

Assignment 2: In-Silico Cloning Strategy: Restriction Analysis, Primer Design, and Vector Construction

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Genetic Engineering: Applications and Principle

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1 Introduction

The objective of this assignment is to design a cloning strategy for the **APOE gene**. This involves constructing a hybrid gene (Promoter + CDS), analyzing restriction sites, and simulating cloning into pBluescript and pET-28a vectors.

2 Task 1: Sequence Retrieval & Construction

Appropriate sequences were retrieved from NCBI. To create an expression-ready construct while retaining regulatory elements, a chimeric approach was used.

2.1 1A. Target Gene Construction (Promoter + CDS)

Instead of using the full genomic sequence (which contains introns), we constructed a hybrid sequence:

- **Promoter Source:** The upstream promoter region (73 bp) was extracted from the Genomic Sequence (**NG_007084**).
- **CDS Source:** The Coding Sequence (CDS) was extracted from the mRNA sequence (**NM_000041**) to ensure an intron-free gene for bacterial expression.
- **Final Construct:** The promoter was ligated upstream of the CDS in-silico.

2.2 1B, 1C, & 1D: Reporter and Vectors

- **GFP (Reporter):** Retrieved from GFP (**GFP sequence by snapgene browser**) for fusion protein creation.
- **Cloning Vector:** pBluescript SK(+) (**X52328 Snapgene**) for initial maintenance.
- **Expression Vector:** pET-28a(+) (**EF442785**) for high-level expression.

3 Vector and Insert Maps

The circular maps of the retrieved sequences are shown below.

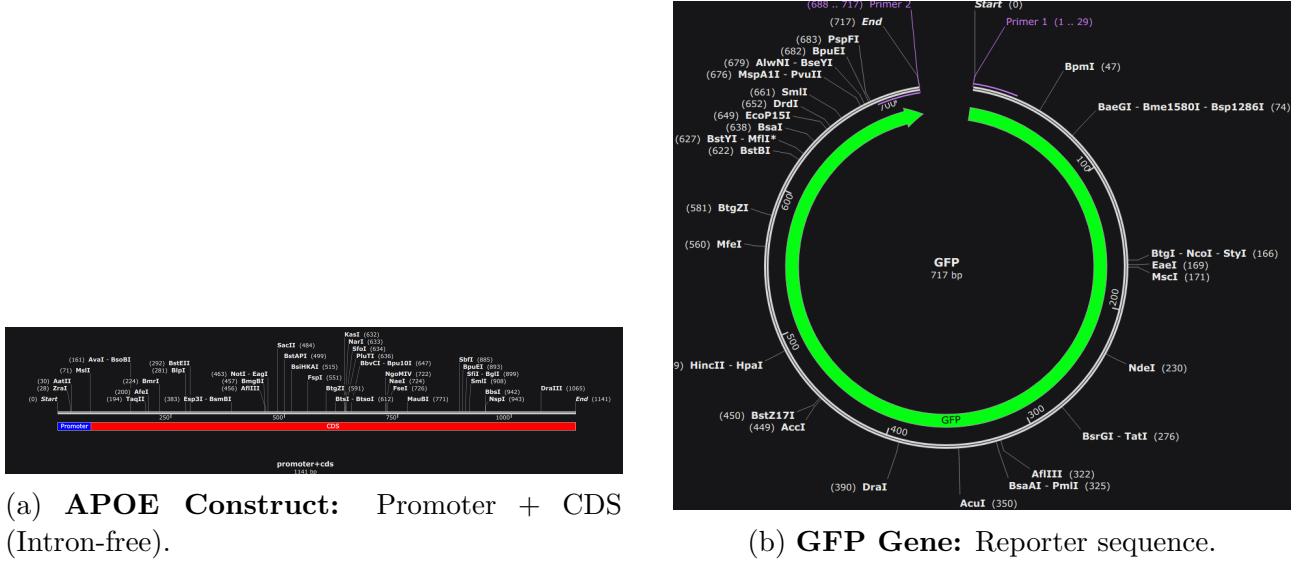


Figure 1: Maps of the Insert DNA sequences used for cloning.

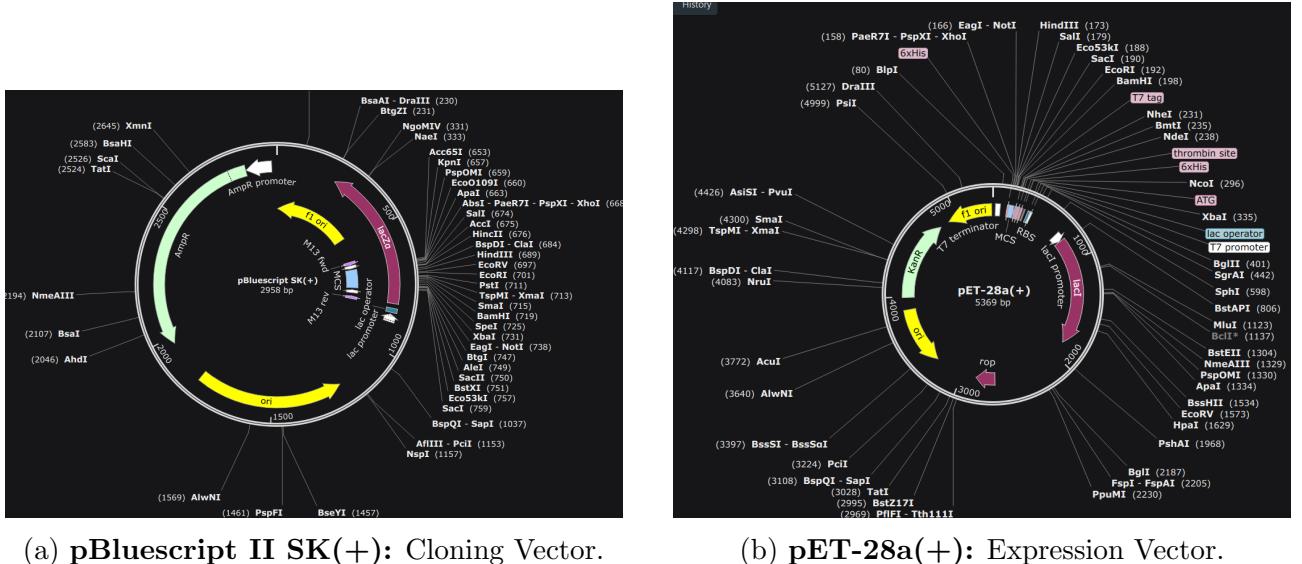


Figure 2: Maps of the Cloning and Expression Vectors.

4 Task 2: Restriction Analysis and Enzyme Selection

To clone the APOE gene and GFP into the vectors, a rigorous restriction site analysis was performed. The goal was to identify enzymes that are:

1. **Unique Cutters (1 cut)** in the Vector's Multiple Cloning Site (MCS).
2. **Zero Cutters (0 cuts)** in the Gene of Interest (APOE + Promoter) and Reporter (GFP).

4.1 Restriction Site Screening

Using SnapGene and NEBcutter tools, we screened common restriction enzymes against all four sequences. The results are summarized below.

Table 1: Comprehensive Restriction Analysis based on sequence scanning. (0 = No Cut, 1 = Unique Cut, >1 = Multiple Cuts)

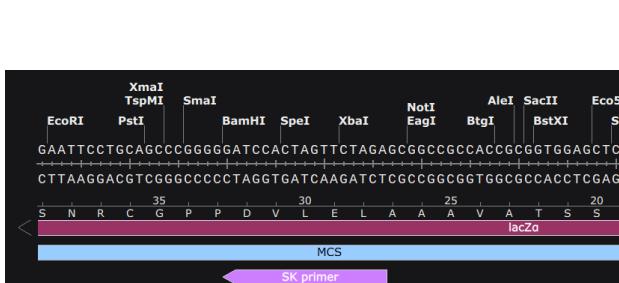
| Enzyme | Site | pBluescript Cuts | pET Vector Cuts | APOE Gene Cuts | GFP Gene Cuts | Cloning Suitability |
|---------|----------|------------------|-----------------|----------------|---------------|----------------------------|
| BamHI | GGATCC | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| HindIII | AAGCTT | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| EcoRI | GAATTC | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| XhoI | CTCGAG | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| SalI | GTTCGAC | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| SacI | GAGCTC | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| XbaI | TCTAGA | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| ClaI | ATCGAT | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| SmaI | CCCGGG | 1 | 1 | 0 | 0 | EXCELLENT (Use this) |
| KpnI | GGTACC | 1 | 0 | 0 | 0 | Good for pBluescript Only |
| SpeI | ACTAGT | 1 | 0 | 0 | 0 | Good for pBluescript Only |
| NotI | GCGGCCGC | 1 | 1 | 1 | 0 | UNSAFE (Cuts APOE Gene) |
| PstI | CTGCAG | 1 | 0 | 5 | 0 | UNSAFE (Cuts APOE Gene) |
| NdeI | CATATG | 0 | 1 | 0 | 1 | UNSAFE (Cuts GFP Gene) |
| ApaI | GGGCC | 1 | 1 | 3 | 0 | UNSAFE (Cuts APOE Gene) |
| SphI | GCATGC | 0 | 1 | 0 | 0 | Unsuitable (No cut in pBS) |

4.2 Final Strategy: Selected Enzymes

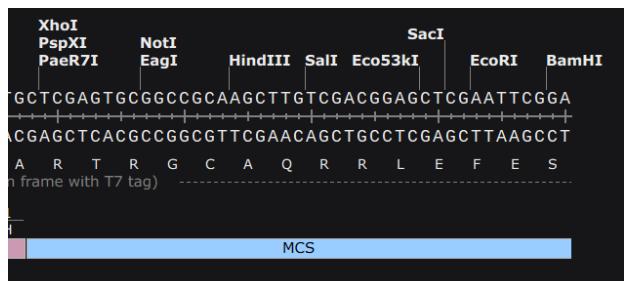
Based on the analysis in Table 1, we selected **BamHI** and **EcoRI** for the cloning strategy.

- **Why BamHI and EcoRI?**

- Both enzymes are absent in the APOE gene (including the promoter region) and the GFP gene, ensuring the inserts remain intact during digestion.
- Both enzymes cut exactly once in the MCS of pBluescript and pET-28a vectors.
- Using two different enzymes allows for **Directional Cloning**, preventing the gene from inserting in the reverse orientation and preventing the vector from self-ligating.



(a) **pBluescript II SK(+)**: Cloning Vector.



(b) **pET-28a(+)**: Expression Vector.

Figure 3: In-silico restriction map showing the unique BamHI and EcoRI sites in the pET-28a MCS, confirming their suitability for cloning.

5 Task 3: Primer Design and PCR Amplification

To amplify the gene of interest (APOE), its promoter, and the reporter gene (GFP) for cloning, specific PCR primers were designed. These primers incorporate the selected restriction sites (**BamHI** and **EcoRI**) to ensure directional cloning.

5.1 Methodology: In-Silico Primer Design & PCR

The primer design and amplification simulation were performed using SnapGene software as follows:

1. Designing Primers in SnapGene

- The sequence file (e.g., APOE mRNA or Promoter) was opened in SnapGene.
- The target region (CDS or specific promoter fragment) was selected.
- From the menu, **Primers** → **Add Primer** was selected.
- We chose "Top Strand" for Forward primers and "Bottom Strand" for Reverse primers.
- **Adding Restriction Sites:** In the insertion tab, the specific enzyme sequence (e.g., GAATTC for EcoRI) and a 3-bp clamp (GCA) were added to the 5' end.
- The software automatically calculated the Melting Temperature (T_m).

2. Simulating PCR

- After designing primers, we selected **Actions** → **PCR...**
- The specific Forward and Reverse primers were selected for each template.
- SnapGene generated a new "PCR Product" file, confirming the correct amplification size.

5.2 3A. Designed Primers

Primers were designed targeting the CDS of the genes (derived from mRNA) and the specific 73 bp core promoter region. Extra base pairs (GCA) were added at the 5' ends for efficient digestion.

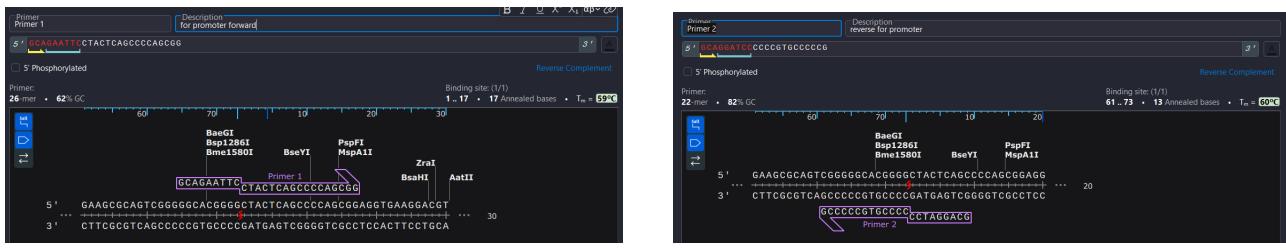
Table 2: List of PCR Primers designed for amplification with Melting Temperatures.

| Target | Primer Name | Sequence (5' → 3') | Enzyme Site | T_m (°C) |
|---------------------|----------------|--|-------------|------------|
| APOE CDS | rev_APOE_BamHI | GCA GGATCC TGAAACTTGGTGAATCTTATTAAACTA | BamHI | 58 |
| | For_APOE_EcoRI | GCA GAATTC ATGAAGGTTCTGTGGGCT | EcoRI | 56.0 |
| Promoter (73 bp) | Fwd_Prom_EcoRI | GCA GAATTC CTACTCAGCCCCAGCGG | EcoRI | 59.0 |
| | Rev_Prom_BamHI | GCA GGATCC CCCCGTGCCCCCG | BamHI | 60.0 |
| GFP CDS | Rev_GFP_BamHI | GCA GGATCC CTATTGTATAGTTCATCCATGCCATGTGT | BamHI | 59.5 |
| | For_GFP_EcoRI | GCA GAATTC ATGAGTAAAGGAGAAGAACTTTCACTGG | EcoRI | 59.0 |

5.3 3B. Visual Confirmation of Primer Binding

SnapGene snapshots confirming the binding location of primers on all three templates are shown below.

i. Promoter Primers (73 bp)

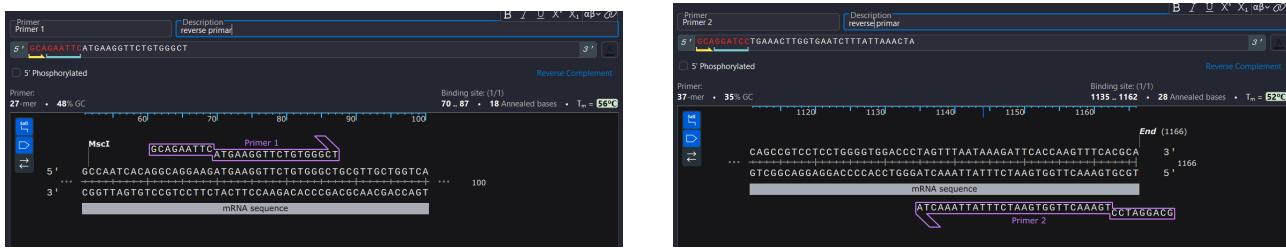


(a) Promoter Forward Primer

(b) Promoter Reverse Primer

Figure 4: Primers binding to the Promoter region.

ii. APOE CDS Primers

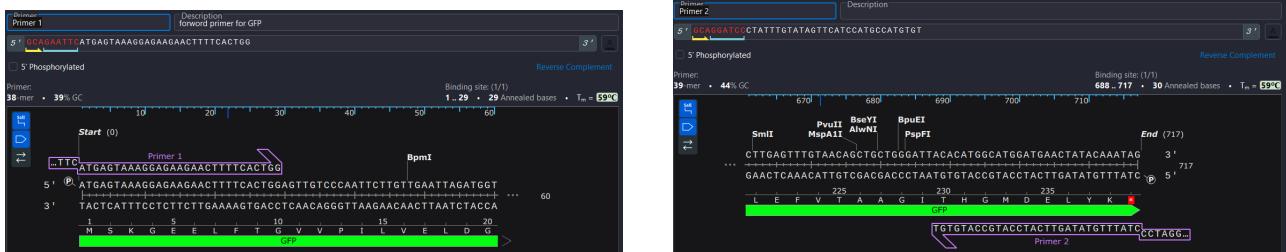


(a) APOE Forward Primer (at Start Codon)

(b) APOE Reverse Primer (at Stop Codon)

Figure 5: Primers binding to the APOE Coding Sequence.

iii. GFP Gene Primers



(a) GFP Forward Primer

(b) GFP Reverse Primer

Figure 6: Primers binding to the GFP Reporter Gene.

5.4 3C. PCR Amplification Programs

Specific PCR cycling programs were designed based on the fragment length.

1. PCR Program for APOE CDS (~1 kb)

(Based on lowest $T_m = 56^\circ\text{C}$)

| Step | Temperature | Time | Cycles |
|----------------------|-------------|--------------|--------|
| Initial Denaturation | 95°C | 5 min | 1 |
| Denaturation | 95°C | 30 sec | 3*30 |
| Annealing | 53°C | 30 sec | |
| Extension | 72°C | 1 min | |
| Final Extension | 72°C | 10 min | 1 |

2. PCR Program for Promoter Fragment (73 bp)

(Based on lowest $T_m = 59^\circ\text{C}$)

| Step | Temperature | Time | Cycles |
|----------------------|-------------|---------------|--------|
| Initial Denaturation | 95°C | 5 min | 1 |
| Denaturation | 95°C | 30 sec | 3*30 |
| Annealing | 55°C | 30 sec | |
| Extension | 72°C | 15 sec | |
| Final Extension | 72°C | 5 min | 1 |

3. PCR Program for GFP CDS (~720 bp)

(Based on lowest $T_m = 59^\circ\text{C}$)

| Step | Temperature | Time | Cycles |
|----------------------|-------------|---------------|--------|
| Initial Denaturation | 95°C | 5 min | 1 |
| Denaturation | 95°C | 30 sec | 3*30 |
| Annealing | 55°C | 30 sec | |
| Extension | 72°C | 45 sec | |
| Final Extension | 72°C | 10 min | 1 |

6 Task 4: Cloning Strategy in pBluescript Vector

In this step, we constructed three different plasmids using the pBluescript II SK(+) vector. The cloning was performed using the **Restriction Enzyme-based Directional Cloning** method using **EcoRI** and **BamHI**.

6.1 Introduction to pBluescript II SK(+) Vector

pBluescript II SK(+) is a widely used high-copy-number phagemid vector derived from pUC19. It is designed for gene cloning, sequencing, and in-vitro transcription. Its mechanism of action relies on three key features:

- **1. Selectable Marker (Ampicillin Resistance):** The plasmid contains the *bla* gene, which confers resistance to the antibiotic Ampicillin. When transformed into *E. coli*, only the cells containing the plasmid survive on Ampicillin-containing agar plates.
- **2. Blue-White Screening (*LacZα*):** The Multiple Cloning Site (MCS) is located within the coding region of the *lacZα* gene.

- **Without Insert:** The functional *lacZα* peptide complements the host cell, producing functional β-galactosidase. This turns the colonies **BLUE** in the presence of X-Gal and IPTG.
- **With Insert (Cloning Success):** When the APOE gene is inserted into the MCS, it disrupts the *lacZα* reading frame (Insertional Inactivation). No functional enzyme is produced, resulting in **WHITE** colonies. This allows for easy visual identification of recombinant clones.
- **3. T3 and T7 Promoters:** The MCS is flanked by bacteriophage T3 and T7 promoters. These allow for the in-vitro transcription of RNA from the inserted gene and facilitate DNA sequencing from both ends.

6.2 Methodology: In-Silico Cloning in SnapGene

The cloning simulation was executed using the "Restriction & Insertion Cloning" tool in SnapGene. The stepwise procedure is described below:

Step 1: Preparation of Insert via PCR

- First, primers carrying **EcoRI** (Forward) and **BamHI** (Reverse) sites were designed.
- A PCR simulation was run on the mRNA/CDS template to generate a linear "**PCR Product**" with sticky ends.

Step 2: Restriction Cloning Interface

We navigated to **Actions → Restriction & Insertion Cloning → Insert Fragment**. This opened a window with three critical tabs, which were configured as follows:

1. **Vector Tab:** We selected the **pBluescript II SK(+) file**. We chose **EcoRI** and **BamHI** to linearize the vector within the Multiple Cloning Site (MCS).
2. **Fragment Tab:** We selected the PCR Product generated in Step 1. The same enzymes (**EcoRI** and **BamHI**) were selected to digest the ends of the insert.
3. **Product Tab:** This tab displayed the final ligated construct. We verified that the insert (CDS/GFP) was in the correct orientation and the reading frame was intact.
4. Finally, we clicked "**Clone**" to generate the circular plasmid map.

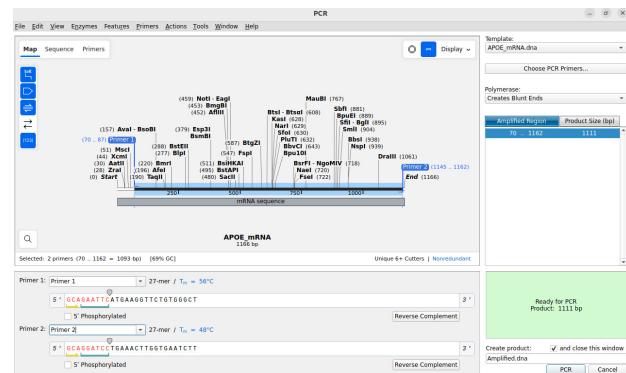
6.3 4A. Cloning APOE CDS in pBS

Objective: To clone the APOE Coding Sequence (derived from mRNA) into pBluescript.

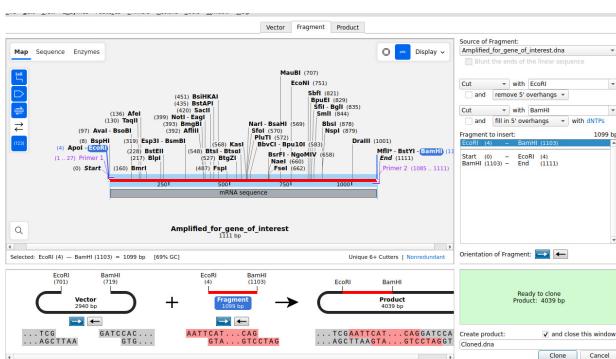
- **Insert Source:** APOE mRNA (NM_000041), amplified from Start Codon (ATG).
- **Enzymes:** **EcoRI** (5' end) and **BamHI** (3' end).



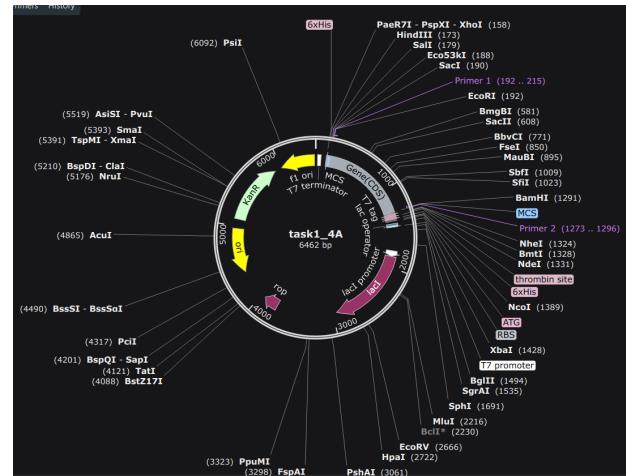
(a) Primer Binding on mRNA (ATG)



(b) Simulated PCR Product (EcoRI-BamHI)



(c) Cloning Window (Product Tab)



(d) Final Plasmid Map 1 (pBS-APOE)

Figure 7: Workflow for cloning the APOE CDS into pBluescript.

6.4 4B. Cloning GFP under APOE Promoter in pBS

Objective: To place the GFP reporter gene under the control of the APOE promoter.

- **Insert:** Promoter (73 bp) fused to GFP.
- **Strategy:** The complete cassette [Promoter-GFP] is flanked by **EcoRI** and **BamHI**.

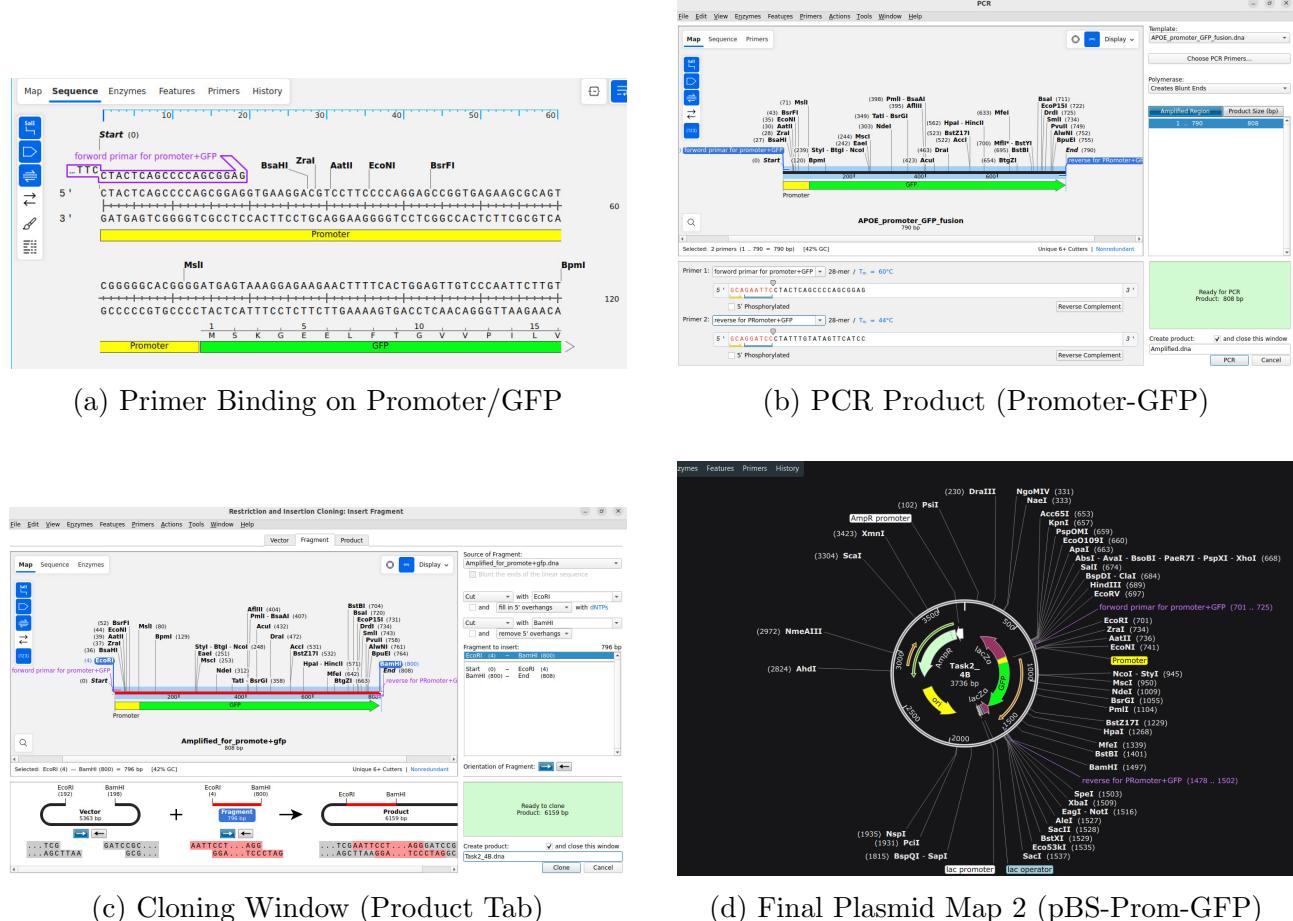


Figure 8: Workflow for cloning GFP driven by the APOE Promoter.

6.5 4C. Cloning Fusion Gene (APOE + GFP) in pBS

Objective: To create a fusion protein (APOE-GFP) where the C-terminus of APOE is linked to the N-terminus of GFP.

- **Insert:** APOE CDS fused to GFP CDS.
 - **Enzymes:** **EcoRI** (Start of APOE) and **BamHI** (End of GFP).

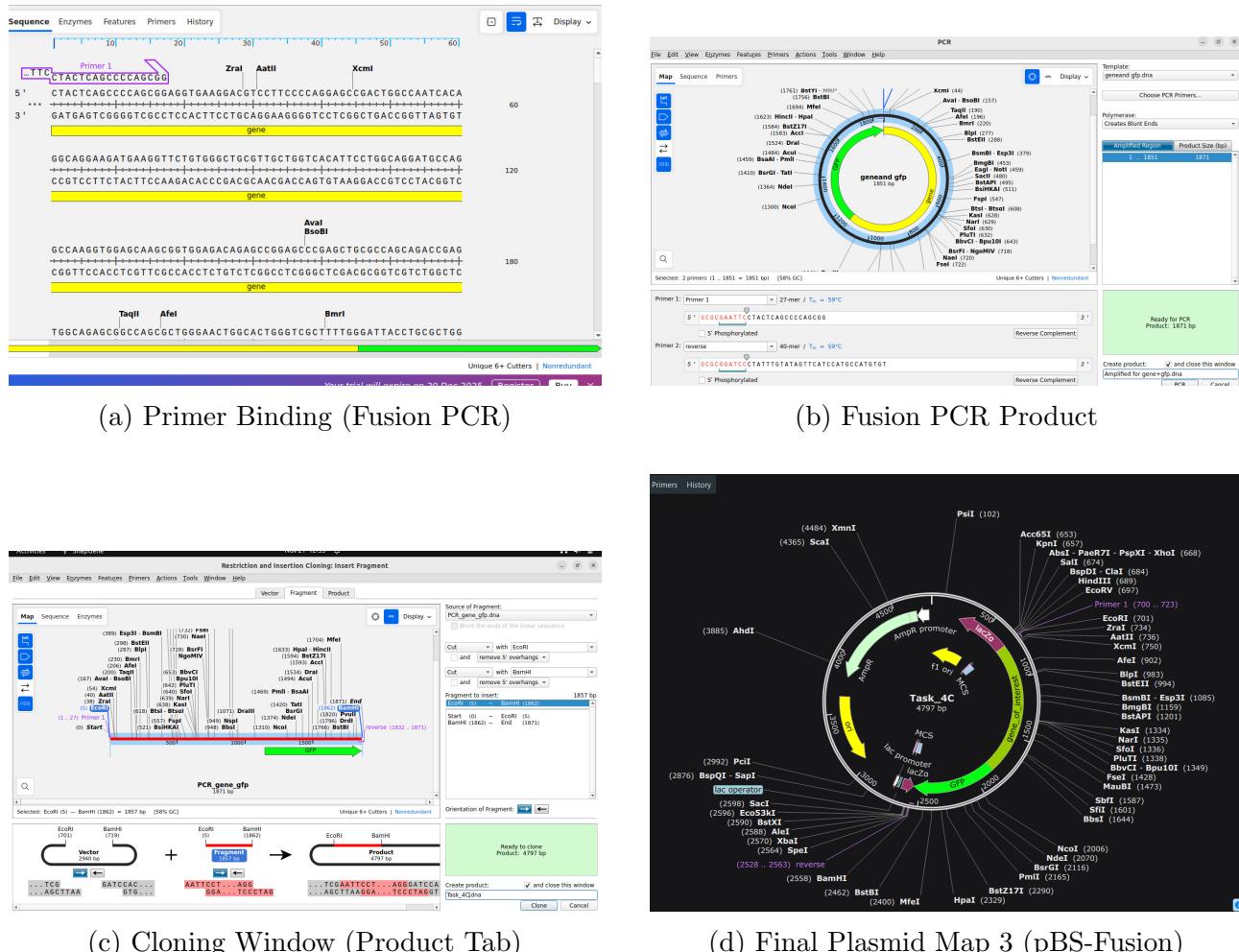


Figure 9: Construction of the APOE-GFP Fusion Plasmid.

7 Task 5: Cloning in pET Expression Vector

After successful cloning in the maintenance vector (pBluescript), the final step is to transfer the gene into an **Expression Vector (pET-28a)** to produce the protein.

7.1 Introduction to pET-28a System

The pET System is the most powerful system for the cloning and expression of recombinant proteins in *E. coli*.

- **T7 Promoter:** It contains a strong bacteriophage T7 promoter, which drives very high levels of transcription.

- **Expression Control:** It is regulated by the *lac* operator, allowing expression only when induced by IPTG.
- **Significance:** This step is crucial to physically manufacture the APOE protein or GFP fusion for functional studies.

7.2 Strategic Change: Re-visiting Restriction Sites

A critical analysis of the pET-28a Multiple Cloning Site (MCS) revealed a difference in enzyme order compared to pBluescript.

- **Observation:** In the pET-28a MCS, following the T7 promoter direction ($5' \rightarrow 3'$), the **BamHI** site appears *before* the **EcoRI** site.
- **The Logic:**
 - In Task 4 (pBS), we used EcoRI (5') and BamHI (3').
 - If we use the same insert for pET, the gene would be inserted in the **Reverse Orientation** (Antisense) relative to the T7 promoter.
- **Solution (Primer Redesign):** To ensure correct Directional Cloning in pET, we swapped the restriction sites on the primers:
 - **Forward Primer (5'):** Now carries the **BamHI** site.
 - **Reverse Primer (3'):** Now carries the **EcoRI** site.

7.3 New Primer Specifications for pET Cloning

Table 3: Redesigned Primers for pET-28a cloning (Enzymes Swapped).

| Target | Primer Name | Enzyme Site added |
|-----------------|----------------|-----------------------|
| APOE CDS | Fwd_APOE_BamHI | BamHI (5' End) |
| | Rev_APOE_EcoRI | EcoRI (3' End) |
| GFP CDS | Fwd_GFP_BamHI | BamHI (5' End) |
| | Rev_GFP_EcoRI | EcoRI (3' End) |

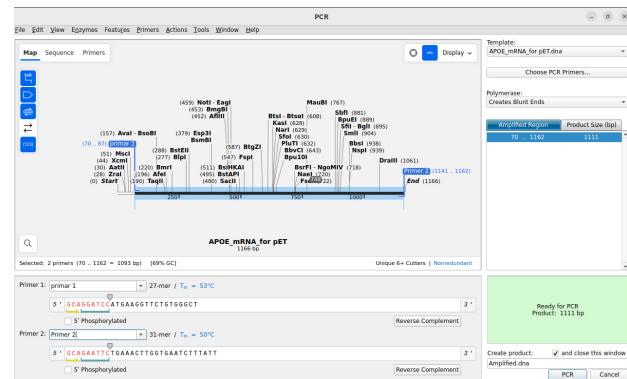
7.4 5A. Cloning APOE Gene into pET-28a

Objective: To express the APOE protein using the T7 promoter system.

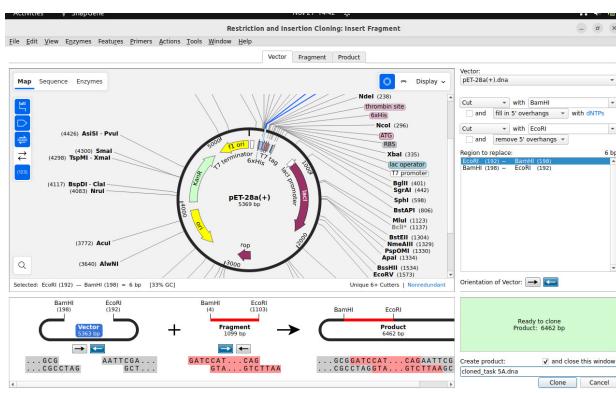
- **Insert:** APOE CDS (Amplified with BamHI-Fwd and EcoRI-Rev).
 - **Direction:** 5'-BamHI → APOE → EcoRI-3'.



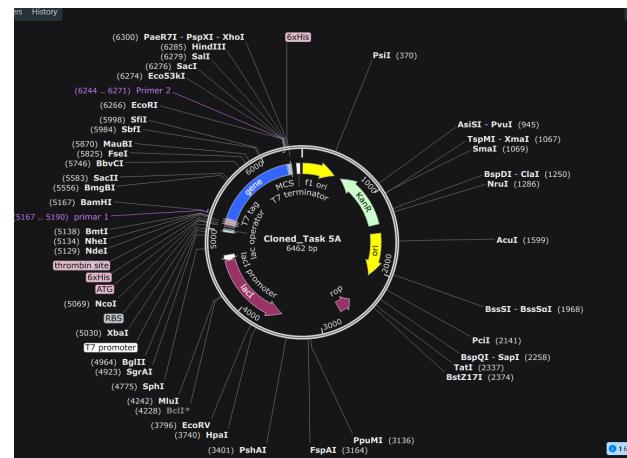
(a) New Primer Binding (BamHI at 5')



(b) Amplified Sequence View (SnapGene)



(c) Cloning Window (Direction Check)



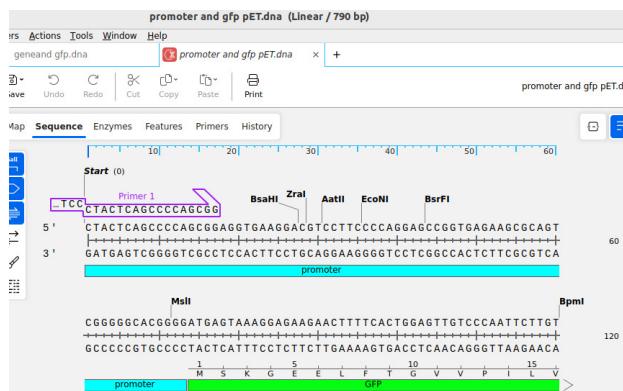
(d) Final Plasmid Map 4 (pET-APOE)

Figure 10: Workflow for cloning APOE into pET-28a with corrected orientation.

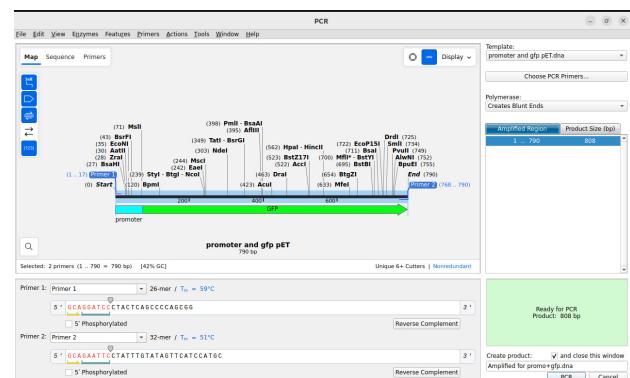
7.5 5B. Cloning Promoter+GFP into pET-28a

Objective: To insert the [APOE Promoter-GFP] cassette into pET. (*Note: Although pET has a T7 promoter, this construct allows testing if the eukaryotic promoter works in alternate hosts or as a control.*)

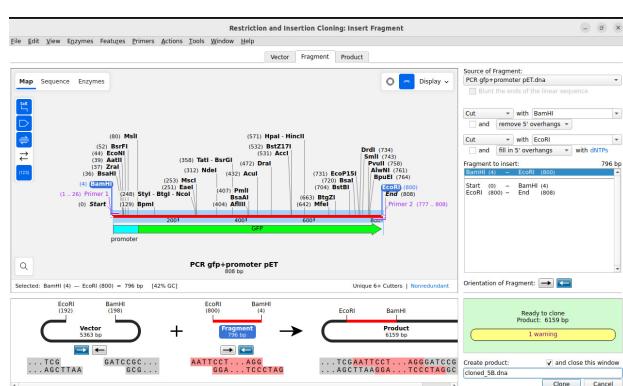
- **Insert:** 73 bp Promoter fused to GFP.
 - **Enzymes:** BamHI (5') and EcoRI (3').



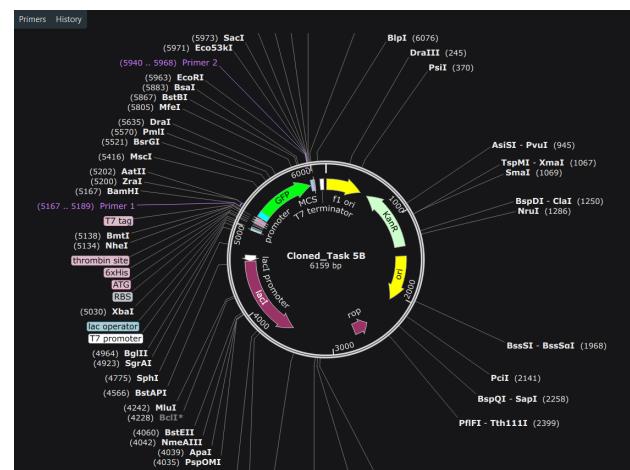
(a) Primer Binding on Cassette



(b) Amplified Sequence View



(c) Cloning Window (Product Tab)



(d) Final Plasmid Map 5 (pET-Prom-GFP)

Figure 11: Cloning the Reporter Cassette into pET Vector.

7.6 5C. Cloning Fusion Gene (APOE+GFP) into pET-28a

Objective: To express the APOE-GFP fusion protein.

- **Insert:** APOE CDS linked to GFP CDS.
 - **Strategy:** The fusion product is flanked by BamHI (Start) and EcoRI (End).

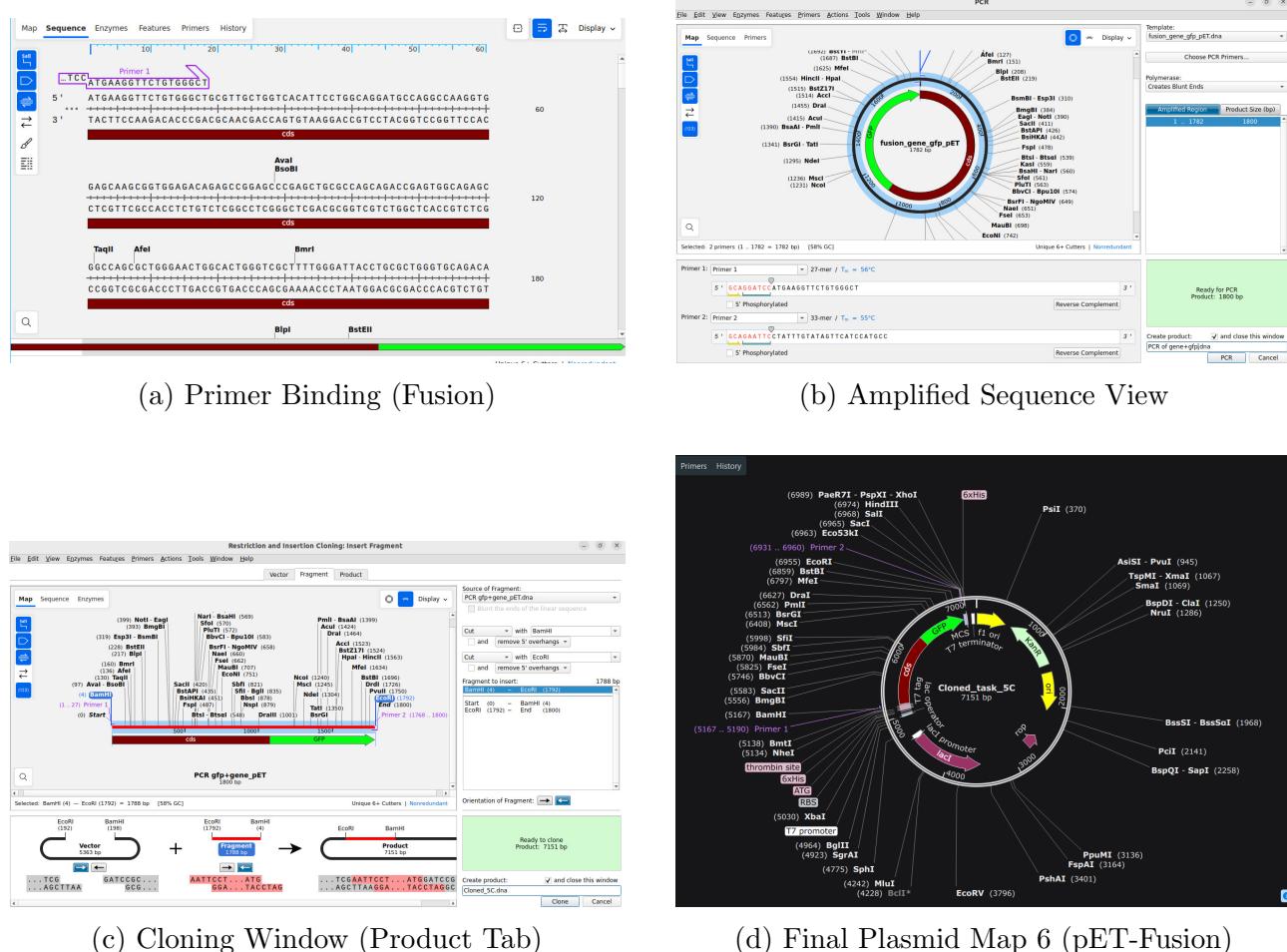


Figure 12: Construction of the Expression-ready Fusion Plasmid.