sgRNA library screening is a powerful technique to identify genes or regulatory regions involved in a specific biological process. The amplification of the enriched sgRNAs, with an amplicon size of 150-300 nt and a target region of ~22 bp, is a crucial step in this process. Here's a revised guideline with comments specific to sgRNA library screening.

### sgRNA Library Screening PCR Amplicon Design (for reference only)

**1. Amplicon Target**

The target for amplification is the sgRNA sequence itself. The goal is to amplify only the integrated sgRNA cassettes from the genomic DNA of cells that have shown a phenotype (e.g., survival, resistance to a drug) during the screen.

Can try Primer3 software for help: https://primer3.org/

**2. Primer Design Considerations**

* **Amplicon Size:** The recommended size of **150-300 nt** is ideal for short-read sequencing platforms like Illumina. This length ensures efficient cluster generation and accurate sequencing. A shorter amplicon (**150-200 bp**) can sometimes provide better sequencing quality and is a good target if feasible.
* **Target Region (~22 bp):** This short region is the variable part of the sgRNA cassette that guides Cas9 to the genomic target. It's the most critical part to read accurately.
* **Location:** The **~22 bp target region should be located near the Read1 priming site** (the 5' end of the amplicon). This is a critical design choice for Illumina sequencing. Placing the variable region at the beginning of the read ensures the highest quality scores and reduces sequencing errors, as base quality typically degrades toward the end of a read. For SR100 run, this can fit anywhere within the Read1 sequence (i.e. First 100 bases).

**3. Primer Design (Revised Guideline)**

* **Primer 1 (Forward Primer):** This primer should bind to the constant region of the sgRNA cassette **upstream** of the variable ~22 bp target.
	+ **GC Clamp:** A GC clamp at the 3' end improves binding efficiency.
	+ **Nextera/Illumina Overhang:** This primer should contain the Read1 sequencing adapter (P5/N5) at its 5' end for the second PCR step.
* **Primer 2 (Reverse Primer):** This primer should bind to the constant region of the sgRNA cassette **downstream** of the variable ~22 bp target.
	+ **GC Clamp:** One GC clamp at the 3' end is also recommended. (avoid GC-stretch)
	+ **Nextera/Illumina Overhang:** This primer should contain the Read2 sequencing adapter (P7/N7) at its 5' end for the second PCR step. *(sequence provided at the end of the file)*

**4. Quality Control**

* **Melting Temperature (Tm​) Balance:** Both primers should have similar Tm​ values (within 5°C) for consistent and efficient amplification.
* **Avoid Secondary Structures:** Use software like OligoAnalyzer (IDT DNA) to check for hairpin loops, self-dimers, and hetero-dimers that could interfere with PCR efficiency. (https://eupages2.idtdna.com/pages/tools/oligoanalyzer)
* **Specificity:** Perform an in-silico PCR to ensure the primers amplify only the sgRNA cassette and not other off-target sequences in the genome.

**Commentary**

The goal of this design is to generate a sequencing library where the variable sgRNA sequence is the primary read. Since sequencing quality is highest at the beginning of a read, placing the ~22 bp target region close to the Read1 start ensures that the most critical information—the specific sgRNA sequence—is captured with the highest possible accuracy. The two-step PCR process is standard for this type of application, as it allows for the initial specific amplification of the sgRNA from genomic DNA, followed by the addition of the necessary sequencing adapters and unique molecular identifiers (UMIs) in a second, cleaner PCR.

\*\* **Nextera/Illumina Overhang:**

1. **Seq**Forward overhang:
　　5’ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG‐[locusspecific sequence]
Reverse overhang:
　　5’ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG‐[locusspecific sequence]
2. **PCR workflow**

