brief final extension and hold at 4 °C.
7. Analyze reaction products. Use agarose gel electrophoresis to assess presence and size or use a fluorescence readout to measure signal increase.
8. Verify control performance. The positive control should show clear amplification and the negative and heat-inactivated controls should show none or minimal signal.
9. Confirm dose dependence across the enzyme serial dilutions. The response should increase within the linear range without evidence of saturation.
10. Record raw data and experimental details. Conclude on enzyme activity as qualitative presence or relative quantitative performance.

Mini-timeline (9 h)

0:00-0:30 - Session Introduction & Planning: review Session 9 results and select samples for analysis. Plan Western blot loading strategy and activity assay design. Discuss safety requirements for detection reagents and imaging equipment.

0:30-1:30 - Sample Preparation & Gel Setup: prepare protein samples for Western blot loading, ensuring appropriate dilutions and controls. Set up transfer apparatus and prepare transfer buffer. Load and run SDS-PAGE gel if not already completed from Session 9.

1:30-2:30 - Protein Transfer: execute electrophoretic transfer of proteins to membrane using appropriate buffer system and transfer conditions. Monitor transfer progress and troubleshoot any issues with membrane contact or buffer conductivity.

2:30-4:30 - Membrane Blocking & Primary Incubation: block membrane to reduce non-specific binding and incubate with primary antibody (anti-His tag or target-specific). Optimize antibody dilution and incubation conditions based on manufacturer recommendations and empirical testing.

4:30-5:30 - Secondary Detection & Imaging: wash membrane, apply secondary antibody, and develop signal using chemiluminescent or colorimetric substrate. Image membrane and capture multiple exposures to ensure optimal signal capture within linear range.

5:30-7:00 - Enzyme Activity Assay: design and execute DNA polymerase activity assay using appropriate template, primers, and detection method. Include comprehensive controls and monitor reaction progress. Analyze results using gel electrophoresis or spectrophotometric methods.

7:00-8:30 - Data Analysis & Documentation: Perform densitometric analysis of Western blot results, quantify activity assay outcomes, and integrate data from both assays. Prepare figures, tables, and summary documentation.

8:30-9:00 - Results Integration: synthesize Western blot and activity data to evaluate overall success of expression and purification.

Table 5. Troubleshooting (Sule et al., 2023)

Issue	Possible Cause	Solution
No signal in Western blot	Transfer failure, antibody problems, low protein concentration.	Verify transfer with check antibody dilutions and specificity, increase sample loading.
High background on blot	Insufficient blocking, antibody cross-reactivity, over-exposure.	Optimize blocking conditions; increase wash stringency, reduce antibody concentration, shorter exposures.
No enzyme activity detected	Enzyme denaturation, missing cofactors.	Check enzyme storage conditions, verify buffer composition.
Activity in negative controls	Contamination, carryover from positive samples.	Use strict sterile technique, prepare fresh controls, check for amplicon contamination.

Poor correlation between blot and activity	Protein misfolding, epitope masking, enzyme inhibition.	Optimize purification conditions, check buffer compatibility, test different activity assay conditions.

Evaluation

By the end of this session, you will be able to:

- Explain Western blot principles and justify technical choices.
- Design and execute a comprehensive Western blot workflow.
- Integrate immunological and functional data for protein validation.
- Apply advanced documentation and analysis practices.

Conclusion

This handbook guided you through a series of workshops in genetic engineering: from the preparation of buffers, solutions, and culture media to the *in-silico* design of constructs, their assembly in-vitro, sequence validation, and functional protein evaluation. Each module paired core theory with standardized procedures, explicit acceptance criteria, and troubleshooting strategies, ensuring both reliable execution and a clear understanding of the underlying design choices.
You designed and optimized genetic constructs, verified reading frames and fusion logic (Benchling/SnapGene), implemented high-fidelity cloning PCRs (primer design, cycling profiles, controls), and carried out in-vitro assemblies (directional restriction/ligation, Gibson, Golden Gate). Bacterial transformation performed by heat shock method, followed by selection and colony-PCR screening, confirmed the identity and integrity of inserts.

For heterologous expression, you performed expression tests varying temperature, incubation time, and inducer concentration (IPTG), then analyzed outcomes by SDS-PAGE. Once the recombinant protein was detected in the soluble fraction, you executed affinity purification (IMAC), followed by Western blotting to evaluate the recombinant protein, and an enzyme activity assay of recombinant (Taq polymerase) using a short PCR cycle to confirm functional activity after heterologous expression.

Beyond technique, the workshops emphasized cross-cutting competences that define professional practice in genetic engineering:

- Experimental design and controls (positive/negative controls).
- Quantitative reasoning (buffer/reagent concentration calculations, insert-to-vector molar ratios).
- Qualitative data evaluation (expected bands, sequence quality, completeness of digestion, transformation efficiency).

Competency checklist (self-assessment)

Before moving on, you should be able to:

- Select and justify a construct and assembly strategy (restriction/ligation, Gibson, or Golden Gate) with explicit acceptance criteria for junction fidelity and reading frame.
- Prepare and verify buffers accurately, including calculations for concentrations, enzyme units, and insert:vector molar ratios.
- Design and run cloning PCRs (primers, cycling profiles, controls) and interpret gels.
- Plan and execute transformations (chemical), choose selection markers, and screen colonies effectively (colony PCR).
- Confirm constructs by Sanger sequencing in silico (primer choice, template prep) and evaluate electropherograms for quality and accuracy.
- Optimize expression sample at constant biomass (inducer, temperature, time).
- Analyse expression/solubility by SDS-PAGE and Western blot.
- Purify His-tagged proteins by IMAC, monitor UV elution, assess purity, and decide on fraction pooling.
- Maintain complete, legible documentation enabling independent reproducibility.
- Apply biosafety and chemical safety requirements to each organism, vector, and reagent.

References

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