TWIST MULTIPLEXED GENE FRAGMENT DESIGN GUIDELINES

Twist Bioscience's silicon-based DNA writing technology enables massively parallel production of high-quality oligos. Leveraging Twist's efficiencies of scale and excellence in DNA synthesis, Twist's Multiplexed Gene Fragments (MGF) offer a unique pooled format for up to hundreds of thousands of gene fragments, between 301 and 500 base pairs in length.

Twist uses phosphoramidite chemistry for synthesis of Multiplexed Gene Fragments. Compared to conventional column-based synthesizers, our synthesis platform miniaturizes the synthesis process, which reduces reaction volumes and increases throughput. This process has been optimized over the years and now allows Twist to print pools of fragments that are up to 500 nucleotides in length. After synthesis, Twist uses an optimized amplification workflow to generate the multiplexed gene fragments in a dsDNA format which mitigates the risk of poor amplification results as PCR amplification of the synthesized material can be very challenging.

The following design guidelines ensure that Twist is able to produce the highest quality MGF pool for you.

1. Fragment length should be between 301-500 bp (including constant flanks)
    a. The total length of the fragment cannot exceed 500 base pairs including constant flanks which will be used for PCR amplification. Flanking sequences must be at least 20-25 bps in length, which will allow for up to 460 bps of variable coding region.

2. Length variation:
    a. The variation of fragment lengths from shortest to longest should not exceed 80 bp (e.g. if the longest fragment is 400 bp, the shortest fragment should be > 320 bp).
    b. As length variation increases, the pool may show bias towards shorter fragment lengths. For example, a Multiplexed Gene Fragment pool of high length variation (including fragments with lengths 375 - 500 bp) was created and showed read counts distributed among a wide range of sequence length. In this wide range of length groups, minimal bias was observed when the variation was within 10%. However, as variation went beyond 10-15%, a noticeable bias was observed towards shorter fragment lengths.

3. Include constant flanks of at least **20-25 bp**, which will serve as primer sites. For increased likelihood of successful amplification, use flanking sequences with a GC content of 35% to 70% and no strong secondary structure.
    a. Please consider the standard rules for primer design:
        i. Length of 20-25 bases
        ii. 35-70% G/C content
        iii. Melting temperature (Tm) of 55-60°C
        iv. Primer pairs should have a Tm within 5°C of each other
        v. Primer pairs should not have complementary regions
        vi. Avoid internal primer binding sites in the variable region
    b. Twist can add standard Twist flanks to your fragments that can be used with restriction cloning if needed.

4. All fragments must include identical constant flanks. **Please do not include sub-pools.**
    a. All fragments in the pool must have the same flank sequences. Multiple sets of flanks can be accomplished by ordering multiple pools. Alternatively, you can design a single pool with internal flanks that are different and external flanks that are universal for Twist to use for amplification. You may then amplify out the subpools; although this does come with inherent risk of skewing uniformity and/or running out of template material.

5. Avoid repeat regions at or near the sequence flanks
    a. This serves as a primer landing site and results in issues with amplification

6. Consider factors that can cause template switching/formation of chimeric products. Though Twist excels at avoiding these types of issues, some are unavoidable. Longer constant regions shared across pool members will result in a higher percentage of chimeric products.
    a. Consider the number of direct repeats in one sequence, as there may be a lower % of correct sequences if your variants contain these components.
        i. Ex: Cas-12a tandem sgRNA with spacers
    b. Consider the length of conserved sequences across fragment members:
        i. Ex: Dual guides with constant linkers between guides

ii. Ex: Deep Mutational Scan libraries (conserved parental sequence with a small number of nucleotide changes per member)
    1. These will have much higher quality with shorter oligos or Twist's Site Saturation Variant Library offering

7. Degenerate nucleotides or barcodes are NOT allowed:
    a. We can only synthesize sequences containing the four standard bases A, C, G and T. The use of degenerate bases (R, Y, S, W, K, M, B, D, H, V, N) are not allowed. However, if you would like to have all four bases (or a subset of bases) at a given position in a fragment, you can design separate fragments, each with a different base at the site of interest.

*Terms and Conditions: Standard turnaround time for Multiplexed Gene Fragments is 8-12 business days. This will vary based on sequence length and complexity. Multiplexed Gene Fragment length is up to 500 base pairs, with up to 460 bp allotted for the variable region with a minimum of 40 bp conserved for the primer region. Quality control tests ensure >90% of gene sequences within a pool will be user defined length.

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