**Primer Designing\_P5**

1. **Introduction to Primer blast :** [**https://www.ncbi.nlm.nih.gov/tools/primer-blast/**](https://www.ncbi.nlm.nih.gov/tools/primer-blast/)
2. **Introduction to Oligo Analyzer :** [**https://www.idtdna.com/calc/analyzer**](https://www.idtdna.com/calc/analyzer)

**Primer design rules**

1. Primer length: **18–24 nt** (sweet spot ~20–22)
2. Tm (nearest-neighbor): **58–62 °C** (pair ΔTm ≤ 1–2 °C)
3. GC content: **40–60%**
4. 3′ GC clamp: **1–2 G/C in last 5 bases**, avoid >3 G/C at 3′ end
5. Homopolymers: avoid runs > **4** identical bases
6. Amplicon size:
   1. **Conventional PCR**: **350–800 bp** (visual gel)
   2. **qPCR**: **80–150 bp** (100–120 ideal)
7. Secondary structures (OligoAnalyzer):
   1. Hairpins/dimers with **ΔG more positive than −9 kcal/mol** are generally acceptable; avoid strong 3′-dimers (≤ −9 is risky)
8. Specificity: zero predicted off-target products with **≥2 perfect 3′-end matches**
9. Human/qPCR extras:
   1. Place primers across **exon–exon junction** (or separate exons with large intron) to avoid gDNA
   2. Avoid known **SNPs** within last 5 bases at 3’ end
10. **Download set of FASTA sequences for Insect COX gene and design a pair of primers to amplify it and fill the table below.**

| **Field** | **Forward Primer** | **Reverse Primer** |
| --- | --- | --- |
| Sequence (5′→3′) |  |  |
| Length (nt) |  |  |
| %GC |  |  |
| Tm (°C, NN) |  |  |
| 3′ clamp (Y/N) |  |  |
| Hairpin ΔG |  |  |
| Self-dimer ΔG |  |  |
| Hetero-dimer ΔG |  |  |
| Amplicon size (bp) |  |  |
| Off-targets (summary) |  |  |
| Exon junction (qPCR) |  |  |
| SNPs in last 5 bases (Y/N) |  |  |