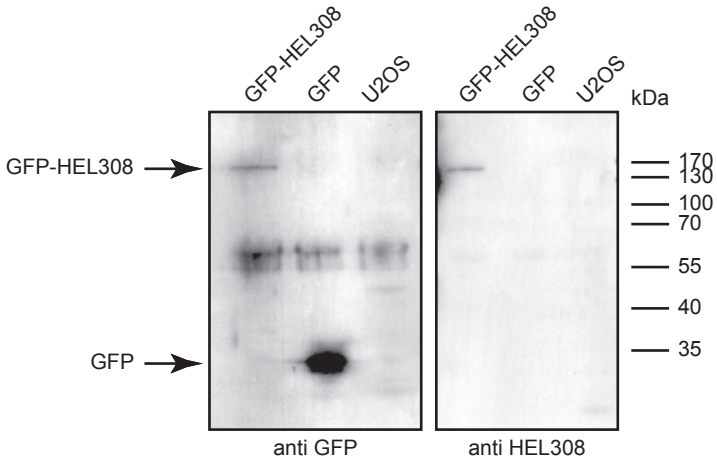
Supplemental Figure Legends

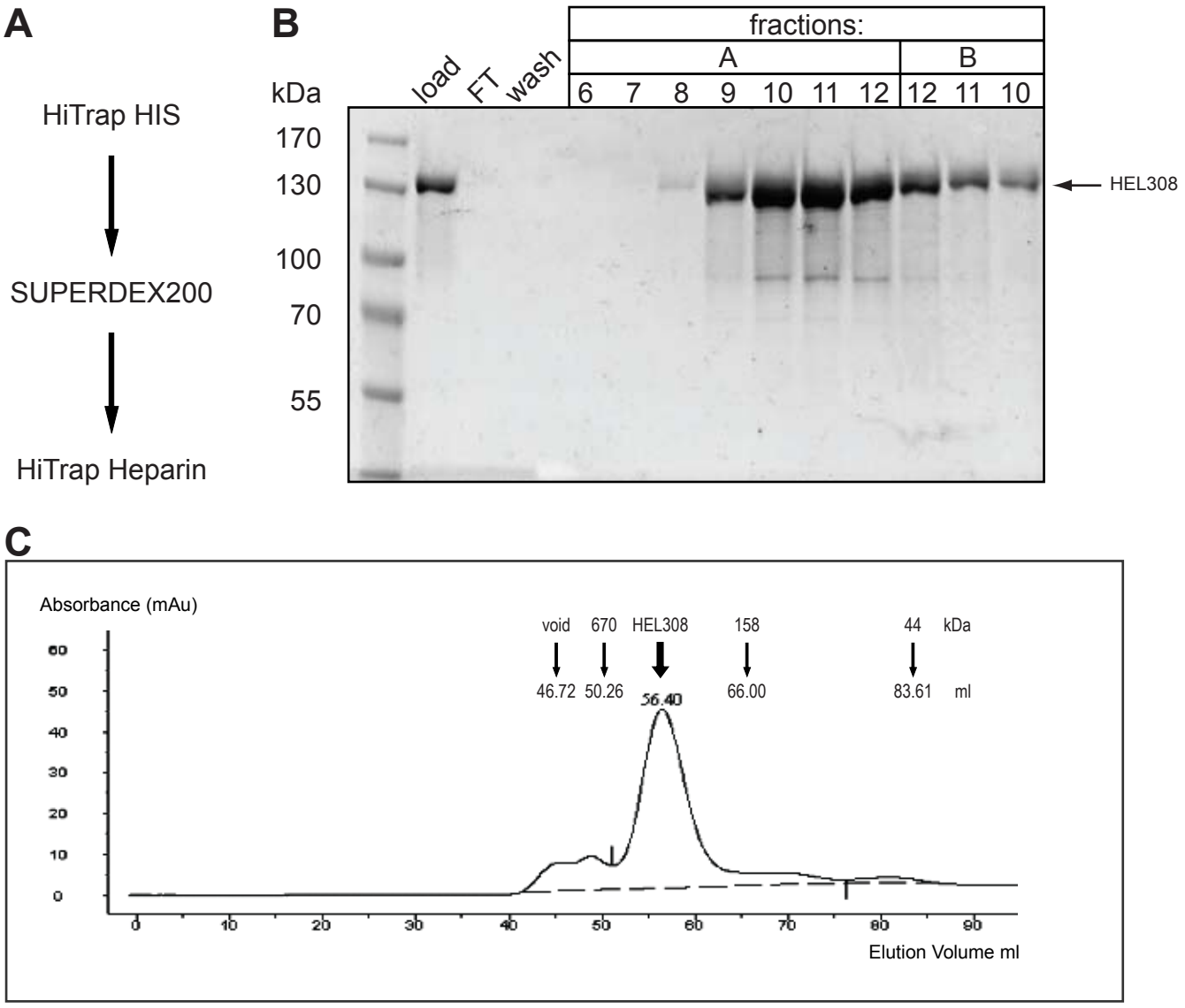
Supplemental Fig. S1. **Generation of stable GFP-HEL308 expressing clones.** Protein extracts from U2OS cells stably transfected with GFP-HEL308 (GFP-HEL308), stably transfected with an empty GFP control construct (GFP) or not transfected (U20S) were run on 7.5 % SDS-PAGE gels and immunoblotted with antibodies directed against GFP (*left-hand panel*) and HEL308 (*right-hand panel*).

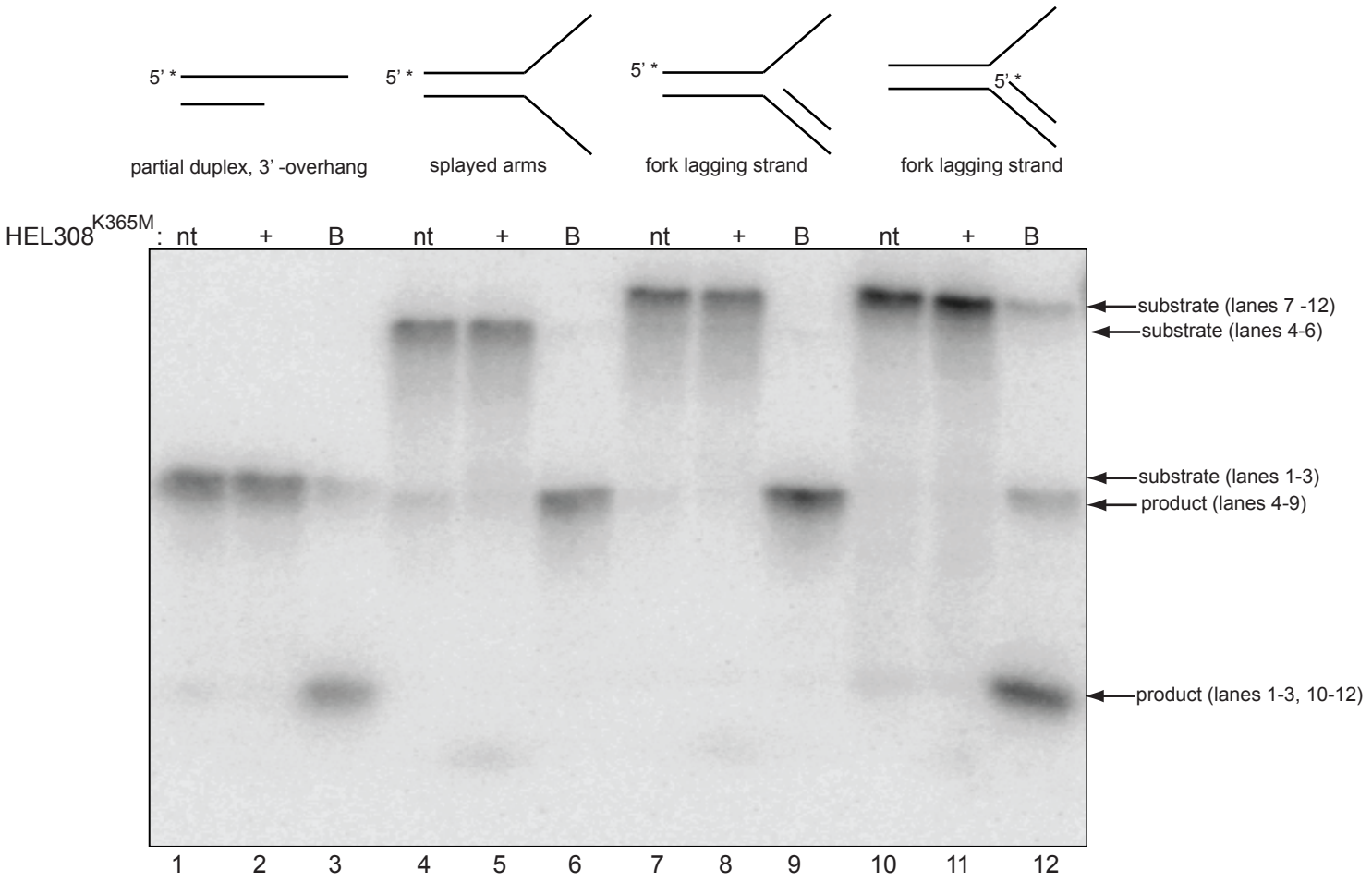
Supplemental Fig. S2. **Purification of human HEL308 from insect cells.** *A.* Schematic representation of sequential columns used to purify HEL308. *B.* Coomassie-stained 7.5 % SDS-PAGE gel containing equal volumes of indicated elution fractions obtained from (final) Heparin column. *Lane* labelled 'load' contains the input material after the SUPERDEX200 purification step, *lane* labelled 'FT' represents the unbound proteins (flow-through), and *lane* labelled 'wash' shows contamination washed off the column. *C.* Gel filtration profile of HEL308 obtained on a SUPERDEX 200 column.

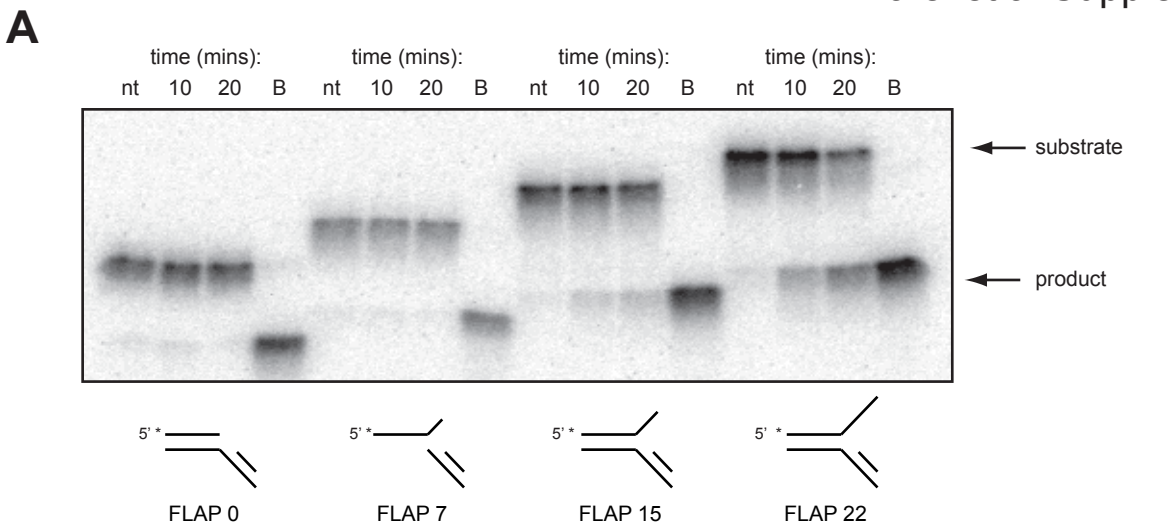
Supplemental Fig. S3. **HEL308^{K365M} displays no unwinding activity.** The 3'-overhang partial duplex (lanes 1-3), splayed arms (lanes 4-6), fork-lagging strand (lanes 7-9) and fork lagging strand with labelled nascent lagging strand (lanes 10-12) substrates listed in Supplemental Table 1 were 5'-end labelled as shown by the position of the asterisk. Substrates were mock-treated (0 pmol/µl lane) in lanes marked nt, treated with 1 pmol/µl of HEL308^{K365M} in lanes marked +, or boiled to fully danature substrates in lanes marked B. For each set of substrates, the position of the substrate and fully unwound/denatured products are marked.

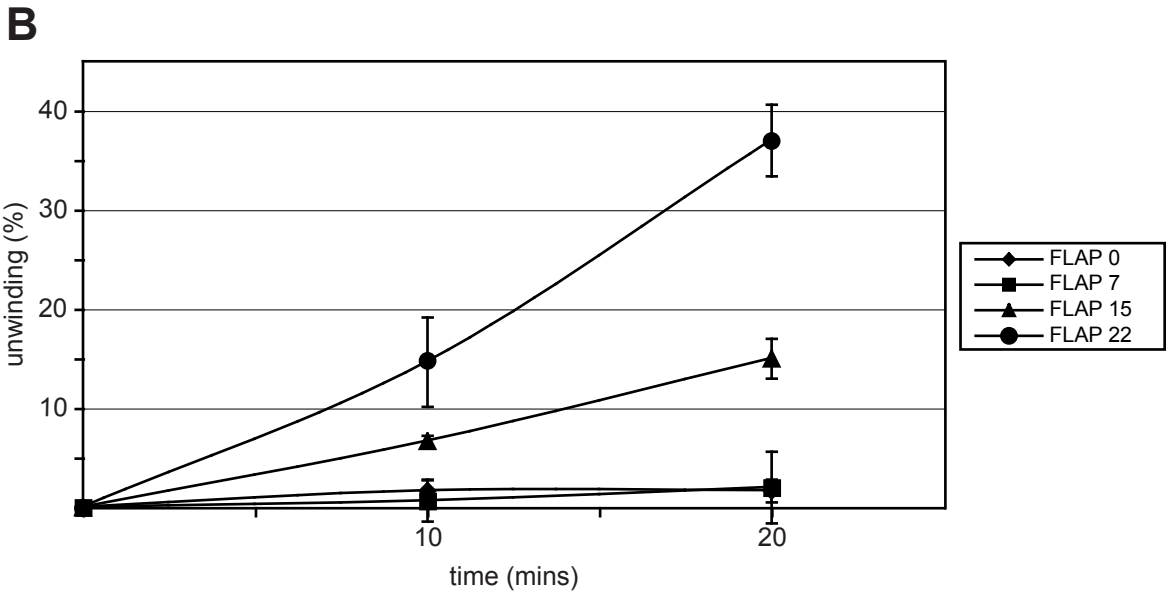
Supplemental Fig. S4. **Defining the minimal length of the 3'-ssDNA region required for unwinding by HEL308.** *A.* Model fork substrates with a 3' ssDNA flap length of 0, 7, 15 or 22 nt were incubated with 0.5 pmol/μl of HEL308 for the stated times. Migration of substrate and product are marked. Lanes marked nt contain control substrate not treated with enzyme, and those marked B boiled substrate. The DNA substrates analyzed are schematically depicted at the bottom of the gel. A 5' asterisk indicates the position of the 5'-end radioactive labelling *B*. Quantification of product formation obtained from experiments shown in *panel A*. The percentage of substrate unwinding is shown as a function of time. Each experiment was performed in triplicate. The error bars represent standard deviation.


GFP-HEL308
GFP
U2OS
GFP-HEL308
GFP
U2OS
kDa
GFP-HEL308
GFP
170
130
100
70
55
40
35
anti GFP
anti HEL308

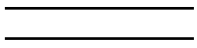

A
HiTrap HIS
SUPERDEX200
HiTrap Heparin
B
kDa
170
130
100
70
55
load
FT
wash
fractions:
A
B
6 7 8 9 10 11 12
12 11 10
HEL308
C
Absorbance (mAu)
void
670
HEL308
158
44
kDa
46.72
50.26
56.40
66.00
83.61
ml
Elution Volume ml


5' *
partial duplex, 3' -overhang
5' *
splayed arms
5' *
fork lagging strand
5' *
fork lagging strand
HEL308K365M: nt + B nt + B nt + B nt + B
substrate (lanes 7 -12)
substrate (lanes 4-6)
substrate (lanes 1-3)
product (lanes 4-9)
product (lanes 1-3, 10-12)
1 2 3 4 5 6 7 8 9 10 11 12

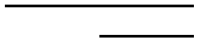

A
time (mins):
nt 10 20 B
time (mins):
nt 10 20 B
time (mins):
nt 10 20 B
time (mins):
nt 10 20 B
substrate
product
5' *
FLAP 0
FLAP 7
FLAP 15
FLAP 22


B
unwinding (%)
40
30
20
10
10
20
time (mins)
FLAP 0
FLAP 7
FLAP 15
FLAP 22

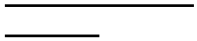
Supplemental Table S1: DNA substrates used in this study - (see Suppl. Table S2 for sequences):



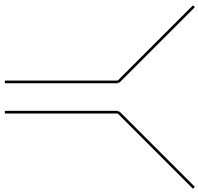
DUPLEX:
OLIGO 1 and DUPLEX



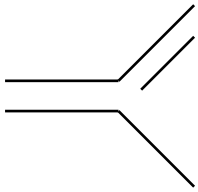
PARTIAL DUPLEX (5' OVERHANG):
OLIGO 1 and 5' OVERHANG/NASCENT LEADING STRAND



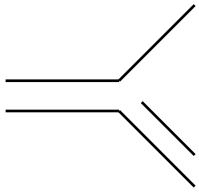
PARTIAL DUPLEX (3' OVERHANG):
OLIGO 4 and 3' OVERHANG/NASCENT LAGGING STRAND



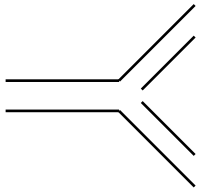
SPLAYED ARMS:
OLIGO 1 and 4



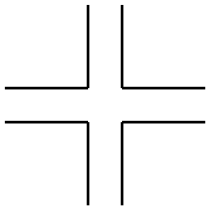
FORK WITH NASCENT LEADING STRAND:
OLIGO 1, 4 and 5' OVERHANG/NASCENT LEADING STRAND



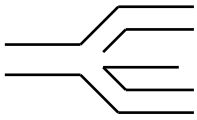
FORK WITH NASCENT LAGGING STRAND:
OLIGO 1, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



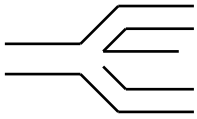
FORK:
OLIGO 1, 4, 5' OVERHANG/NASCENT LEADING STRAND AND
3' OVERHANG/NASCENT LAGGING STRAND



HOLLIDAY JUNCTION (MOBILE):
OLIGO 1, 2, 3 and 4

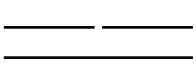


CHICKEN FOOT (5' OVERHANG):
OLIGO 1, DUPLEX, NASCENT LEADING STRAND - MISMATCH and
NASCENT LAGGING STRAND - LONG

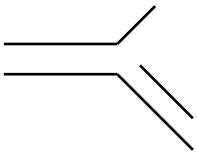


CHICKEN FOOT (3' OVERHANG):
OLIGO1, DUPLEX, NASCENT LEADING STRAND - LONG and
NASCENT LAGGING STRAND - MISMATCH

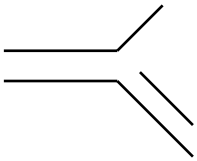
Supplemental Table S1 - DNA substrates used in this study (continued) - (see Suppl. Table S2 for sequences):



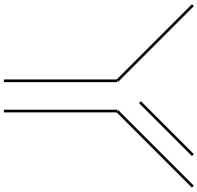
FLAP 0 :
OLIGO FLAP 0, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



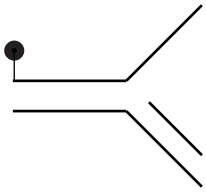
FLAP 7 :
OLIGO FLAP 7, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



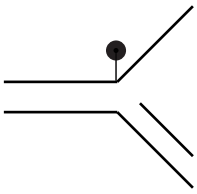
FLAP 15 :
OLIGO FLAP 15, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



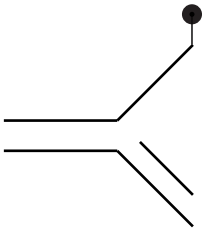
FLAP 22 :
OLIGO 1, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



STREP 1 :
OLIGO STREP 1, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



STREP 34 :
OLIGO STREP 34, 4 and 3' OVERHANG/NASCENT LAGGING STRAND



STREP 56 :
OLIGO STREP 56, 4 and 3' OVERHANG/NASCENT LAGGING STRAND

Supplemental Table S2: Oligonucleotide sequences of substrates used in this study:

OLIGO 1:
5'GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAAGCTTACGTTAACCCA

OLIGO 2:
5'TGGGTTAACGTAAGCTTAATAGATGTCCTAGCAAGACGAGATATCATTATGAGCCT

OLIGO 3:
5'AGGCTCATAATGATATCTCGTCTTGCTAGGACATGACCCTCTAGATGCAATTTCTT

OLIGO 4:
5'AAGAAATTGCATCTAGAGGGTCATGTCCTAGCAATCCAGAAGAATTCGGCAGCGTC

DUPLEX:
5'TGGGTTAACGTAAGCTTAATAGATGTCCTAGCAATCCAGAAGAATTCGGCAGCGCT

5' OVERHANG/NASCENT LEADING STRAND:
5'TGGGTTAACGTAAGCTTAATAG

NASCENT LEADING STRAND MISMATCH:
5' TGGGTTAACGTAAGCTTAATAT

NASCENT LEADING STRAND LONG:
5' TGGGTTAACGTAAGCTTAATATGACCCTCAGATGCAATTTCTT

3' OVERHANG/NASCENT LAGGING STRAND:
5'GACCCTCTAGATGCAATTTCTT

NASCENT LAGGING STRAND MISMATCH:
5'TTATTAAGCTTACGTTAACCCA

NASCENT LAGGING STRAND LONG:
TTATTAAGCTTACGTTAACCCAGTTATTAAGCTTACGTTAACCCA

FLAP 0:
5'GACGCTGCCGAATTCTTCTGGATTGCTAGGACAT

FLAP 7:
5' GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAA

FLAP 15:
5' GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAAGCT

STREP 1:
5'[Biotin]GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAAGCTTACGTTA
ACCCA

STREP 34:
5'GACGCTGCCGAATTCTTCTGGATTGCTAGGACA[Biotin]TCTATTAAGCTTACGTTA
ACCCA

STREP 56:
5'GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAAGCTTACGTTA
ACCCA [Biotin]

LINEAR DUPLEX:

5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAAGCTTACGTTAACCCA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGTAGATAATTCGAATGCAATTGGGT

5’ OVERHANG:

5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACATCTATTAAGCTTACGTTAACCCA
GATAATTCGAATGCAATTGGGT

3’ OVERHANG:

GACCCTCTAGATGCAATTTCTT
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGTACTGGGAGATCTACGTTAAAGAA

SPLAYED ARMS:

CTATTAAGCTTACGTTAACCCA
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACAT
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGTA
CTGGGAGATCTACGTTAAAGAA

FORK LEADING STRAND:

CTATTAAGCTTACGTTAACCCA
TGATAATTCGAATGCAATTGGGT
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
A
CTGGGAGATCTACGTTAAAGAA

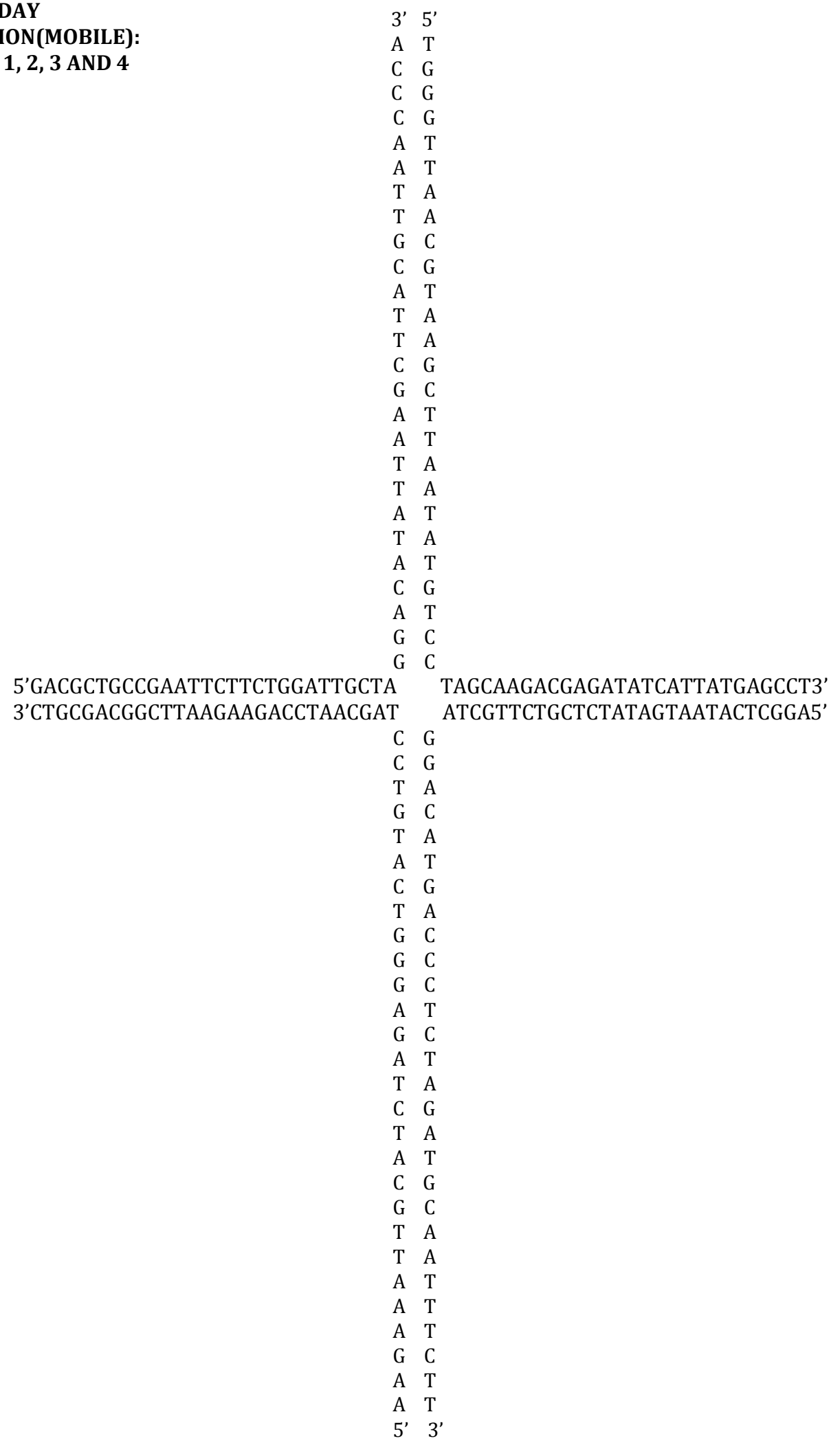
FORK LAGGING STRAND:

CTATTAAGCTTACGTTAACCCA
T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
AGACCCTCTAGATGCAATTTCTT
CTGGGAGATCTACGTTAAAGAA

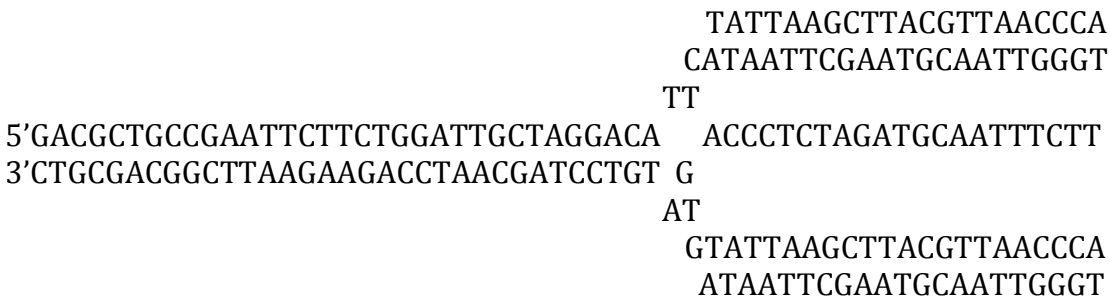
FORK:

CTATTAAGCTTACGTTAACCCA
TGATAATTCGAATGCAATTGGGT
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
AGACCCTCTAGATGCAATTTCTT
CTGGGAGATCTACGTTAAAGAA

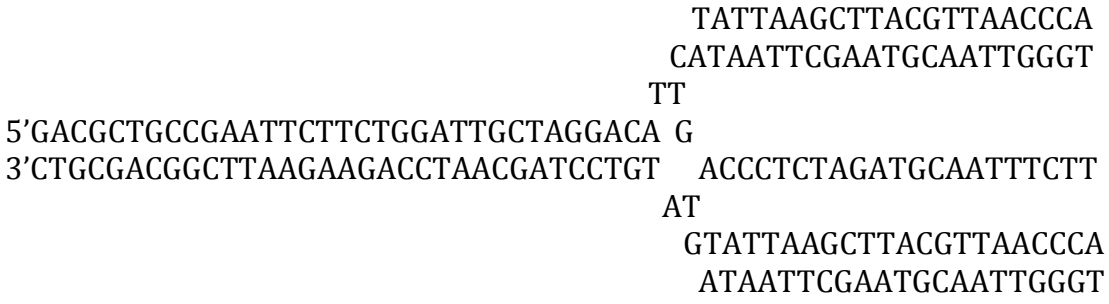
HOLLIDAY JUNCTION(MOBILE): OLIGO 1, 2, 3 AND 4



CHICKEN FOOT 5’ OVERHANG:



CHICKEN FOOT 3’ OVERHANG:



FORK LAGGING STRAND:

FLAP 0:

```
                                        T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
                                        AGACCCTCTAGATGCAATTTCTT
                                         CTGGGAGATCTACGTTAAAGAA
```

FLAP 7:

```
                                         CTATTAA
                                        T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
                                        AGACCCTCTAGATGCAATTTCTT
                                         CTGGGAGATCTACGTTAAAGAA
```

FLAP 15:

```
                                         CTATTAAGCTTACGT
                                        T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
                                        AGACCCTCTAGATGCAATTTCTT
                                         CTGGGAGATCTACGTTAAAGAA
```

FORK LAGGING STRAND BIOTIN:

```
                                         CTATTAAGCTTACGTTAACCCA
  B                                     T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
                                        AGACCCTCTAGATGCAATTTCTT
                                         CTGGGAGATCTACGTTAAAGAA

                                        BCTATTAAGCTTACGTTAACCCA
                                        T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
                                        AGACCCTCTAGATGCAATTTCTT
                                         CTGGGAGATCTACGTTAAAGAA

                                                                B
                                         CTATTAAGCTTACGTTAACCCA
                                        T
5’GACGCTGCCGAATTCTTCTGGATTGCTAGGACA
3’CTGCGACGGCTTAAGAAGACCTAACGATCCTGT
                                        AGACCCTCTAGATGCAATTTCTT
                                         CTGGGAGATCTACGTTAAAGAA
```

B = Biotin