A manual for pGreen3-based CRISPR ternary vector system

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A pGreen3 CRISPR binary vector list

Since morphogenic regulator (MR) genes and their promoters are usually species-specific, MR components from maize may not work in other plants, especially in dicot plants. However, ternary vector systems harboring no MR modules are still able to greatly enhance transformation of plants, such as sorghum (Che et al. Plant Biotechnol J. 2018, 16: 1388-1395). Therefore, we generated a set of pGreen3 CRISPR/Cas9 binary vectors harboring no MR genes (Figure 1 and Table 1, Zhang et al. Plant Physiol, 2019, DOI: 10.1104/pp.19.00767, and unpublished data). These vectors and the virulence helper pVS1-VIR2 can constitute a ternary vector system for genome editing in a variety of dicot and monocot plants. In pG3B/H/K-U6SC/-U6EC1, the U6:sgRNA cassettes can be replaced by digestion with *Hin*dIII and *Spe*I, the *Cas9* promoters can be replaced by digestion with *Spe*I and *Xba*I and Gibson assembly, and *SpCas9* can be replaced by digestion with *Xba*I and *Sac*I. Thus, the pGreen3 vectors can be easily modified to meet users' requirement.

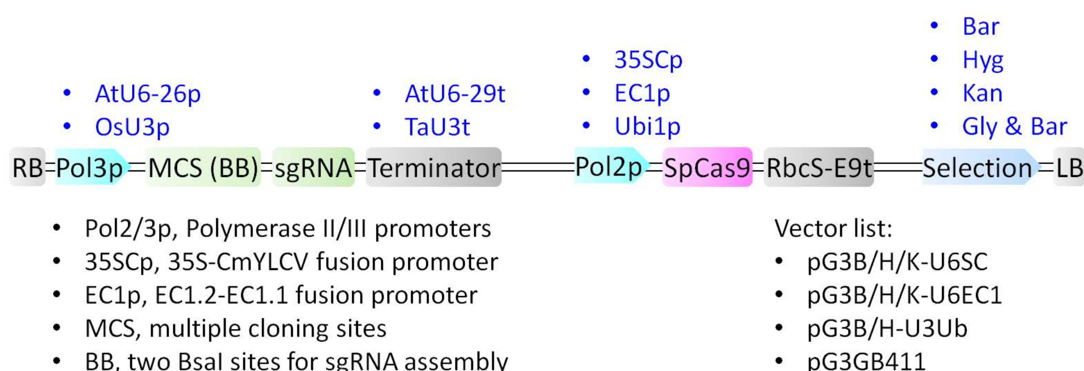

Figure 1. T-DNA structures of pGreen3 CRISPR binary vectors harboring no MR genes. These vectors and the virulence helper pVS1-VIR2 can constitute a ternary vector system for genome editing in a variety of dicot and monocot plants.

Table 1. The pGreen3 CRISPR/Cas9 binary vectors

Vector	Selection	sgRNA cassette	Cas9 cassette	MR module
pG3B-U6SC	Bar	U6_26p-BB-sgRNA-U6_29t	35SCp:Cas9	−
pG3H-U6SC	Hyg	U6_26p-BB-sgRNA-U6_29t	35SCp:Cas9	−
pG3K-U6SC	Kan	U6_26p-BB-sgRNA-U6_29t	35SCp:Cas9	−
pG3B-U6EC1	Bar	U6_26p-BB-sgRNA-U6_29t	EC1p:Cas9	−
pG3H-U6EC1	Hyg	U6_26p-BB-sgRNA-U6_29t	EC1p:Cas9	−
pG3K-U6EC1	Kan	U6_26p-BB-sgRNA-U6_29t	EC1p:Cas9	−
pG3H-U3Ub	Hyg	OsU3p-BB-sgRNA-TaU3t	Ubi1p:Cas9	−
pG3B-U3Ub	Bar	OsU3p-BB-sgRNA-TaU3t	Ubi1p:Cas9	−
pG3GB411	Gly & Bar	OsU3p-BB-sgRNA-TaU3t	Ubi1p:Cas9	−
pG3GB411-BWM	Gly & Bar	OsU3p-BB-sgRNA-TaU3t	Ubi1p:Cas9	+

Two additional pCambia2 control vectors, pGB411 and pGB411-BWM (unpublished data) have the same T-DNA structures as pG3GB411 and pG3GB411-BWM, respectively. 35SCp, 35S-CmYLCV fusion promoter; EC1p, EC1.2-EC1.1 fusion promoter.

Simplified protocol of assembly of one or two sgRNA cassettes

1. Search for target sites on websites, such as http://crispor.tefor.net/. Select those targets with both high specificity score and high editing efficiency score.
2. Order two 23-nt oligos for generation of a single sgRNA cassette, or order four primers for generation of two sgRNA cassettes. See the corresponding parts of this manual for oligo design, primer design, and PCR reaction.
3. Set up Golden Gate reactions as follows:

Component	Volume	Reaction conditions
Inserts (0.05 µM) or purified PCR framents (~25 ng/µl)	2 µl	5 hours at 37°C
Vectors (~100 ng/µl)	2 µl	5 min at 50°C
10× T4 DNA Ligase Buffer (NEB)	1.5 µl	10 min at 80°C
10× BSA	1.5 µl	
*Bsa*I (NEB)	1 µl	NOTE: It is essential to use a
T4 DNA Ligase (HC, NEB)	1 µl	High Concentration (HC) Ligase
ddH$_2$O	6 µl	(2 million units/ml, NEB)
Total volume	15 µl	

4. Transform *E.coli* competent cells with 5 µl of reaction mixture, and select positive clones on kanamycin LB agar plates.
5. Identify correct clones by colony PCR and verify them by sequencing.

Oligos or primers for generation of one or two sgRNA cassettes for dicots

Two 23-nt oligos are required for generation of a single sgRNA cassette, and four primers are required for generation of two sgRNA cassettes.

Sequences of two 23-nt oligos for generation of a single sgRNA cassette

oDT-F: 5'-ATTGNNNNNNNNNNNNNNNNNNN
oDT-R: 5'-AAACNNNNNNNNNNNNNNNNNNN

Notes:

1. The 19-nt N in oDT-F represent a 19-nt target sequence in front of PAM (NGG), whereas those in oDT-R represent reverse complement sequence of target in oDT-F.
2. No phosphorylation is required for the oligos.
3. An insert with the compatible ends is generated by annealing the two oligos.

Sequence of one sgRNA expression cassette for dicots

(U6-26p)-(<u>Target-1</u>)-(sgRNA-Sc)-(U6-29t/S)

CGACTTGCCTTCCGCACAATACATCATTTCTTCTTAGCTTTTTTTCTTCTTCTTCGTTCATACAGTTTTTTTTTGTTTATCAGCTT
ACATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTTAACTTTCCATTCGGAGTTTTTGTATCTTGTTTCATAGTT**TGTCCCAG**
GATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAA
AGGCCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTA
AGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAG
TGATTG<u>*NNNNNNNNNNNNNNNNNNN*</u>GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC
AACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGGATAGAATTTCCCAGCTTTTTT**GCGTGTTTCAGCTCTCATGATC**
CTT

Notes:

1. Underlined letters come from the insert generated by annealing the two oligos, while the rest come from the binary vectors.
2. Boxed letters indicate primer sites.
3. Primer sequences are as follows:

Colony PCR primers (5'→3'):
U6-26p-F: TGTCCCAGGATTAGAATGATTAGGC
U6-29t-R: AAGGATCATGAGAGCTGAAACACGC
(U6-26p-F + U6-29t-R = 413 bp)

Sequencing primers (5'→3'):
U6-26p-F

Sequences of a PCR fragment and primers for generation of two sgRNA cassettes

A PCR fragment:

(Target-1)-(sgRNA-Sc)-(U6-26t/S)-(U6-29p)-(Target-2)

ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
CGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGCAAAATTTTCCAGATCGATTTCTTCTTCCTCTGTTCTTC
GGCGTTCAATTTCTTTAATCCAAACTACTGCAGCCTGACAGACAAATGAGGATGCAAACAATTTTAAAGTTTATCTAACGCT
AGCTGTTTTGTTTCTTCTCTCTGGTGCACCAACGACGGCGTTTTCTCAATCATAAAGAGGCTTGTTTTACTTAAGGCCAATA
ATGTTGATGGATCGAAAGAAGAGGGCTTTTAATAAACGAGCCCGTTTAAGCTGTAAACGATGTCAAAAACATCCCACATC
GTTCAGTTGAAAATAGAAGCTCTGTTTATATATTGGTAGAGTCGACTAAGAGATTGNNNNNNNNNNNNNNNNNNNG
TTTAGAGACCAATAAT

Length: 496-bp

Template: pCBC-DT1T2.2 (a modified version of pCBC-DT1T2 with U6-26t shortened)

Primers:

DT1-BsF: ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNGTT

DT1-F0: GNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC

DT2-R0: NNNNNNNNNNNNNNNNNNNCAATCTCTTAGTCGACTCTAC

DT2-BsR: ATTATTGGTCTCGAAACNNNNNNNNNNNNNNNNNNNCAA

Notes: The 19-nt N in DT1-BsF/-F0 represent a 19-nt target sequence in front of PAM (NGG for SpCas9), whereas those in DT2-BsR/-R0 represent reverse complement sequence of another target.

A representative PCR reaction:

Component	Volume	Cycling conditions
10× KOD plus Buffer	5 µl	1. One cycle: 94 °C, 2 min. 2. 30 cycles: 94 °C, 15 sec; 60 °C, 30 sec; 68 °C, 30 sec. 3. One cycle: 68 °C, 5 min
MgSO4 (25mM)	3 µl	
dNTPs (2mM, Toyobo)	4 µl	
KOD plus (Toyobo)	1 µl	
pCBC-DT1T2.2 (10 ng/µl)	1 µl	
DT1-BsF (20 µM)	1 µl	
DT1-F0 (1 µM)	1 µl	
DT2-R0 (1 µM)	1 µl	
DT2-BsR (20 µM)	1 µl	
ddH_2O	32 µl	
Total volume	50 µl	

Sequence of two sgRNA expression cassettes for dicots

(U6-26p)-(Target-1)-(sgRNA-Sc)-(U6-26t/S)-(U6-29p)-(Target-2)-(sgRNA-Sc)-(U6-29t/S)

CGACTTGCCTTCCGCACAATACATCATTTCTTCTTAGCTTTTTTTCTTCTTCTTCGTTCATACAGTTTTTTTTTGTTTATCAGCTT
ACATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTTAACTTTCCATTCGGAGTTTTTGTATCTTGTTTCATAGTT**TGTCCCAG**
GATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAA
AGGCCCCTGGGAAT**CTGAAAGAAGAGAAGCAGGCCCATT**TATATGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTA
AGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAG
TGATTGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACT
TGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGCAAAATTTTCCAGATCGATTTCTTCTTCCTCTGTTCTTCGGCGTTCAATT
TCTTTAATCCAAACTACTGCAGCCTGACAGACAAATGAGGATGCAAACAATTTTAAAGTTTATCTAACGCTAGCTGTTTTGT
TTCTTCTCTCTGGTGCACCAACGACGGCGTTTTCTCAATCATAAAGAGGCTTGTTTTACTTAAGGCCAATAAT**GTTGATGG**
ATCGAAAGAAGAGGGCTTTTAATAAACGAGCCCGTTTAAGCTGTAAACGATGTCAAAAACATCCCACATCGTTCAGTTGA
AAATAGAAGCTCTGTTTATATATTGGTAGAGTCGACTAAGAGATTGNNNNNNNNNNNNNNNNNNNGTTTTAGAG
CTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGGATAGA
ATTTCCCAGCTTTTTTGCGTGTTTCAGCTCTCATGATCCTT

Notes:

1. Underlined letters come from the PCR fragment, while the rest come from the binary vectors, such as pG3B/H/K-U6SC and pG3B/H/K-U6EC1.
2. Boxed letters indicate primer sites.
3. Primer sequences are as follows:

Colony PCR primers (5'→3'):

U6-26p-F2: CTGAAAGAAGAGAAGCAGGCCCATT

U6-29p-R: AGCCCTCTTCTTTCGATCCATCAAC

(U6-26p-F2 + U6-29p-R = 490 bp)

Sequencing primers (5'→3'):

U6-26p-F2

Oligos or primers for generation of one or two sgRNA cassettes for monocots

Two 23-nt oligos are required for generation of a single sgRNA cassette, and four primers are required for generation of two sgRNA cassettes.

Sequences of two 23-nt oligos for generation of a single sgRNA cassette

oMT-F: 5'-GGCGNNNNNNNNNNNNNNNNNNN

oMT-R: 5'-AAACNNNNNNNNNNNNNNNNNNN

Notes:

1. The 19-nt N in oMT-F represent a 19-nt target sequence in front of PAM (NGG), whereas those in oMT-R represent reverse complement sequence of target in oMT-F.
2. No phosphorylation is required for the oligos.

Sequence of one sgRNA expression cassette for monocots

(OsU3p)-(Target-1)-(sgRNA-Sc)-(TaU3t)

AGTAATTCATCCAGGTCACCAAGTTCTAGGATTTTCAGAACTGCAACTTATTTTATCAAGGAATCTTTAAACATACGAACAGAT
CACTTAAAGTTCTTCTGAAGCAACTTAAAGTTATCAGGCTTGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCAT
AGCACAA**GACAGGCGTCTTCTACTGGTGCTAC**CAGCAAATGCTGGAAGCCGGGAACACTGGGTACGTTGGAAACCACGTG
ATGTGAAGAAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATG
GGCCG**GCCCATTACGCAATTGGACGACAAC**AAAGTCTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTT
AAAAGAGTTGTGCAGATGATCCGTGGCG*NNNNNNNNNNNNNNNNNNN*GTTTTAGAGCTAGAAATAGCAA
GTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTGTCCTTCTGTTTTTTTAGT
CAGTCTCTTTTTT**CAGAAGTACAACATCTT**

Notes:

1. Underlined letters come from the insert generated by annealing the two oligos, while the rest come from the binary vectors.
2. Boxed letters indicate primer sites.
3. Primer sequences are as follows:

Colony PCR primers (5'→3'):

OsU3p-F3: GACAGGCGTCTTCTACTGGTGCTAC

TaU3t-R: AACCACCCAAGATGTTGTACTTCTG

(OsU3p-F3 + TaU3t-R = 427 bp)

Sequencing primers (5'→3'):

OsU3p-F3

Sequences of a PCR fragment and primers for generation of two sgRNA cassettes

A PCR fragment:

(Target-1)-(sgRNA-Sc)-(OsU3t/S)-(TaU3p)-(Target-2)

ATTATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG
TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTCGTTTTGCATTGAGTTTTCTCCGTCGCATGTTTGC
AGCATGAATCCAAACCACACGGAGTTCAAATTCCCACAGATTAAGGCTCGTCCGTCGCACAAGGTAATGTGTGAATATTATAT
CTGTCGTGCAAAATTGCCTGGCCTGCACAATTGCTGTTATAGTTGGCGGCAGGGAGAGTTTTAACATTGACTAGCGTGCTGA
TAATTTGTGAGAAATAATAATTGACAAGTAGATACTGACATTTGAGAAGAGCTTCTGAACTGTTATTAGTAACAAAAATGGAA
AGCTGATGCACGGAAAAAGGAAAGAAAAAGCCATACTTTTTTTTAGGTAGGAAAAGAAAAAGCCATACGAGACTGATGTC
TCTCAGATGGGCCGGGATCTGTCTATCTAGCAGGCAGCAGCCCACCAACCTCACGGGCCAGCAATTACGAGTCCTTCTAAAA
GCTCCCGCCGAGGGGCGCTGGCGCTGCTGTGCAGCAGCACGTCTAACATTAGTCCCACCTCGCCAGTTTACAGGGAGCAG
AACCAGCTTATAAGCGGAGGCGCGGCACCAAGAAGCGNNNNNNNNNNNNNNNNNNNGTTTAGAGACCAATAAA
T

Length: 722-bp

Template: pCBC-MT1T2.2 (a modified version of pCBC-MT1T2 with OsU3t shortened)

Primers:

MT1-BsF: ATATTATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNGTT

MT1-F0: GNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC

MT2-R0: AACNNNNNNNNNNNNNNNNNNNCGCTTCTTGGTGCC

MT2-BsR: ATTTATTGGTCTCTAAACNNNNNNNNNNNNNNNNNNNC

Notes: The 19-nt N in DT1-BsF/-F0 represent a 19-nt target sequence in front of PAM (NGG for SpCas9), whereas those in DT2-BsR/-R0 represent reverse complement sequence of another target.

A representative PCR reaction:

Component	Volume	Cycling conditions
10× KOD plus Buffer	5 µl	
MgSO4 (25mM)	3 µl	
dNTPs (2mM, Toyobo)	4 µl	
KOD plus (Toyobo)	1 µl	1. One cycle: 94 °C, 2 min.
pCBC-MT1T2.2 (10 ng/µl)	1 µl	2. 30 cycles: 94 °C, 15 sec;
MT1-BsF (20 µM)	1 µl	60 °C, 30 sec; 68 °C, 30 sec.
MT1-F0 (1 µM)	1 µl	3. One cycle: 68 °C, 5 min
MT2-R0 (1 µM)	1 µl	
MT2-BsR (20 µM)	1 µl	
ddH_2O	32 µl	
Total volume	50 µl	

Sequence of two sgRNA expression cassettes for monocots

(OsU3p)-(Target-1)-(sgRNA-Sc)-(OsU3t/S)-(TaU3p)-(Target-2)-(sgRNA-Sc)-(TaU3t/S)

AGTAATTCATCCAGGTCACCAAGTTCTAGGATTTTCAGAACTGCAACTTATTTTATCAAGGAATCTTTAAACATACGAACAGAT
CACTTAAAGTTCTTCTGAAGCAACTTAAAGTTATCAGGCTTGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCAT
AGCACAA**GACAGGCGTCTTCTACTGGTGCTAC**CAGCAAATGCTGGAAGCCGGGAACACTGGGTACGTTGGAAACCACGTG
ATGTGAAGAAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATG
GGCCG**GCCCATTACGCAATTGGACGACAAC**AAAGTCTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTT
AAAAGAGTTGTGCAGATGATCCGTGGCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTA
AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTCGTTTTGCATTGAGTTTTCTCCG
TCGCATGTTTGCAGCATGAATCCAAACCACACGGAGTTCAAATTCCCACAGATTAAGGCTCGTCCGTCGCACAAGGTAATGTG
TGAATATTATATCTGTCGTGCAAAATTGCCTGGCCTGCACAATTGCTGTTATAGTTGGCGGCAGGGAGAGTTTTAACATT**GAC**
TAGCGTGCTGATAATTTGTGAGAAATAATAATTGACAAGTAGATACTGACATTTGAGAAGAGCTTCTGAACTGTTATTAGTAA
CAAAAATGGAAAGCTGATGCACGGAAAAAGGAAAGAAAAAGCCATACTTTTTTTTAGGTAGGAAAAGAAAAAGCCATACG
AGACTGATGTCTCTCAGATGGGCCGGGATCTGTCTATCTAGCAGGCAGCAGCCCACCAACCTCACGGGCCAGCAATTACGAG
TCCTTCTAAAAGCTCCCGCCGAGGGGCGCTGGCGCTGCTGTGCAGCAGCACGTCTAACATTAGTCCCACCTCGCCAGTTTAC
AGGGAGCAGAACCAGCTTATAAGCGGAGGCGCGGCACCAAGAAGCGNNNNNNNNNNNNNNNNNNNGTTTTAGA
GCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTGTCC
TTCTGTTTTTTTAGTCAGTCTCTTTTTTCAGAAGTACAACATCTT

Notes:

1. Underlined letters come from the PCR fragment, while the rest come from the binary vectors, such as pG3B/H-U3Ub, pG3GB411, and pG3GB411-BWM.
2. Boxed letters indicate primer sites.
3. Primer sequences are as follows:

Colony PCR primers (5'→3'):
OsU3p-F4: GCCCATTACGCAATTGGACGACAAC
TaU3p-R: CTCACAAATTATCAGCACGCTAGTC
(OsU3p-F4 + TaU3p-R = 421 bp)

Sequencing primers (5'→3'):
OsU3p-F4

Identify correct clones of MR vectors by additional colony PCR

The > 8-kb MR modules may cause the binary vectors to become unstable during assembly process of sgRNA cassettes. Therefore, additional colony PCR reactions are required for identification of positive clones of MR vectors. Please note: small colonies are more probable to be positive clones than large colonies if these two types of colonies co-exist in the same LB agar plates. Sequences of primers for additional colony PCR reactions for identification of MR vectors, such as pG3GB411-BWM, are as follows:

zCas9-IDF: cggcctcgatattgggactaactct
zCas9-IDR: cttatctgtggagtccacgagcttc
zCas9-IDF + zCas9-IDR = 424-bp

Rab17-IDF: agttgtagaaactacacttagaacc
Rab17-IDR: ggattttaccggtgctcatccattattc
Rab17-IDF + Rab17-IDR = 450-bp

mCherry-IDF2: gcaggacggcgagttcatctac
HSPt-IDR: ccatagtccataccatagcacatac
mCherry-IDF2 + HSPt-IDR = 615-bp

Gly-IDF: cgatgggccgcgtgttgaac
Gly-IDR: caaggcgcgggttgatgact
Gly-IDF + Gly-IDR = 505-bp

Bar-IDF: ctgcaccatcgtcaaccactac
Bar-IDR: cagaaacccacgtcatgccagt
Bar-IDF+Bar-IDR = 429-bp