



Experimental Package

Oligonucleotide Sheet and Execution Milestones

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Experimental Package and Oligonucleotide Sheet

1. Overview

Bulk pause assays compare a consensus arrest template against an iso-thermal scrambling control. The two templates differ only in the order of the 12 nucleotides at the centre; flanking sequence, base composition and nearest-neighbour melting temperature are held constant.

2. Oligonucleotide Sequences

A. Consensus template

FIELD	VALUE
Sequence (5'→3')	GCGTCGTACGTCAGTCGAT TATAATTAATTT GCAGTCGTAGCTGCAGTC
Length	49 nt
Motif position	20–31 (1-indexed)
Motif composition	5 A, 7 T, 0% GC
Motif T_m	12.94 °C (conditions below)

B. Iso-thermal scrambling control

FIELD	VALUE
Sequence (5'→3')	GCGTCGTACGTCAGTCGAT TTAAATATTTAT GCAGTCGTAGCTGCAGTC
Length	49 nt
Motif composition	5 A, 7 T, 0% GC — identical to consensus
Motif T_m	12.94 °C — ΔT_m 0.000 °C
Difference from consensus	Nucleotide order only

HOW THIS CONTROL WAS SELECTED

Melting temperature is a sum over nearest-neighbour dinucleotide stacking, not a function of base composition. Two sequences can share composition exactly and still differ in T_m . All 792 permutations of 5 A and 7 T were enumerated and filtered on four criteria, in order: matched nearest-neighbour T_m (ΔT_m below 0.005 °C); no match, on either strand, to a known regulatory or insertion motif; no homopolymer run longer than three; and the shortest substring shared with the consensus or its reverse complement. Thirty-six permutations passed the motif screen, and the longest shared substring achievable among them is four nucleotides. Three candidates tied at that floor and the tie was broken on dissimilarity at the 5' end.

MOTIFS EXCLUDED FROM THE CONTROL, AND WHY THIS MATTERS

A scramble matched only on thermodynamics can still land on a biologically active sequence. The screen above excludes, on both strands: the LINE-1 / ORF2p endonuclease insertion consensus 5'-TTTTT↓AA and its TTTTT core, the *E. coli* σ^{70} -10 element TATAAT, the TATA-box core TATAAA, the polyadenylation signal AATAAA and its ATTAAA variant, and A- or T-tracts of four or more. The ORF2p site is the consequential one, because Document 2 assays ORF2p variants: a control carrying the enzyme's own insertion consensus is not a negative control.

DISCLOSURE: THE CONSENSUS ITSELF CONTAINS TATAAT

The first six nucleotides of the consensus motif are TATAAT, the canonical *E. coli* σ^{70} -10 promoter element. This is a property of the motif under test and is therefore not something the control design can remove. It is stated here so that it is not mistaken for an oversight, and because any interpretation of a positive result has to address the substantial existing literature on polymerase behaviour at AT-rich promoter elements.

THERMODYNAMIC CONDITIONS

T_m figures use the SantaLucia 1998 unified nearest-neighbour parameters, computed for the isolated 12-mer as a free duplex at 250 nM total strand concentration in 50 mM Na^+ , which matches the annealing buffer in Document 2, step 2. Absolute T_m depends on these choices. ΔT_m does not: the two sequences share length and composition, so the salt and concentration corrections are identical and cancel. ΔT_m of 0.000 °C is therefore the load-bearing figure and holds under any buffer.

C. Fluorescent primer

FIELD	VALUE
Sequence (5'→3')	[6-FAM] GACTGCAGCTACGACTGC
Length	18 nt

GC content	61%
Binding site	Template positions 32–49, immediately 3' of the motif
Modification	5' 6-carboxyfluorescein
Purification	PAGE (required for the labelled oligo)

The primer binds downstream of the motif on both templates, so extension proceeds toward the template 5' end and passes through the motif. The same primer is used for consensus and control.

3. Ordering Specification

OLIGO	SYNTHESIS SCALE	MINIMUM DELIVERED YIELD	PURIFICATION	QC
Consensus template, 49 nt	25 nmol	5 nmol	PAGE	MALDI-TOF
Control template, 49 nt	25 nmol	5 nmol	PAGE	MALDI-TOF
6-FAM primer, 18 nt	25 nmol	5 nmol	PAGE (HPLC acceptable)	MALDI-TOF
Unlabelled primer, 18 nt	25 nmol	—	Desalted	Standard

SYNTHESIS SCALE IS NOT DELIVERED YIELD

Vendors quote synthesis scale. The quantity that arrives is lower than the scale, and lower again after purification. A 25 nmol synthesis of an unmodified 25–50-mer carries a 10 nmol guaranteed yield, and both PAGE purification and a 5' fluorophore reduce recovery below that figure. Order against the minimum delivered yield in the column above rather than against scale alone, and raise the scale if a vendor will not commit to that yield for the purified or labelled oligonucleotide.

WHAT THE MATERIAL HAS TO COVER

At 100 nM in a 50 µL reaction, one reaction consumes 5 pmol of primer. The full comparative series in Document 2, five conditions in triplicate, consumes 75 pmol. A 5 nmol delivery therefore supports approximately 65 complete runs, which leaves room for optimisation and for repeat experiments without a second order.

FOURTH OLIGONUCLEOTIDE

An unlabelled primer of identical sequence is included so that labelled and unlabelled reaction kinetics can be compared directly. This comparison is not required to interpret the bulk result, because the consensus and control templates are run with the same labelled primer. It is a prerequisite for single-molecule work, and Document 2, section 5, specifies it under that heading.

4. Expected Products

SPECIES	LENGTH	CONDITION
Unextended primer	18 nt	No extension
Arrest products	19–30 nt	Stop within the motif
Full-length	49 nt	Read-through

5. Execution Milestones

1 Oligonucleotide QC

Verify purity and mass by MALDI-TOF on delivery. Confirm the 6-FAM primer mass includes the label.

2 Cell-free expression

Express RT / ART variants by PURExpress per Document 2, step 1. Confirm protein synthesis before proceeding.

3 Comparative time course

Run consensus and control templates in parallel across the 15 s to 10 min series, with the full control set from Document 2, section 4.

4 Quantification

Report arrest fraction as band intensity in the 19–30 nt window divided by total lane intensity, for each template at each timepoint.

6. Decision Point

A reproducible difference in arrest fraction between consensus and control templates, surviving the controls in Document 2, is the result that justifies progressing to single-molecule measurement. Absent that

difference, the sequence-specific arrest hypothesis is not supported by this assay and single-molecule work would not resolve it.