

Multiplexed Gene Fragments (MGFs) Design Guidelines

For Research Use Only (RUO). Not for use in diagnostic procedures.

This guide provides detailed instructions and best practices for designing and ordering high-quality Multiplexed Gene Fragments (MGFs) from Twist Bioscience. By following these guidelines, you can ensure optimal performance, including high yield and better uniformity, for your pooled Multiplexed Gene Fragments.

DON'T SETTLE FOR LESS IN TARGETED SEQUENCING.

Get in touch at sales@twistbioscience.com or learn more at twistbioscience.com/products/ngs



OVERVIEW OF MULTIPLEXED GENE FRAGMENTS (MGFS)

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 (MGFs) offer a unique pooled format for up to hundreds of thousands of gene fragments between 301 and 500 base pairs in length. After synthesis of full length oligos up to 500nt in length, Twist uses an optimized amplification workflow to generate the multiplexed gene fragments in a dsDNA format which mitigates the risk of poor amplification.

FRAGMENT DESIGN GUIDELINES

REQUIREMENT	SPECIFICATION	DETAIL
Total Length	301–500 base pairs (bp)	This includes the variable region and both the 5' and 3' constant flanks.
Variable Region Length	Up to 460 bp	Since flanking sequences must be at least 20–25 bp each, your variable region can be up to 460 bp.
Length Variation	≤ 15%*	The shortest fragment in your pool must be no more than 15% shorter than the longest fragment. (e.g., if the longest fragment is 400 bp, the shortest must be ≥ 340 bp).

*Note on Length Variation: Pools exceeding 15% variation often show a bias toward shorter fragment lengths. If your order exceeds this specification, you will be prompted to split your pool or re-upload your order.

POOLS WITH LENGTH VARIATION

Pools exceeding 15% variation often show a bias toward shorter fragment lengths and result in poor uniformity. To maintain the high uniformity, we will not accept pools with more than 15% variation from the longest fragment (e.g., if the longest fragment is 400 bp, the shortest must be ≥ 340 bp) and pools outside of this variation will require modification. The following strategies can be used to resolve length variation issues:

- Split into sub-pools
 - The eCommerce interface will automatically suggest splitting an order into multiple pools to reduce variation.
 - Contact your Twist Account Manager to explore options for discounts on sub-pool designs
- Increase the length of shorter fragments by the inclusion of a random stuffer inside your constant flanks.

AVOIDING CHIMERA-PRONE DESIGNS

To maintain a high percentage of correct sequences, consider the following factors that can cause template switching or the formation of chimeric products:

- Long Constant Regions: Pools with long conserved regions internal to variable regions may form chimeric products because of template switching during PCR. The amount of chimera formed will be proportional to the length and number of conserved regions contained within the variable portion of the pool. (e.g., Dual guides with constant linkers, paired CDR variants with constant framework sequences).
- Direct Repeats: Direct repeats are challenging to amplify and will reduce the pool quality (e.g., Cas-12a tandem sgRNA with spacers).
- Protein Mutation Libraries: For libraries with a conserved parent sequence and small nucleotide changes, consider using Twist's Site Saturation Variant Library offering or shorter Oligo Pools for higher quality.

NUCLEOTIDE AND BARCODE USAGE

Allowed Bases: Only the four standard bases (A, G, C, and T) are allowed.

Degenerate Bases: The use of degenerate bases is not allowed.

FLANK DESIGN GUIDELINES

Constant 5' and 3' flanks are required for every sequence ordered, as they are essential for PCR. We have found that flanks that meet the following requirements will produce higher quality MGFs (better yield and uniformity). All fragments within a single pool must include identical constant flanks. Multiple sets of flanks can be accomplished by ordering multiple pools.

FLANK INPUT

Flanks are accepted by our eCommerce platform either by direct input (as shown in **Figure 1**), or they can be automatically identified if your sequences are uploaded with ≥ 20 bp of identical sequence on the 5' and 3' end. After the input of your flanks, select 'update' to verify your flanks. Any errors identified will show directly below the flank. Errors associated with specific fragment sequences will be identified directly in the sequence list (e.g. repeated flank sequence within variant sequence).

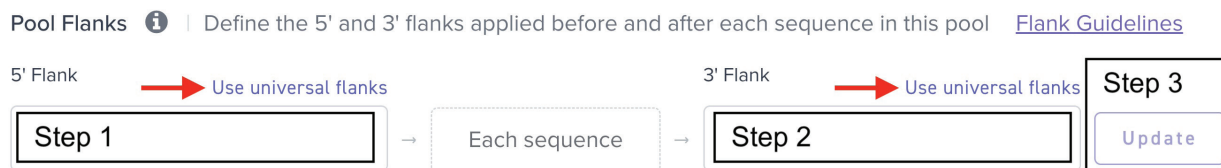


Figure 1. Pool Flank Input region. Each Pool requires its own set of constant 5' and 3' flanks to be input. Flank input steps are as follows: Step 1) input 5' flank sequence, Step 2) input 3' flank sequence, and Step 3) Select "Update" to run the Flank Screener. Any flank errors will show up within the region outlined with a dotted line. If your downstream process is not sensitive to specific flanks, we suggest that you select "Twist universal flanks" (→)

MANDATORY FLANK REQUIREMENTS

Your flanks must meet the following criteria to be accepted by the eCommerce Flank Screener:

- 1. Length:** Each flank must be ≥ 20 bp in length with no exceptions.
- 2. Variable Region Match:** 8 nucleotides (nt) from either the 5' or 3' flank is permitted within the variable region.
- 3. GC Content:** Each flank must have a GC content of 35–70%.
- 4. Melting Temperature (T_m):** Each flank must have a T_m ≥ 60 °C*.
- 5. Homodimer ΔG :** Flank homodimers must have a $\Delta G < -9000$ cal/mol*.
- 6. Hairpin ΔG :** Flank hairpins must have a $\Delta G < -3000$ cal/mol*.
- 7. Heterodimer ΔG :** Any heterodimer involving a flank must have a $\Delta G < -9000$ cal/mol*.

*T_m and ΔG is calculated using Primer3 with the following reaction-condition parameters (dv_conc: 2.5nM, dna_conc: 500nM, dntp_conc: 0.8nM, formamide_conc: 0.0%)



SUGGESTED FLANK CRITERIA (FOR BEST RESULTS)

We recommend that your flanks also meet these criteria to further optimize pool quality:

Dinucleotide Repeats: Flanks should not contain more than two consecutive dinucleotide repeats (e.g., ATATAT).

Identical Nucleotides: Flanks should not contain more than three consecutive identical nucleotides (e.g., AAAA).

Repeat Regions: Avoid repeat regions at or near the sequence flanks, as these can cause off-target priming and/or polymerase slippage.

SUGGESTED FLANKS

If your downstream application (e.g., cloning) is not sensitive to the specific 5' and 3' DNA sequences, we highly recommend using the following Twist universal flanks. These sequences have been routinely used to create high-quality MGFs:

5' Suggested Flank: CAATCCGCCCTCACTACAACCG

3' Suggested Flank: CTACTIONTGGCGTCGATGAGGGA

COMPATIBLE WORKFLOWS

- Restriction digest/assembly workflows are always compatible with Twist universal flanks. Adding restriction sites within your sequences will cleave off any unwanted external flanks.
- Common Gibson Assembly kits often have strand displacing activity and will likely still work with slightly lower reaction efficiency if internal homology is included.



AMPLIFICATION OF MULTIPLEXED GENE FRAGMENTS

Twist Bioscience does not recommend performing any PCR amplification on your final MGFs product before use, as this can create chimeras and bias the pool towards mid-GC containing sequences (40–60%). If you choose to amplify your MGFs, we can no longer guarantee the quality of your pool.

If amplification is necessary, you must adhere to the following:

- Use the Recommended Protocol: Follow only the recommended amplification protocol found in the Amplifying Twist Multiplexed Gene Fragments Guide.
- Maximum Cycles: Adhere to a strict maximum of 8 amplification cycles. Fewer cycles are always better for maintaining high MGF pool quality.

YIELD OPTIONS

To limit the need for amplification, MGFs are offered in:

Standard Yield: 200 ng minimum

Upgraded Yield: 2 µg or 5 µg (available for an additional fee)*

*MGFs are a custom product and while upgraded yield can be requested, we cannot guarantee that any specific pool will be able to meet the requested yield. To increase the likelihood of receiving an upgraded yield, we strongly recommend carefully designing your flanks to meet all specification requirements or using the Twist suggested flanks. If the yield is not met, you will not be charged any additional fees for yield options.

QUALITY CONTROL OPTIONS

MGFs are offered with either standard or upgraded Quality Control (QC). While both QC options should meet the following specifications only orders with the upgraded Quality Control will have sequencing data included:

Standard QC specifications: Fragment Analysis to ensure $\geq 90\%$ of fragments fall within 20% of the expected peak size. If fragments of multiple lengths are submitted, Fragment Analysis to ensure that $>90\%$ of fragments fall within -20% of the shortest oligo length and +20% of the longest oligo length.

Upgraded QC specifications: Fragment Analysis along with Oxford Nanopore Technology (ONT) sequencing data.

HAVE FURTHER QUESTIONS?

For additional support please contact Twist Bioscience's support team at customersupport@twistbioscience.com.