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# *The Impact of Rational Primer Design on PCR Experimental Outcomes*

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***Abstract:*** Precision cancer modeling relies on high-fidelity PCR technology for validating genetically engineered animal models. However, suboptimal primer design frequently causes non-specific amplification and reduced detection sensitivity. In this study, we employed traditional primer design principles to develop target-specific primers for validating Smad4 and Cre gene knockout in a colorectal cancer mouse model. Through optimized PCR amplification and DNA gel electrophoresis, we achieved high specificity in genotyping with clear band separation and confirmed tissue-specific recombinase activity. These results demonstrate that rational primer design ensures reliable amplification efficiency and minimizes off-target effects. Furthermore, we discuss the limitations of conventional design strategies in complex genomic contexts and propose computational approaches for enhancing primer specificity in future studies.

## 1. Introduction

Cancer remains one of the leading causes of global mortality, with its pathogenesis involving a complex network of biological processes, including gene mutations, epigenetic modifications, and tumor microenvironment regulation [1]. To unravel these mechanisms and develop effective therapeutic strategies, researchers rely heavily on high-fidelity and reproducible animal models. In recent years, advancements in gene-editing technologies such as base editing and prime editing have revolutionized the construction of tumor models [2, 3]. By precisely knocking out tumor suppressor genes or activating proto-oncogenes, these models successfully recapitulate the genetic heterogeneity and microenvironmental features of human tumors. However, the reliability of these models—including phenotypic stability and experimental reproducibility—depends largely on the accuracy of downstream molecular biology techniques, among which polymerase chain reaction (PCR) stands as a cornerstone.

PCR is indispensable in molecular biology, enabling the amplification of specific DNA sequences for applications ranging from gene expression analysis to mutation detection. Its performance is fundamentally determined by primer design. Primers—short oligonucleotides that initiate DNA synthesis—dictate the specificity, sensitivity, and efficiency of amplification. Suboptimal primer design can lead to non-specific amplification, primer dimers, or failed reactions, undermining the

validity of experimental results. Therefore, rational primer design is not just a technical step but a key determinant of PCR success, especially in complex scenarios such as cancer research where detection of rare mutations or analysis of multiple genomic loci is required [4].

The effectiveness of primers is influenced by a multitude of parameters. GC content, for instance, affects thermodynamic stability: maintaining GC content between 45% and 55% balances hybridization efficiency and reduces non-specific binding, with a ΔG of -20.9 kcal/mol at 25℃ for 55% GC content [2]. Primer length (typically 18–25 bp) correlates linearly with melting temperature (Tm), allowing precise control of ΔG (<-3 kcal/mol) and suppressing stem-loop formation by 42% in multiplex PCR systems [5]. Annealing Tm, too, must be optimized to align with primer Tm, as mismatches between primers and templates can drastically reduce amplification specificity. Shen et al. constructed a PCR Primer Specificity Checking system and found that when the primer mismatch rate exceeds 15%, the risk of non-specific amplification increases threefold, highlighting the critical role of sequence complementarity [1].

This study systematically validated the genotype of mice harboring knockout genes associated with colorectal cancer by rationally designing primers and combining PCR amplification with DNA gel electrophoresis analysis to assess the effectiveness of target gene knockout and the reliability of the experimental method. Furthermore, we discussed the limitations of traditional primer design in complex experimental environments and the potential advantages of novel design strategies in improving amplification specificity and detection accuracy, providing feasibility analysis for subsequent application in similar genotype validation experiments.

## 2. Materials and Methods

### 2.1 Primer Design

Primers were designed based on the target and reference gene sequences obtained from the NCBI database. Primer3 software was used to generate candidate primer pairs, adhering to general design guidelines: primer length was controlled between 18–25 nucleotides, GC content was maintained at 45%–55%, melting Tm range was 55–65 ℃, Tm difference between forward and reverse primers was less than 2 ℃, and self-complementary sequences were minimized to avoid hairpin formation and primer dimers. The specificity of each primer pair was verified across the mouse genome (Mus musculus) using the NCBI Primer-BLAST tool to ensure single target amplification.

### 2.2 DNA extraction

#### 2.2.1 Experimental materials

1) Experimental Instruments: EP tubes, test tubes, metal bath heaters, heat-resistant gloves, tweezers, pipettes

2) Experimental Reagents: $H_2O$, 2 primers, extracted DNA reagents , Dissolve 5 g of NaOH crystals, 150 μL of 5% NaOH solution

#### 2.2.2 Experimental step

1) Preparation of tissue lysis buffer: We dissolve 5 g of NaOH crystals and 0.12 g of EDTA in 400 mL of distilled water to prepare 5× alkaline lysis buffer A. Separately, we dissolve 9.69 g of Tris powder in 400 mL of distilled water and adjust the pH to 5.5 with dilute hydrochloric acid to prepare 5× neutralization buffer B. Before use, we dilute both solutions A and B 5-fold with distilled water.

2) First, we carefully remove the labeled mouse from its cage using gentle handling to minimize stress. Then, we cleanly excise the tail tip (approximately 0.5–1 cm) using a sterile scalpel or sharp

scissors and immediately place the excised tissue into a pre-labeled 1.5 mL EP tube. Each tube is pre-filled with 150 μL of 5% NaOH solution, an alkaline lysis buffer that effectively lyses the tail tissue and releases DNA. Tubes are then clearly numbered to ensure sample identification throughout the process.

3) The EP tubes containing the mouse tail tissue and NaOH solution were carefully transferred and placed into a preheated metal bath heater set to the appropriate Tm (typically 95-100 ℃). The samples were incubated for a specified duration (usually 30-60 minutes) to allow complete tissue digestion and DNA release through alkaline lysis.

4) After the incubation period, we carefully remove the EP tube from the metal bath using heat-resistant gloves or forceps to avoid burns. We allow the tube to cool slightly before adding an equal volume of 5× Neutralizing Solution B (pH 5.5) to neutralize the alkaline solution, or proceed with DNA precipitation according to the subsequent protocol.

## 2.3 PCR

### 2.3.1 Experimental materials

1) Experimental instruments: Centrifuges, pipettes, PCR tubes, single-row test tubes, EP tubes

2) Experimental reagents: DNA labeling solution, primer-FW, primer-RV, anhydrous ethanol, $ddH_2O$

### 2.3.2 Experimental steps

1) We calculate the amount of each component in the PCR reaction mixture according to a certain ratio and record them in order (Table 1).

Table 1 Comparison of PCR Reaction Systems for NSCL2 and LGR5Cre

| Component | PCR System (μL) |
|---|---|
| Nuclease-free water | 6.0 |
| Forward primer | 0.5 |
| Reverse primer | 0.5 |
| Taq premix | 7.5 |
| Mouse DNA template | 0.5 |
| Total volume | 15 |

2) We place the EP tube into the centrifuge and centrifuge at a speed of 12000 rpm for 2 seconds.

3) We use a pipette to transfer the centrifuged mixture into a single row of tubes for PCR and number them sequentially.

4) We add 2 μL of gDNA sample to the corresponding PCR tube; add 2 μL of nuclease-free water ($ddH_2O$) to the negative/blank control tube.

5) The single-row tubes were placed in a PCR instrument for amplification reactions, including initial denaturation, denaturation, annealing, and extension cycles, with approximately 30–35 cycles and a total reaction time of approximately 2 h.

## 2.4 DNA Gel Electrophoresis

### 2.4.1 Experimental materials

1) Experimental instruments: Single row tubes, centrifuges, electronic balances, weighing paper, conical flasks, graduated cylinders, microwave ovens, combs, plastic boxes, electrophoresis tanks, PCR instruments, pipettes, gel imagers

2) Experimental reagents: Agarose, nucleic acid dye, DNA labeling solution, TAE buffer

### 2.4.2 Experimental steps

1) We check whether the single-row tube is sealed, balance the liquid level, and place it in a centrifuge for centrifugation at 4 ℃, 12,000 rpm, and 15 minutes.

2) To make the gel, we first take out the electronic balance and turn it on. Take out a piece of weighing paper, place it on the scale and peel it. Open the agarose jar and use a medicine spoon to take 2.0 g of agarose powder onto the weighing paper.

3) We transfer the powder to an Erlenmeyer flask, use a graduated cylinder to measure 100 mL of 1 × TAE buffer, add it to the Erlenmeyer flask, and shake until evenly distributed.

4) We place the Erlenmeyer flask in a microwave and heat until the liquid boils. We shake well and repeat 2–3 times. We observe the solution change from turbid to clear. We take it out and rinse the bottle with cold water until the liquid has tiny bubbles. We add an appropriate amount of nucleic acid dye and shake well. We pour the dissolved solution into a plastic box with a comb inserted and wait for 20 minutes until the gel solidifies.

5) We gently place the gel into the electrophoresis tank, add enough 1 × TAE buffer to completely submerge the gel, set the voltage to 120 V, and run for approximately 40 minutes.

6) We remove the gel and pipette the DNA labeling solution into the sample wells, leaving empty wells between samples.

7) We take out the single-row tube from the PCR instrument and transfer the product into the sample well using a pipette, making sure the number corresponds to the sample position.

8) We close the electrophoresis tank lid, turn on the power, maintain constant voltage conditions (5–10 V/cm, typically 120 V), and run for approximately 40 minutes.

9) After electrophoresis, the gel was removed and placed in a gel imaging system for imaging. The original image was saved and the results were analyzed to complete the genotype PCR identification.

## 3. Result

### 3.1 Primer Design and Specificity Verification

To verify the effectiveness of Smad4 and Cre gene knockout in a colorectal cancer model, primer design strictly adheres to parameter standards. The core design criteria include: controlling the primer GC content within 45–55% (actual values: 50% for Smad4 - F, 55% for Smad4 - R; 48% for villin - F, 52% for cre - R), maintaining the melting Tm within 55–65 ℃ (60 ±1 ℃ for the Smad4 primer pair, 58 ℃ for the Cre primer pair), and ensuring 3' - end stability (strictly avoiding self - complementary sequences of > 3 bp). Specificity verification was performed on the Mus musculus genome via NCBI Primer - BLAST, confirming that all primers have no off - target binding sites. The relevant primer sequences and characteristics are detailed in the Table 2:

Table 2 Primer Information

| Primer Name | Sequence (5'→3') | Length (bp) | GC (%) | Tm (℃) | Target Band Size |
|---|---|---|---|---|---|
| Smad4-F | GGGCAGCGTAGCATA TAAGA | 20 | 50 | 59.2 | WT: 400 bp |
| Smad4-R | GACCCAAACGTCAC CTTCTAC | 20 | 55 | 60.1 | Mut: 500 bp |
| villin-F | GTGTTTGGTTTGGTTCCTCTGCA TAAGA | 29 | 48 | 63.5 | ~300 bp |
| cre-R | GCAGGCAAATTTGGTGTACCGTCA | 25 | 52 | 58.8 | ~300 bp |

### 3.2 DNA Gel Electrophoresis Analysis

To ensure that the experimental mice possessed the desired conditional knockout genotype, we performed PCR typing of the Smad4 and Cre genes in the resulting offspring. Accurate identification of the Smad4 gene is crucial for distinguishing wild-type (+/+), heterozygous (flox/+), and homozygous flox (flox/flox). Cre gene testing confirms whether the individual carries the tissue-specific recombinase, thereby determining whether Smad4 is knocked out in the target tissue.

We extracted genomic DNA from mouse tail biopsies and performed PCR amplification using Smad4 typing primers and Cre-specific primers (Table 2). For Smad4 identification, genotype was determined by the presence of a 400 bp (wild-type) and 500 bp (flox-type) band on electrophoresis. For Cre detection, Cre gene expression was determined by the presence of a ~300 bp band (villin-Cre amplification product). Amplified products were separated on a 1.5% agarose gel and compared to the expected bands.

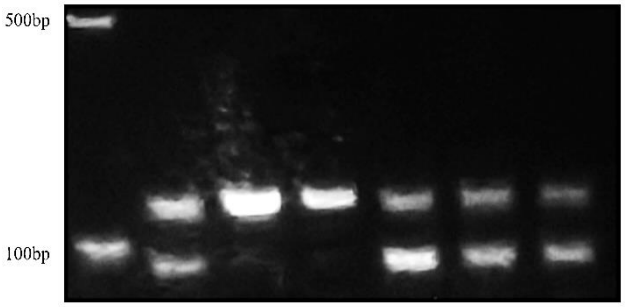


Figure 1 Genotyping of Smad floxed by DNA Gel Electrophoresis.

Figure 1 presents the electrophoresis results of Smad4 genotyping across 6 lanes, with the DNA ladder markers (500 bp, 250 bp, 100 bp) on the left as molecular weight references. Lane 1, with the x/flox (heterozygote) genotype, shows a clear target band; Lane 2 and Lane 3, both with the flox/flox (homozygote) genotype, display a single, distinct 250 bp target band at a position consistent with Lane 1 (with Lane 3's band characteristics matching Lane 2 exactly, verifying the stability of the homozygote genotype); and Lanes 4–6, all having the x/flox (heterozygote) genotype, each show a 250 bp target band identical to that of Lane 1, indicating the consistency of the heterozygote genotype. Since the target bands all lie at 250 bp, matching the expected amplification product size from primer design, and the band patterns of homozygotes (flox/flox) and heterozygotes (x/flox) differ significantly, effective differentiation between the genotypes is achievable.

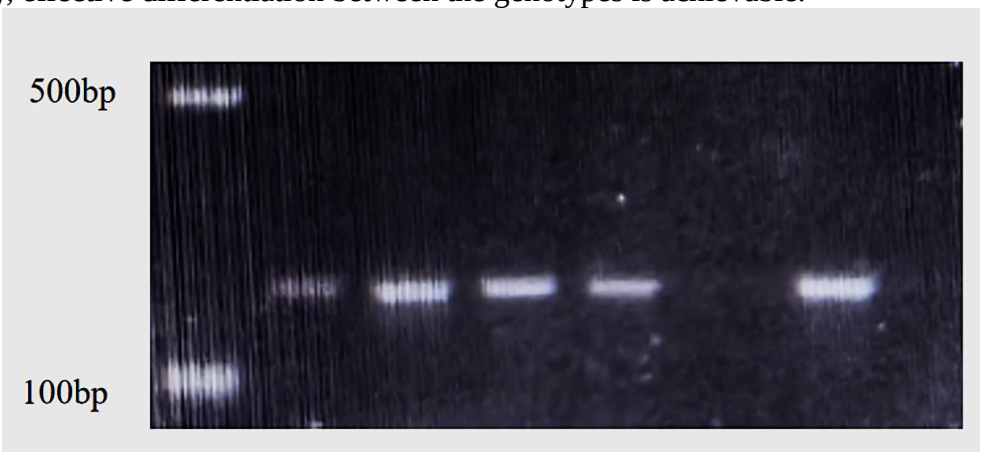


Figure 2 Genotyping of Cre by DNA Gel Electrophoresis.

Figure 2 illustrates the detection of the Cre transgene. In the electrophoretic gel image, lanes 1–4 and lane 6 (Cre+ samples) each show a distinct 250 bp specific band, indicating functional recombinase expression in these samples; lane 5 (Cre- control sample), however, lacks this band, confirming the specificity of Cre gene expression. All samples exhibit sharp, well-resolved bands with uniform fluorescence intensity, demonstrating high-quality DNA templates and efficient PCR amplification, which provides a reliable experimental foundation for subsequent genetic analyses.

These results collectively validate a robust experimental framework for colorectal cancer research. Figure 1 demonstrates successful generation of Smad4flox/flox homozygotes, with distinct electrophoretic patterns confirming precise loxP site integration and absence of off-target amplification, essential for reliable tissue-specific gene knockout. Figure 2 shows Cre transgene expression in sample 1-4 and 6 mice, verified by specific 250 bp bands, ensuring spatiotemporal control over Smad4 deletion via the Cre-loxP system. The clarity of bands and lack of smearing across all lanes reflect high-quality PCR and DNA templates, underpinning result reliability. Together, these findings establish validated genetic models enabling precise manipulation of Smad4 function, providing a critical tool for dissecting its role in colorectal tumorigenesis, particularly in cell-type-specific deletion studies.

## 4. Conclusion

This study systematically validated the genotype of colorectal cancer-related gene knockout mice through rational primer design, PCR amplification, and DNA gel electrophoresis, aiming to assess the effectiveness of target gene knockout and the reliability of the experimental methods employed. The findings not only confirm the feasibility of the established protocol but also highlight critical considerations for optimizing molecular assays in complex genetic contexts, particularly in cancer research.

First, the rational design of primers proved foundational to the success of PCR amplification. Guided by standard criteria—including primer length (18–25 bp), GC content (45%–55%), melting Tm, (55–65 ℃), and minimal self-complementarity—primers generated using Primer3 software and validated via NCBI Primer-BLAST exhibited high specificity for the target and reference genes in the mouse genome (Mus musculus). This specificity was further corroborated by PCR results, where amplification products of expected sizes were consistently obtained for both NSCL2 and LGR5Cre genes, with no evidence of non-specific bands or primer dimers in gel electrophoresis. The absence of such artifacts indicates that the primers effectively initiated DNA synthesis at the intended loci, supporting the validity of the genotype validation process. These results align with prior research emphasizing that adherence to primer design guidelines directly enhances PCR sensitivity and reproducibility, particularly in detecting rare or low-abundance sequences critical for cancer model validation [4,5].

Second, the integrated workflow—encompassing DNA extraction, PCR, and gel electrophoresis—demonstrated robustness and reproducibility. DNA extraction from mouse tail tissue using alkaline lysis (5% NaOH) followed by neutralization (Tris-HCl, pH 5.5) efficiently released genomic DNA, as evidenced by successful PCR amplification. The standardized PCR reaction system (15 μL total volume) with consistent ratios of nuclease-free water, primers, Taq premix, and DNA template yielded reliable amplification across multiple samples. Notably, the inclusion of negative controls (nuclease-free water) confirmed the absence of contamination, further validating the integrity of the experimental setup. Subsequent DNA gel electrophoresis, conducted using 2% agarose gels in 1× TAE buffer with nucleic acid dye, clearly resolved PCR products, allowing for straightforward genotype identification. The uniformity of band intensity and migration patterns across replicate samples underscored the reproducibility of the method, a key requirement for preclinical research

where consistent results are essential for translating findings to therapeutic development.

However, despite the overall success of the protocol, this study also highlighted limitations inherent to traditional primer design strategies in complex genetic environments. Traditional approaches, while effective for simple loci, often struggle with regions of high sequence homology, repetitive elements, or epigenetic modifications—common features in cancer-related genes. For instance, colorectal cancer-associated genes such as NSCL2 and LGR5Cre may reside in genomic regions with pseudogenes or paralogous sequences, increasing the risk of cross-amplification even with primers that meet standard design criteria. Although Primer-BLAST validation mitigated this risk in the current study, broader application to more genetically diverse or poorly annotated loci could compromise specificity. Additionally, traditional design does not account for dynamic factors such as secondary structure formation during amplification or variations in DNA methylation, which can alter primer binding efficiency in tumor models characterized by epigenetic dysregulation. These limitations align with observations by Shen et al., who noted that primer mismatch rates exceeding 15% drastically increase non-specific amplification risk, a challenge exacerbated in complex genomes [1].

To address these limitations, emerging primer design strategies integrating bioinformatic advances and machine learning offer promising solutions. For example, machine learning models trained on large datasets of PCR outcomes can predict primer performance by accounting for contextual factors such as genomic neighborhood, epigenetic marks, and thermodynamic stability under varying reaction conditions [6]. These models outperform traditional rules-based design by identifying subtle sequence features that influence specificity, such as hidden complementarity or context-dependent Tm shifts. Another innovation is the use of multiplex primer design algorithms that optimize primer sets for concurrent amplification of multiple loci, reducing cross-reactivity through iterative refinement of primer-pair interactions [7]. Such approaches are particularly valuable in cancer research, where simultaneous detection of multiple mutations or gene expression signatures is often required.

The principles underlying these novel strategies revolve around a shift from static criteria to dynamic, context-aware optimization. Traditional methods rely on fixed parameters (e.g., GC content ranges), whereas machine learning models leverage statistical patterns to weight factors based on their actual impact on amplification success. For instance, a model might prioritize avoiding complementarity with known pseudogenes over strict adherence to a Tm range if the former more strongly correlates with specificity in a given genomic region. Multiplex algorithms, meanwhile, use thermodynamic simulations to predict and minimize primer-primer interactions, ensuring efficient amplification of all targets in a single reaction.

Despite their potential, these advanced strategies have limitations. They require large, high-quality training datasets, which may not exist for poorly studied genes or non-model organisms. Additionally, the computational complexity of machine learning models can hinder accessibility for researchers without bioinformatic expertise, potentially limiting widespread adoption. There is also a risk of overfitting to specific experimental conditions, reducing generalizability across different PCR protocols or sample types.

Looking forward, several avenues for progress are evident. First, expanding training datasets for machine learning models to include diverse genomic contexts—including cancer-specific genetic and epigenetic features—will enhance their predictive power for tumor-related loci. Second, developing user-friendly tools that democratize access to advanced design algorithms will enable broader implementation in laboratories with limited computational resources. Third, integrating real-time PCR data feedback loops into design pipelines could allow for adaptive optimization, where primer performance in initial experiments is used to refine subsequent designs. Finally, combining primer design with CRISPR-based validation—e.g., using guide RNAs to confirm target specificity—could

provide an orthogonal check for complex loci, ensuring that amplified products truly correspond to the intended gene.

In conclusion, this study demonstrates that rational primer design, combined with standardized PCR and gel electrophoresis, provides a reliable method for genotyping colorectal cancer-related gene knockout mice. The success of the protocol validates the effectiveness of traditional primer design for well-characterized loci but also underscores the need for advanced strategies in complex genetic environments. By embracing innovations in machine learning and bioinformatics, future research can overcome the limitations of traditional methods, enhancing the specificity, efficiency, and reproducibility of PCR-based assays in cancer model validation. Such advances will be critical for accelerating our understanding of tumor pathogenesis and developing more precise therapeutic strategies.

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