Advanced Cell Biology & Biotechnology

Biotechnology Project Lab

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& Isabella Tarulli

The lecture of November 22nd 2021 is about to begin….

Biotechnology Project – V lesson

0 – Summary of the previous lesson

1 - Subcloning NRG1-III-β3 from pCR-Blunt II-TOPO into the expression vector pEGFP-C3

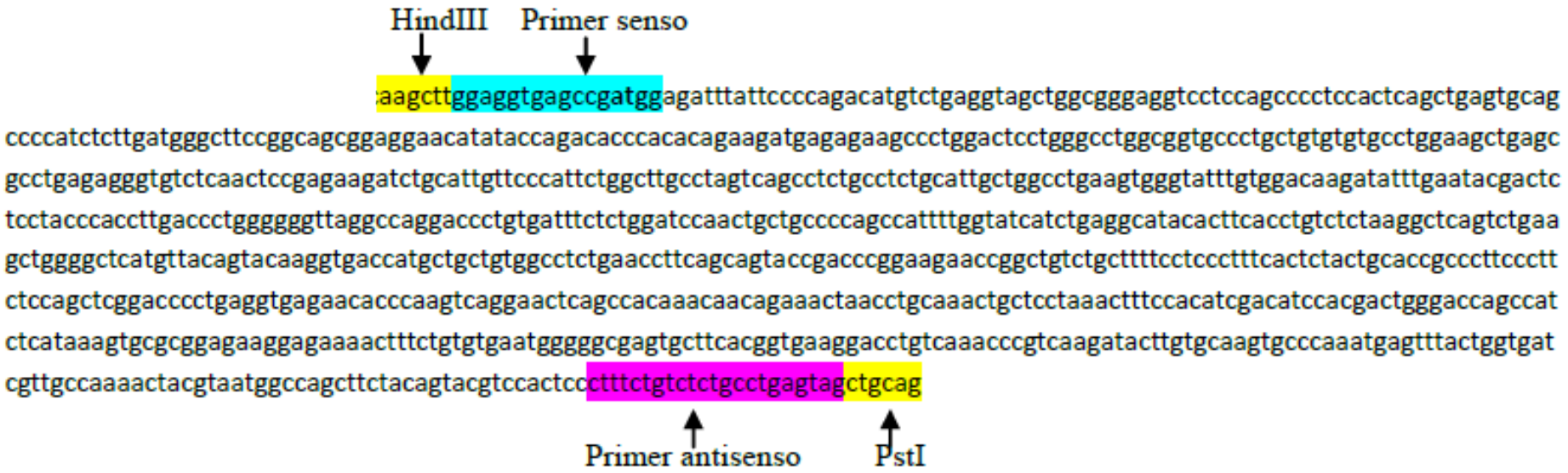
2 - Subcloning NRG1-III-β3 from pCR-Blunt II-TOPO or pEGFP-C3 into the expression vector pCMV-Tag4

5′-CGTTAACTTGACC**ATG**TGCATC**TAG**CTCCATGGCATGC-3′

5′-CGTTAACTTGACC**ATG**TGCATC**TAG**CTCCATGGCATGC-3′
3′-GCAATTGAACTGGTACACGTAGATCGAGGTACCGTACG-5′

Primer sense: 5′-AAGCTTCGTTAACTTG-3′
Primer antisense: 5′-CTGCAGGCATGCCATG-3′

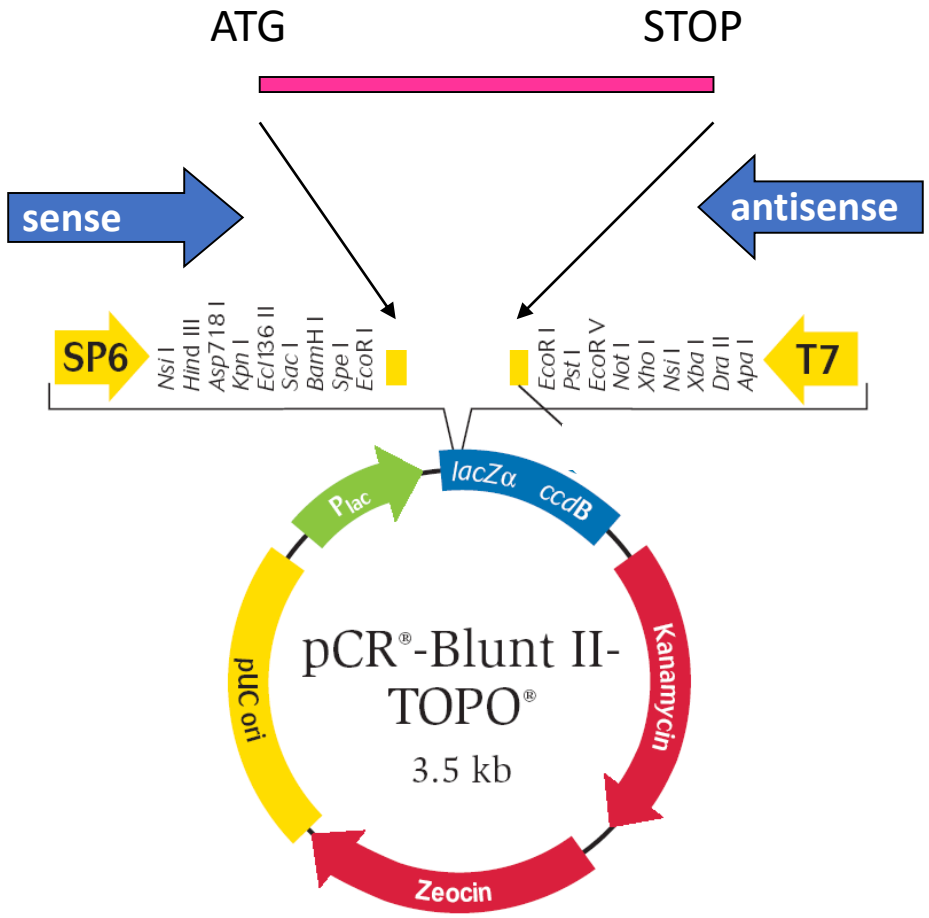
5′-AAGCTTCGTTAACTTGACCATGTGCATCTAGCTCCATGGCATGCCTGCAG-3′
3′-TTCGAAGCAATTGAACTGGTACACGTAGATCGAGGTACCGTACGGACGTC-5′



Is NRG1 cloned sense or antisense within pCR-Blunt II-TOPO vector?

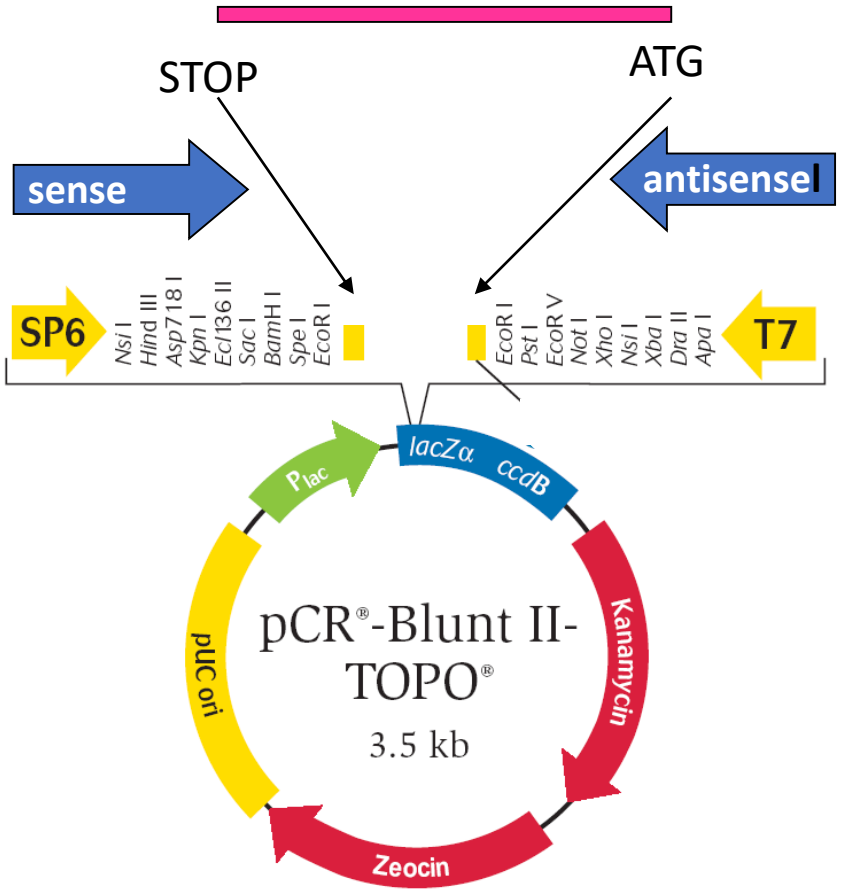
INSERT cloned in SENSE orientation

5′-**ATG**CGTACCTTTAACTCG**TAG**-3′
3′-TACGCATGGAAATTGAGCATC-5′



INSERT cloned in ANTISENSE orientation

5′-CTACGAGTTAAAGGTACGCAT-3′
3′-**GAT**GCTGAATTTCCATGC**GTA**-5′



200bp 100bp

SENSE ORIENTATION

sense

5′-**ATG**CGTACCTTTAACTCG**TAG**-3′

3′-TACGCATGGAAATTGAGCATC-5′

antisense

SENSE: NNNNNN**ATG**CGTACCTTTAACTCG**TAG**NNNNNN

ANTISENSE: NNNNNNCTACGAGTTAAAGGTACGCATNNNNNN

BLAST:

Query: 1 **ATG**CGTACCTTTAACTCG**TAG** 21

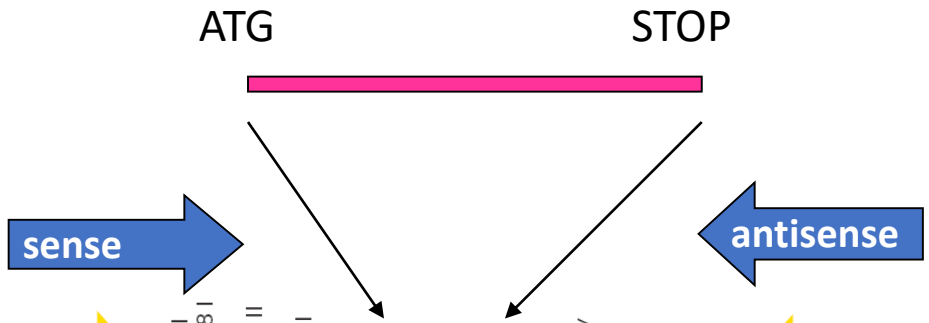
||||||||||||||||||||

Subjct Sense : 201 **ATG**CGTACCTTTAACTCG**TAG** 221

Query: 1 **ATG**CGTACCTTTAACTCG**TAG** 21

||||||||||||||||||||

Subjct Antisense: 121 **ATG**CGTACCTTTAACTCG**TAG** 101



200bp 100bp

ANTISENSE ORIENTATION

sense

5′-CTACGAGTTAAAGGTACGCAT-3′

3′-**GAT**GCTGAATTTCCATGC**GTA**-5′

antisense

SENSE: NNNNNNNCTACGAGTTAAAGGTACGCATNNNNNNN

ANTISENSE: NNNNNNN**ATG**CGTACCTTTAACTCG**TAG**NNNNNNN

BLAST:

Query: 1 **ATG**CGTACCTTTAACTCG**TAG** 21

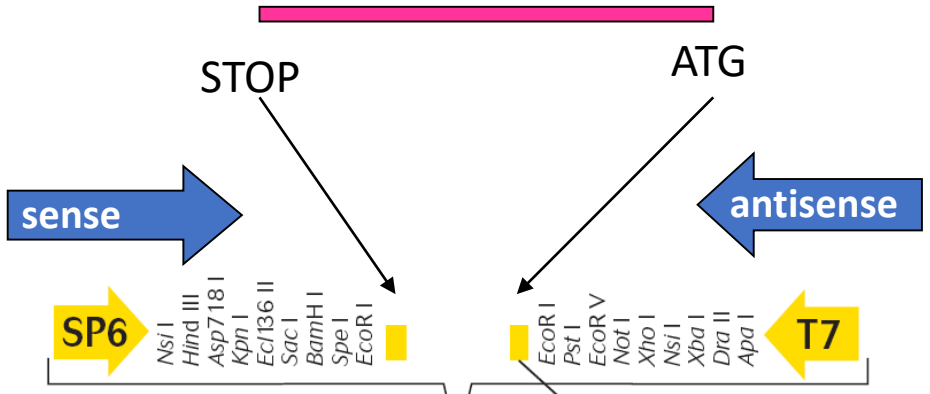
||||||||||||||||||||

Subjct Sense: 221 **ATG**CGTACCTTTAACTCG**TAG** 201

Query: 1 **ATG**CGTACCTTTAACTCG**TAG** 21

||||||||||||||||||||

Subjct Antisense: 101 **ATG**CGTACCTTTAACTCG**TAG** 121



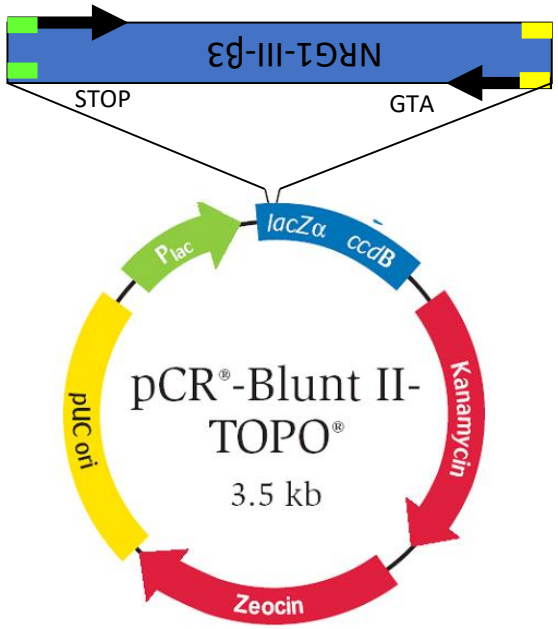
Biotechnology Project – V lesson

0 – Summary of the previous lesson

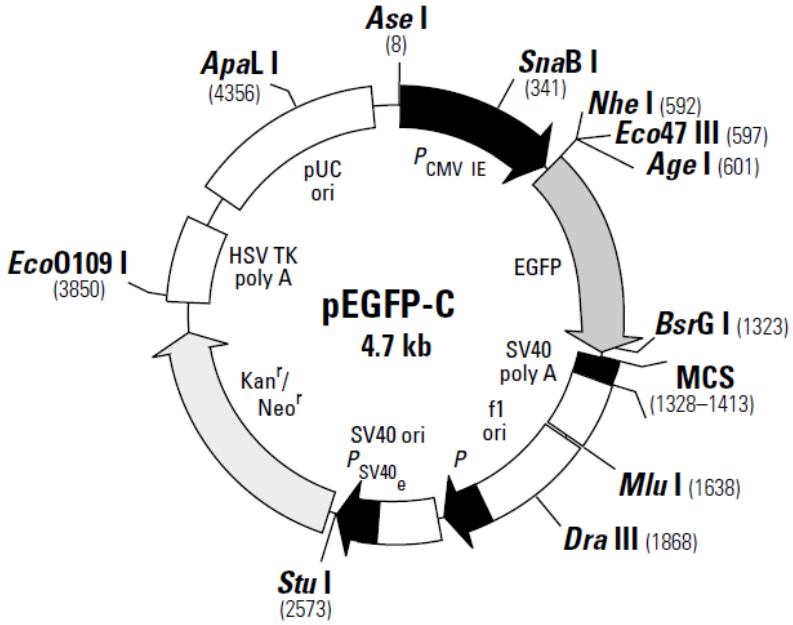
1 - Subcloning NRG1-III-β3 from pCR-Blunt II-TOPO into the expression vector pEGFP-C1, 2 or 3

2 - Subcloning NRG1-III-β3 from pCR-Blunt II-TOPO or pEGFP-C3 into the expression vector pCMV-Tag4

1-Subcloning NRG1-III-β3 from pCR-Blunt II-TOPO into pEGFP-C

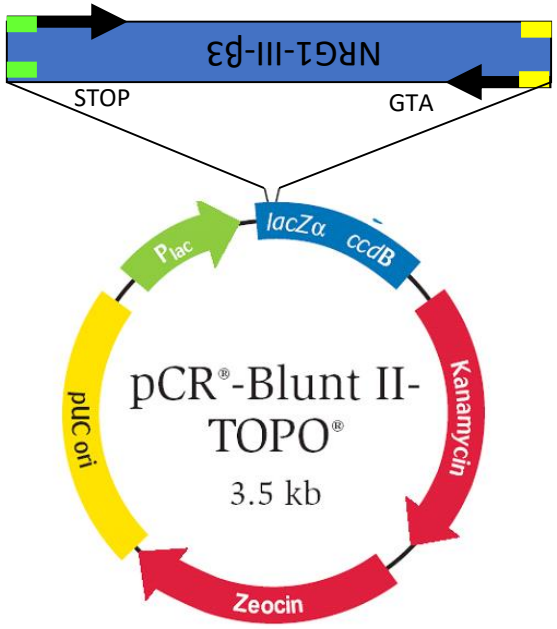


pCR-bluntII-TOPO-NRG1β3



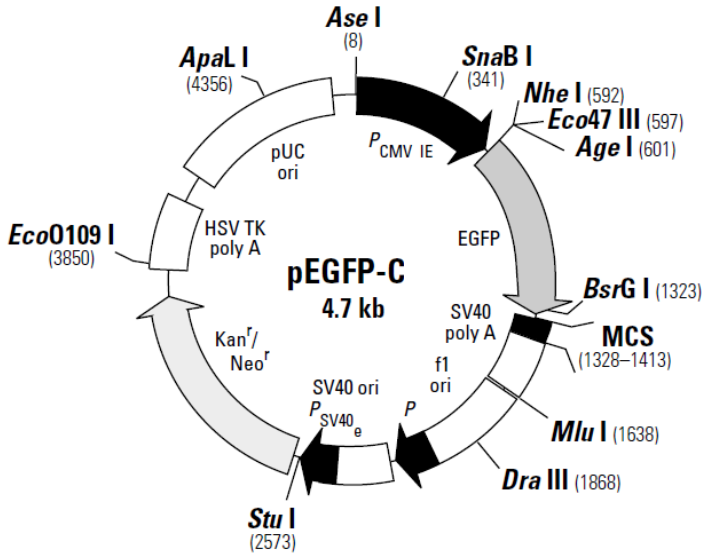
pEGFP-C

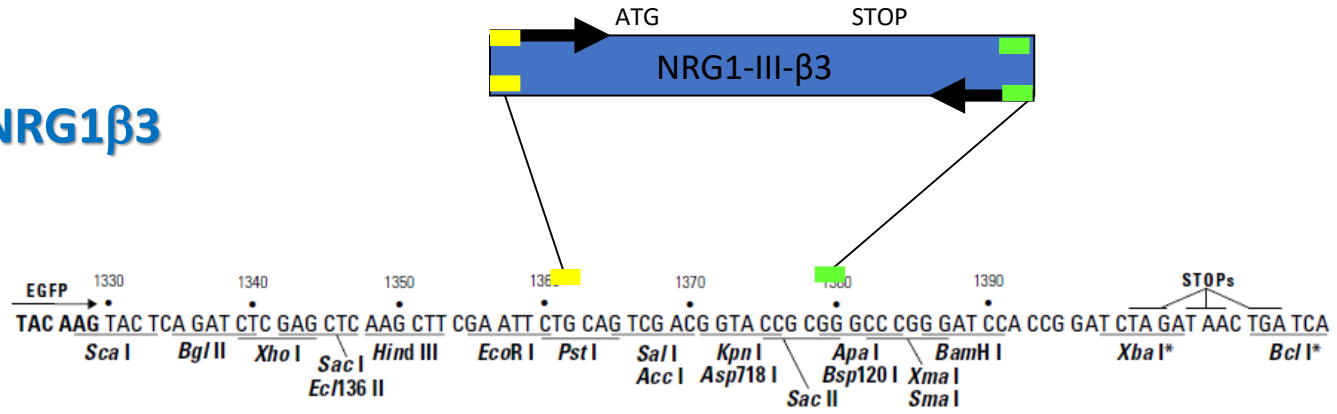
both c/d and g/h were cloned antisense



pCR-bluntII-TOPO-NRG1β3

pEGFP-C

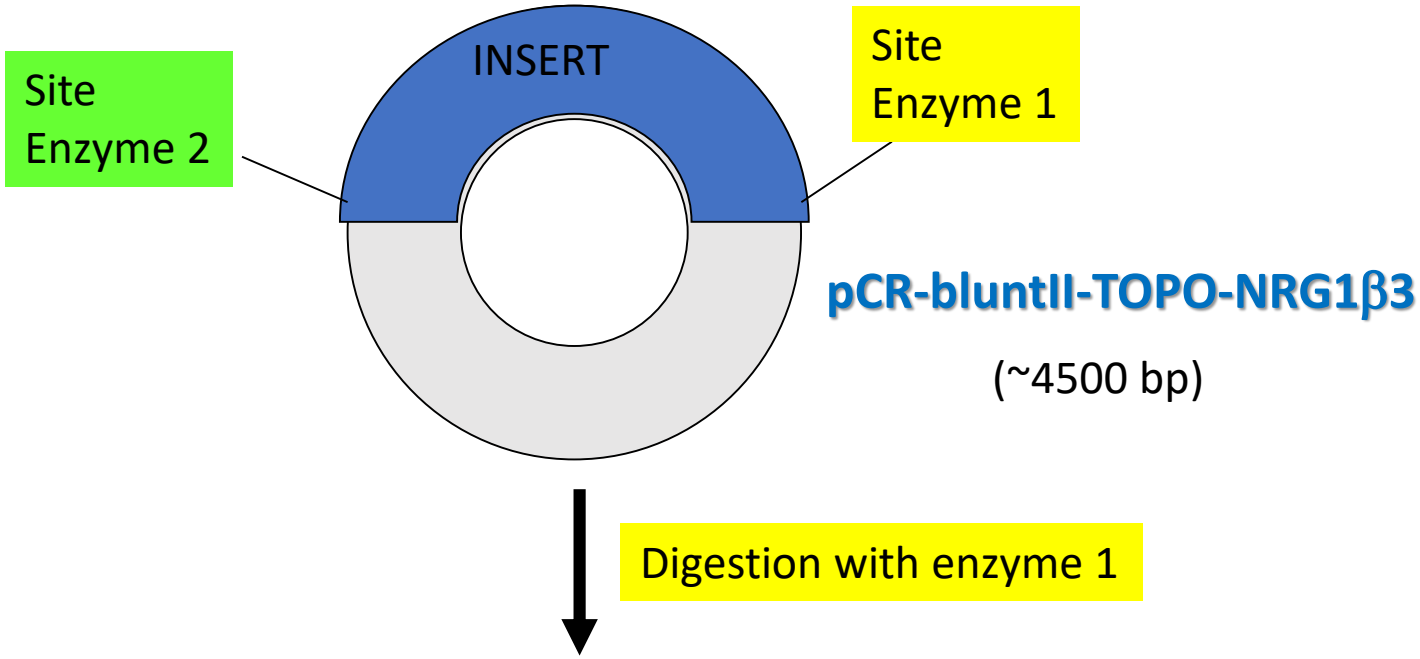


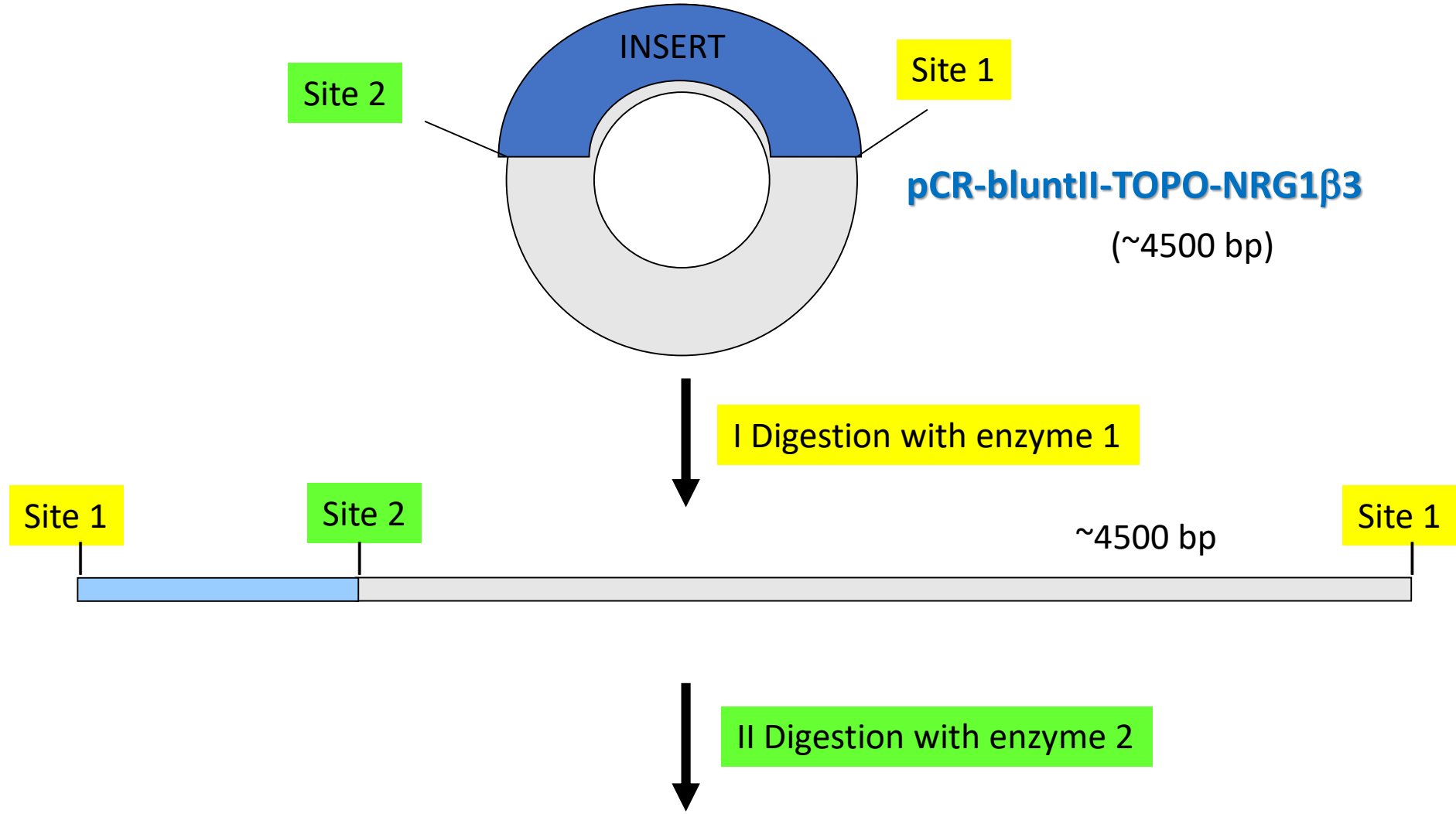


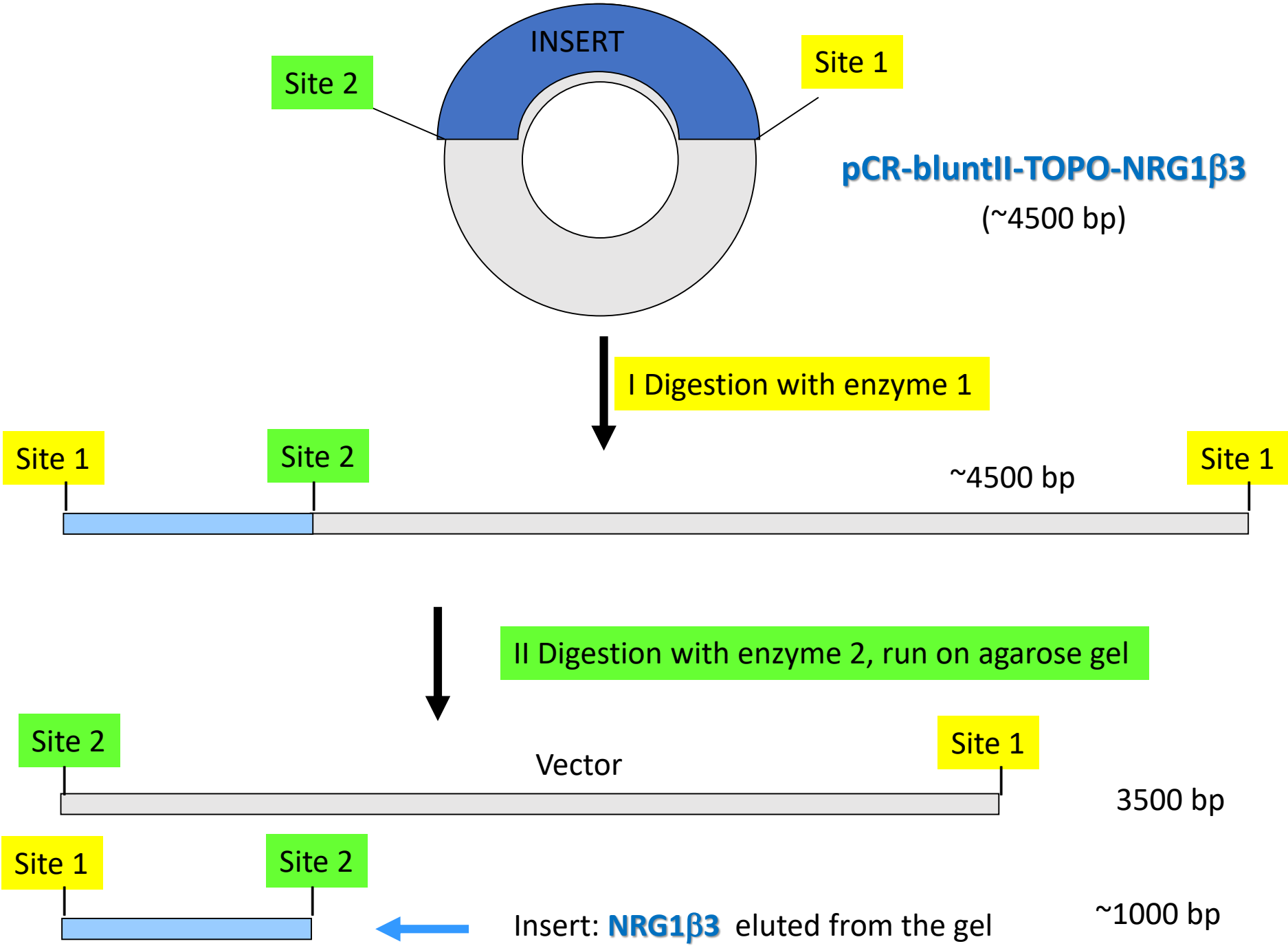
To transfer the insert (cDNA coding NRG1IIIβ3) from the vector **pCR-blunt II TOPO** into the new vector **pEGFP-C** you have to digest both vectors:

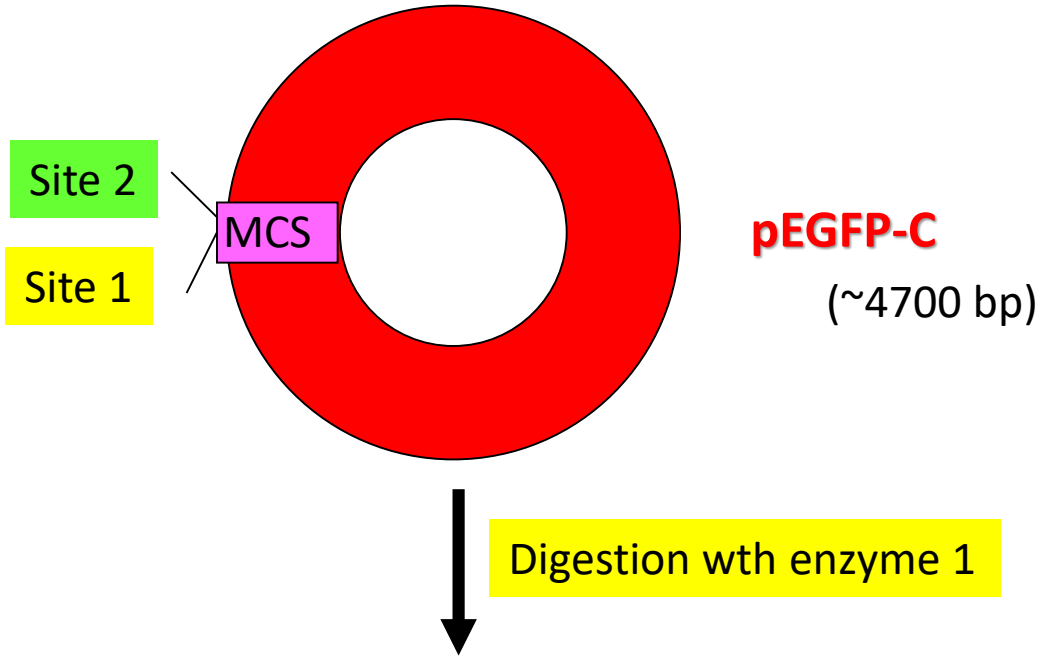
1- with the restriction enzyme that cuts on the upstream primer (**yellow**);

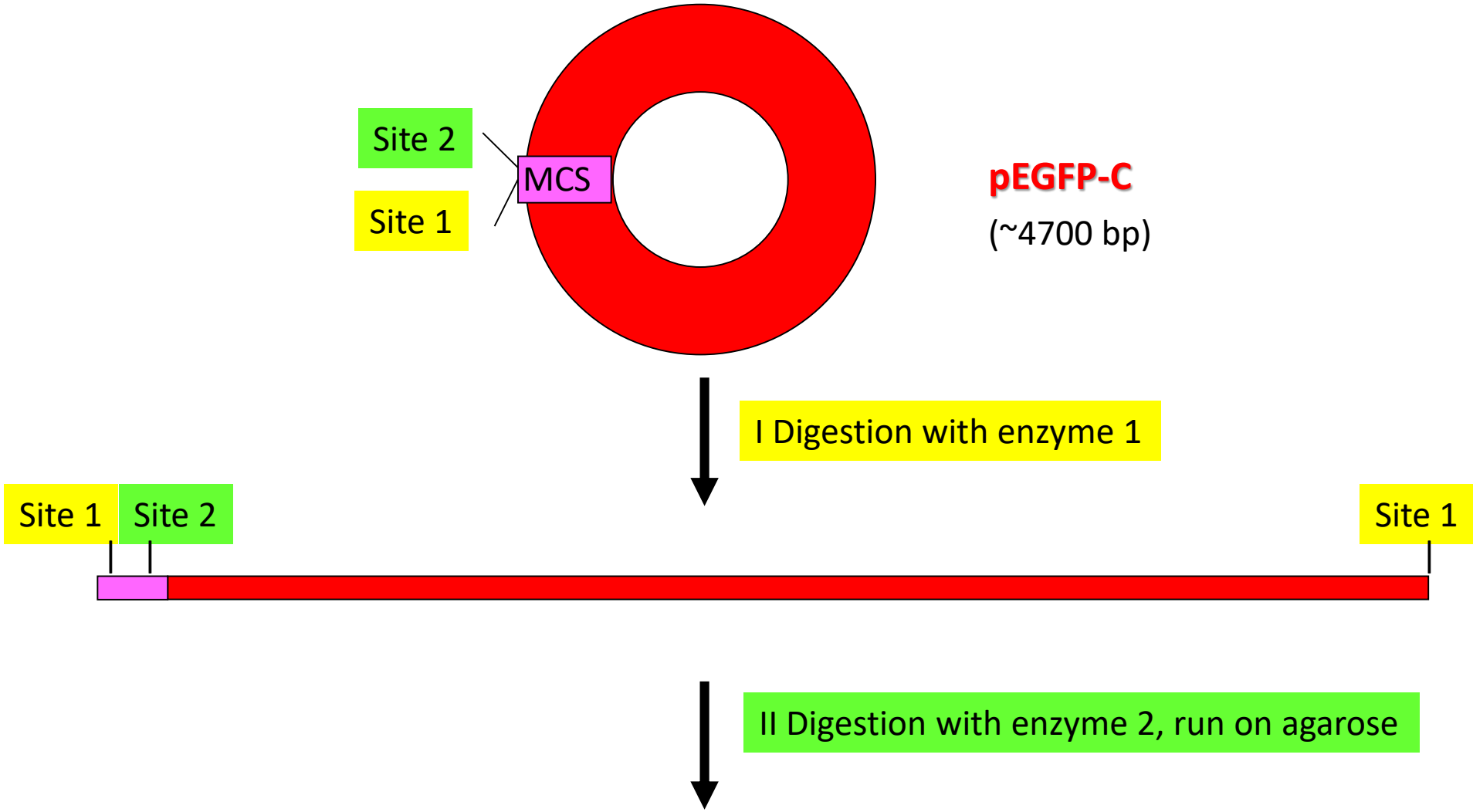
2- with the restriction enzyme that cuts on the downstream primer (**green**).

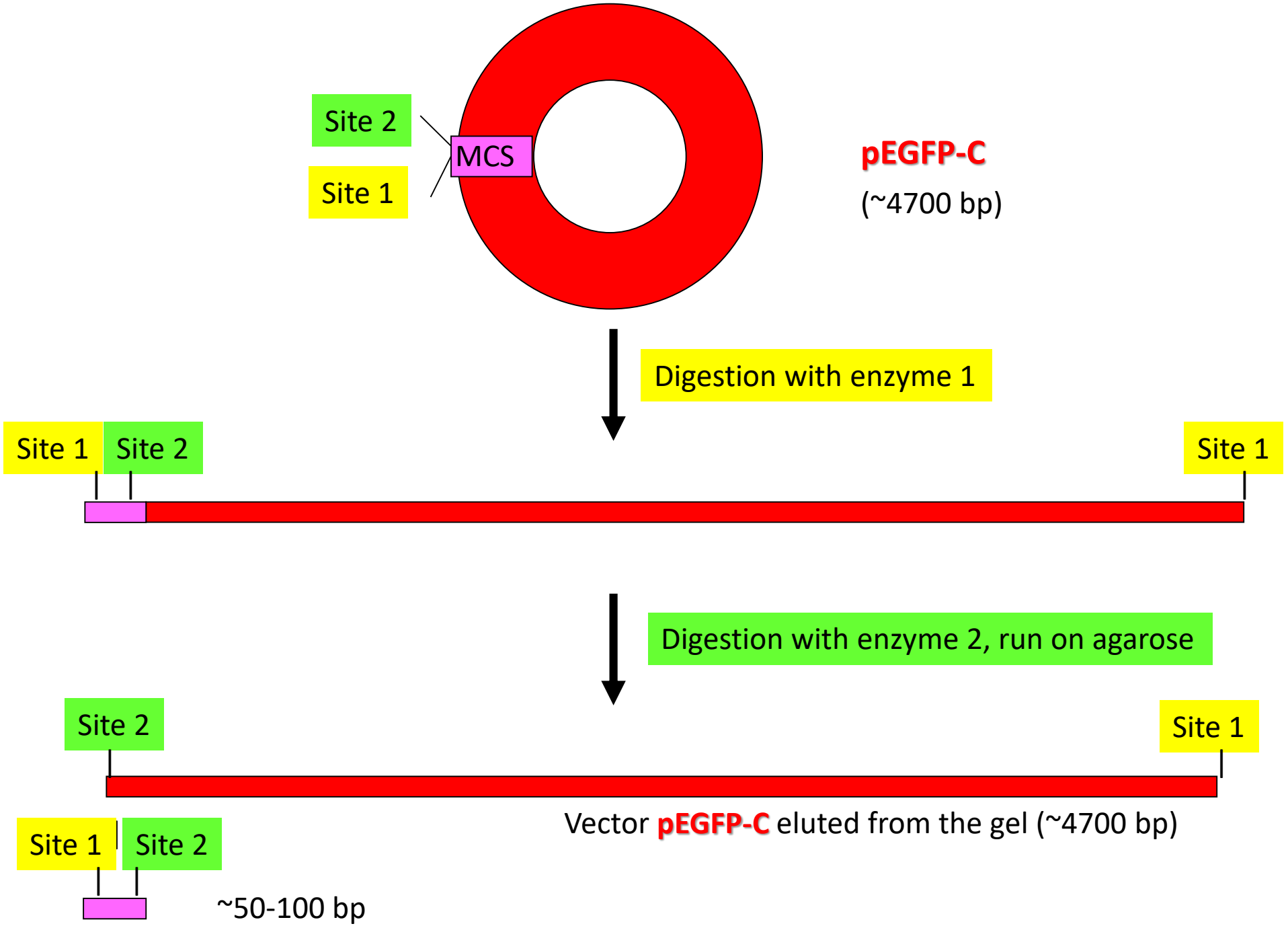

INSERT
Site
Enzyme 2
Site
Enzyme 1
pCR-bluntII-TOPO-NRG1β3
(~4500 bp)
Digestion with enzyme 1


INSERT
Site 2
Site 1
pCR-bluntII-TOPO-NRG1β3
(~4500 bp)
I Digestion with enzyme 1
Site 1
Site 2
~4500 bp
Site 1
II Digestion with enzyme 2


INSERT
Site 2
Site 1
pCR-bluntII-TOPO-NRG1β3
(~4500 bp)
I Digestion with enzyme 1
Site 1
Site 2
~4500 bp
Site 1
II Digestion with enzyme 2, run on agarose gel
Site 2
Vector
Site 1
3500 bp
Site 1
Site 2
Insert: NRG1β3 eluted from the gel
~1000 bp

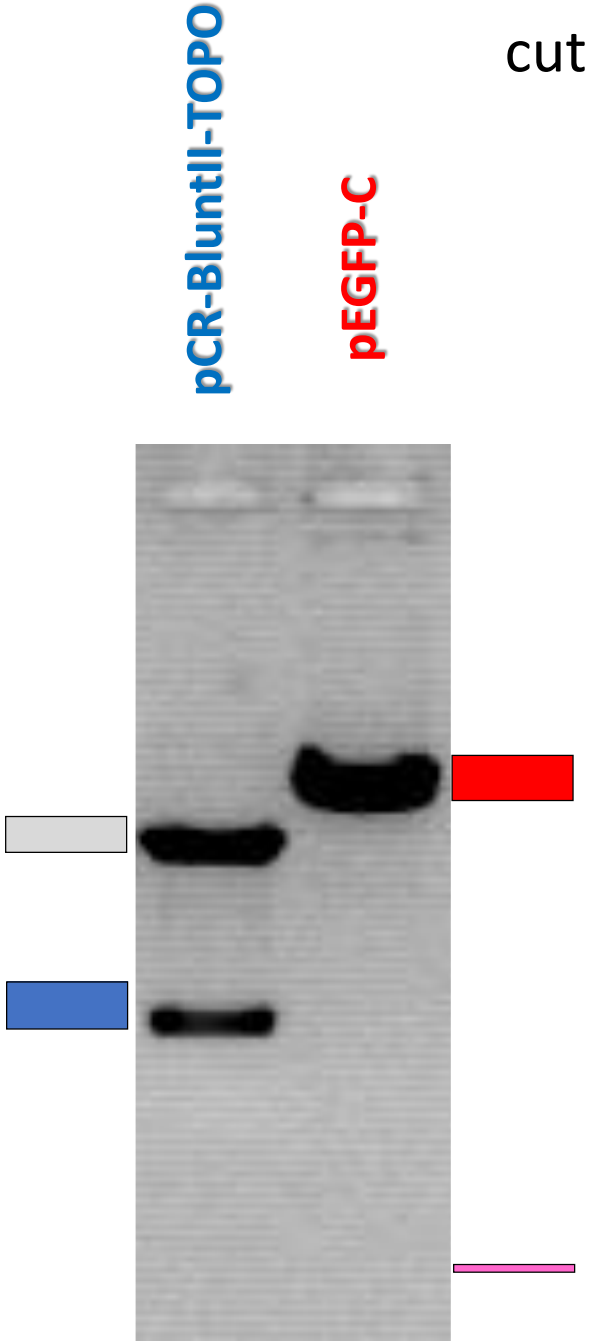

Site 2
MCS
Site 1
pEGFP-C
(~4700 bp)
Digestion wth enzyme 1

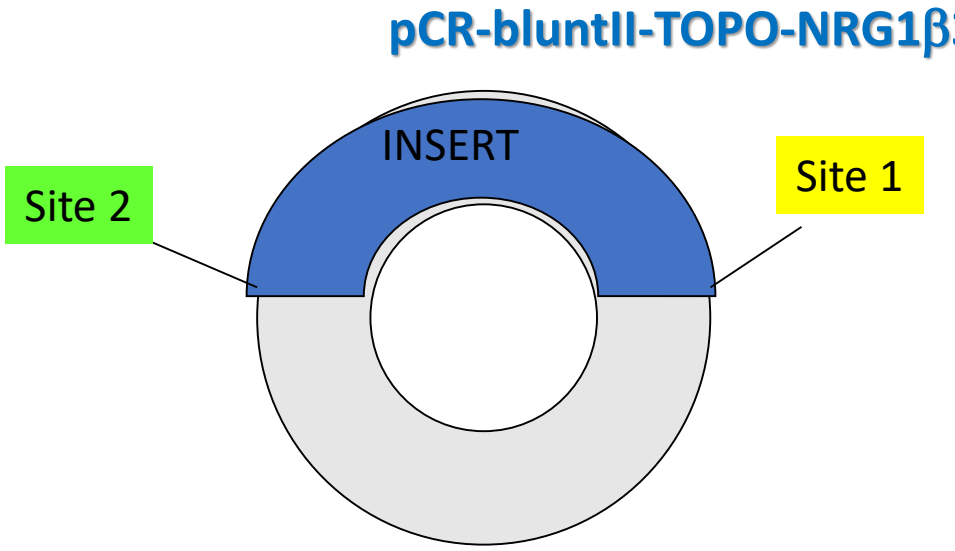

Site 2
MCS
Site 1
pEGFP-C
(~4700 bp)
I Digestion with enzyme 1
Site 1
Site 2
Site 1
II Digestion with enzyme 2, run on agarose

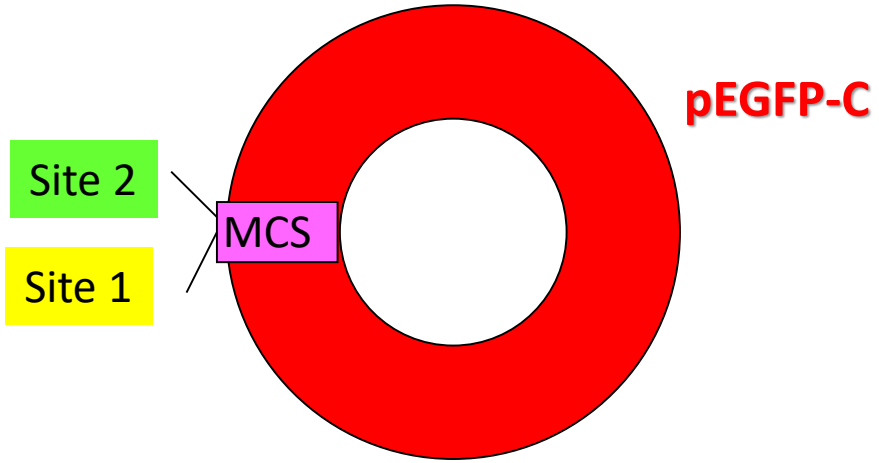

Site 2
MCS
Site 1
pEGFP-C
(~4700 bp)
Digestion with enzyme 1
Site 1
Site 2
Site 1
Digestion with enzyme 2, run on agarose
Site 2
Site 1
Vector pEGFP-C eluted from the gel (~4700 bp)
Site 1
Site 2
~50-100 bp

pCR-Blunt II-TOPO & pEGFP-C

cut with the enzyme 1 and enzyme 2







I digestion

pCR-BluntII-TOPO-NRG1-III-β3 (2,5 µg/µl)		µl	-> 20 µg
buffer 10x (which?)		µl	-> 1x
Enzyme I (which?)		µl	-> 1u/µg
BSA 10x (1 µg/µl)		µl	-> 1x (0,1 µg/µl)
H2O			
total	50	µl	

pEGFP-C (1, 5 µg/µl)		µl	-> 5 µg
buffer 10x (which?)		µl	-> 1x
Enzyme I (which?)		µl	-> 1u/µg
BSA 10x (1 µg/µl)		µl	-> 1x (0,1 µg/µl)
H2O			
total	50	µl	

- after digestion with the first enzyme, verify that digestion is complete by running 5 µl on agarose gel

- purify DNA by loading it on a column

- elute purified DNA in 50µl water

- proceed with the second digestion

II digestion

pCR-BluntII-TOPO-NRG1-III-β3 µl **(how many µl?)**

(already digested with enzyme I)

buffer 10x (which?)	µl	-> 1x
Enzyme II (which?)	µl	-> 1u/µg
BSA 10x (1 µg/µl)	µl	-> 1x (0,1 µg/µl)
H2O		

---

total µl **(how many µl?)**

pEGFP-C µl **(how many µl?)**

(already digested with enzyme I)

buffer 10x (which?)	µl	-> 1x
enzyme II (which?)	µl	-> 1u/µg
BSA 10x (1 µg/µl)	µl	-> 1x (0,1 µg/µl)
H2O		

---

total µl **(how many µl?)**

- after digestion with the second enzyme, verify that digestion is complete by running 5 µl on agarose gel

- run the digestion on an agarose gel and purify the insert and the vector for the following ligase reaction

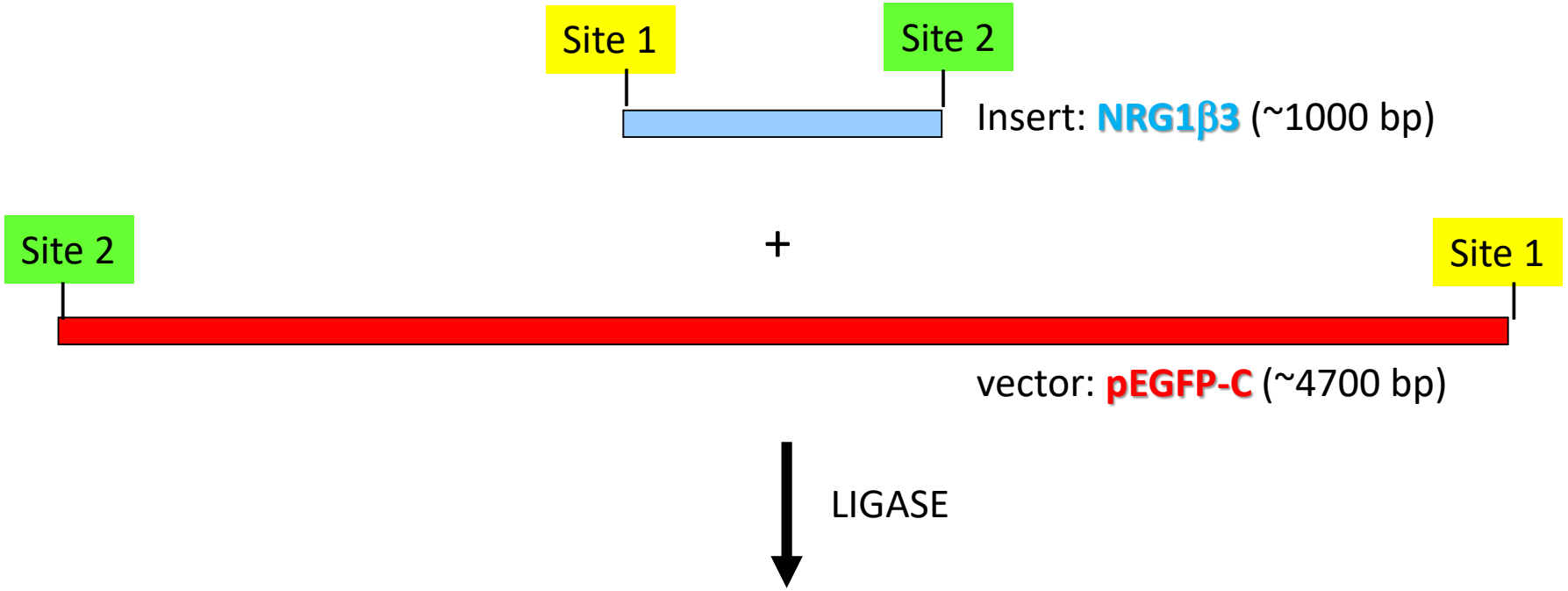
If the two enzymes cut DNA in the same buffer,
you can digest DNA with both enzymes together

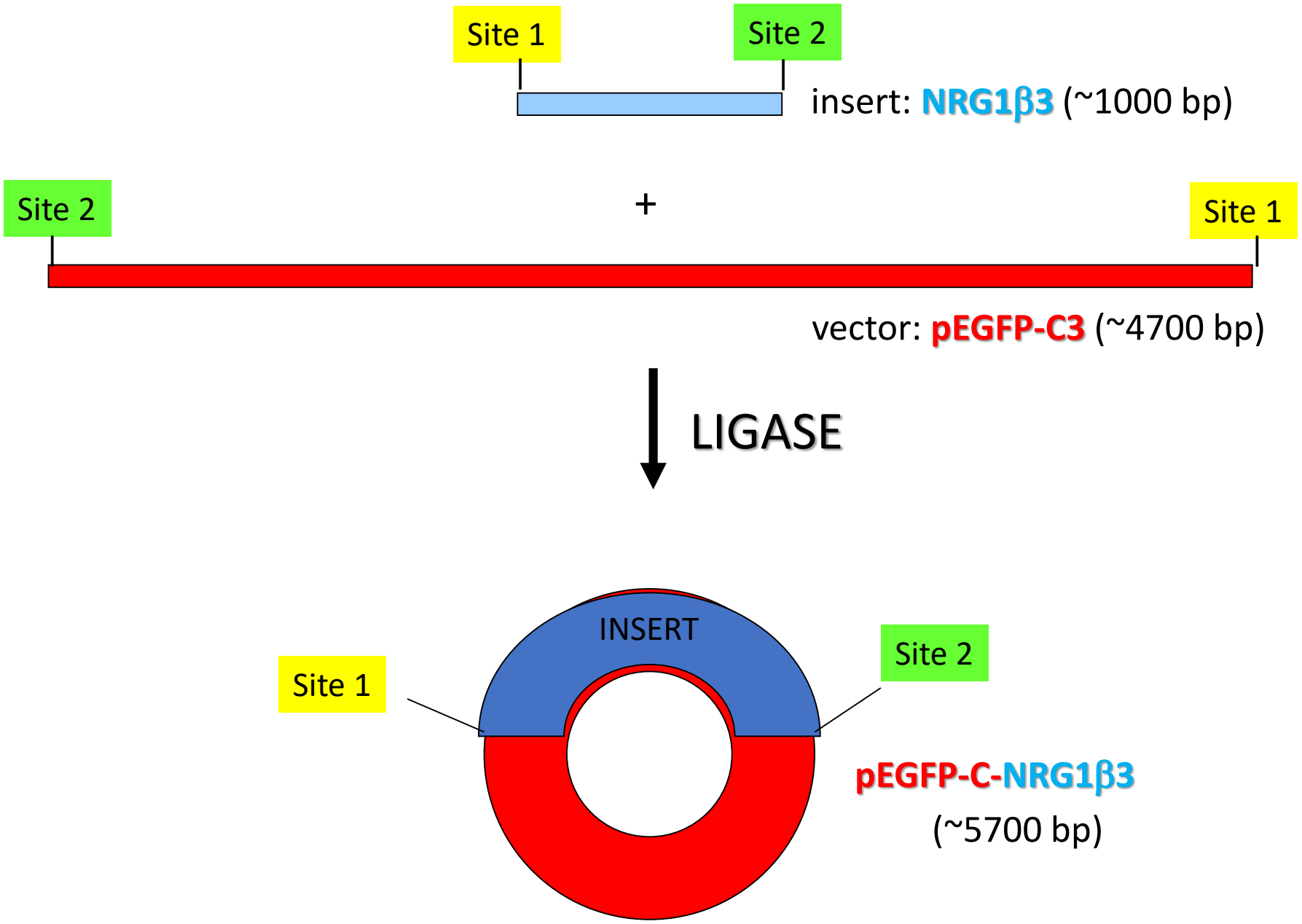
Single digestion (enzime I + enzime II)

pCR-BluntII-TOPO-NRG1-III-β3 (2,5 µg/µl)		µl	-> 20 µg
buffer 10x (which?)		µl	-> 1x
Enzyme I (which?)		µl	-> 1u/µg
Enzyme II (which?)		µl	-> 1u/µg
BSA 10x (1 µg/µl)		µl	-> 1x (0,1 µg/µl)
H2O			
total	50	µl	

Use about 1 unit (u) enzyme/µg DNA

pEGFP-C (1,5 µg/µl)		µl	-> 5 µg
buffer 10x (which?)		µl	-> 1x
Enzyme I (which?)		µl	-> 1u/µg
Enzyme II (which?)		µl	-> 1u/µg
BSA 10x (1 µg/µl)		µl	-> 1x (0,1 µg/µl)
H2O			
total	50	µl	


Site 1
Site 2
Insert: NRG1β3 (~1000 bp)
Site 2
+
Site 1
vector: pEGFP-C (~4700 bp)
LIGASE


Site 1
Site 2
insert: NRG1β3 (~1000 bp)
Site 2
+
Site 1
vector: pEGFP-C3 (~4700 bp)
LIGASE
INSERT
Site 1
Site 2
pEGFP-C-NRG1β3
(~5700 bp)

I Exercise:

• identify the buffers to digest with enzyme 1 and enzyme 2 the vector with the insert (**pCR-bluntII-TOPO**) and the vector for the following subcloning (**pEGFP-C**)

As we do in the in notebook in the lab, write the protocol for all necessary passages to digest the vector with the insert (pCR-bluntII-TOPO) and the vector for the following subcloning (pEGFP-C3):

• first digestion (write all ingredients of the first reaction)
• purification of the DNA?
• second digestion (write all ingredients of the second reaction)

OR

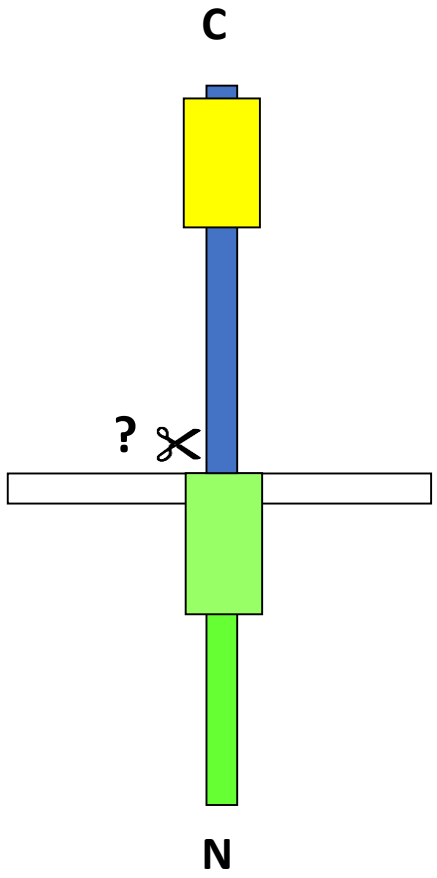
• first & second digestion together (write all ingredients of the reaction)

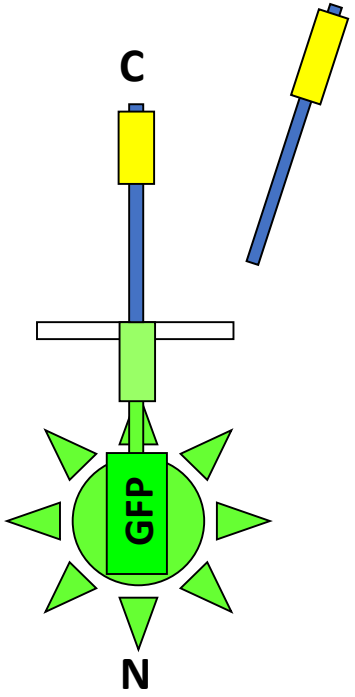
Biotechnology Project – V lesson

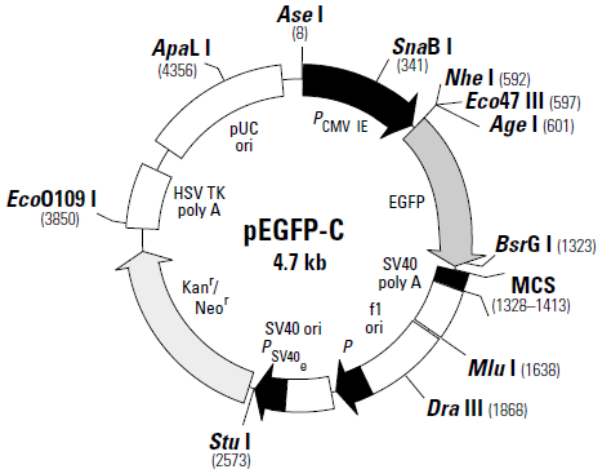
0 – Summary of the previous lesson

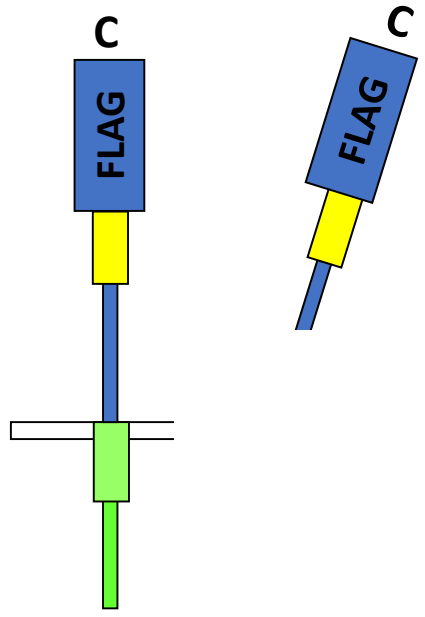
1 - Subcloning NRG1-III-β3 from pCR-Blunt II-TOPO into the expression vector pEGFP-C3

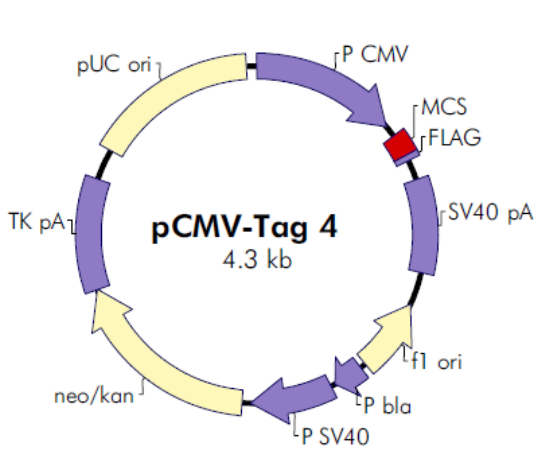
2 - Subcloning NRG1-III-β3 from **pCR-Blunt II-TOPO** or **pEGFP-C** into the expression vector **pCMV-Tag4**



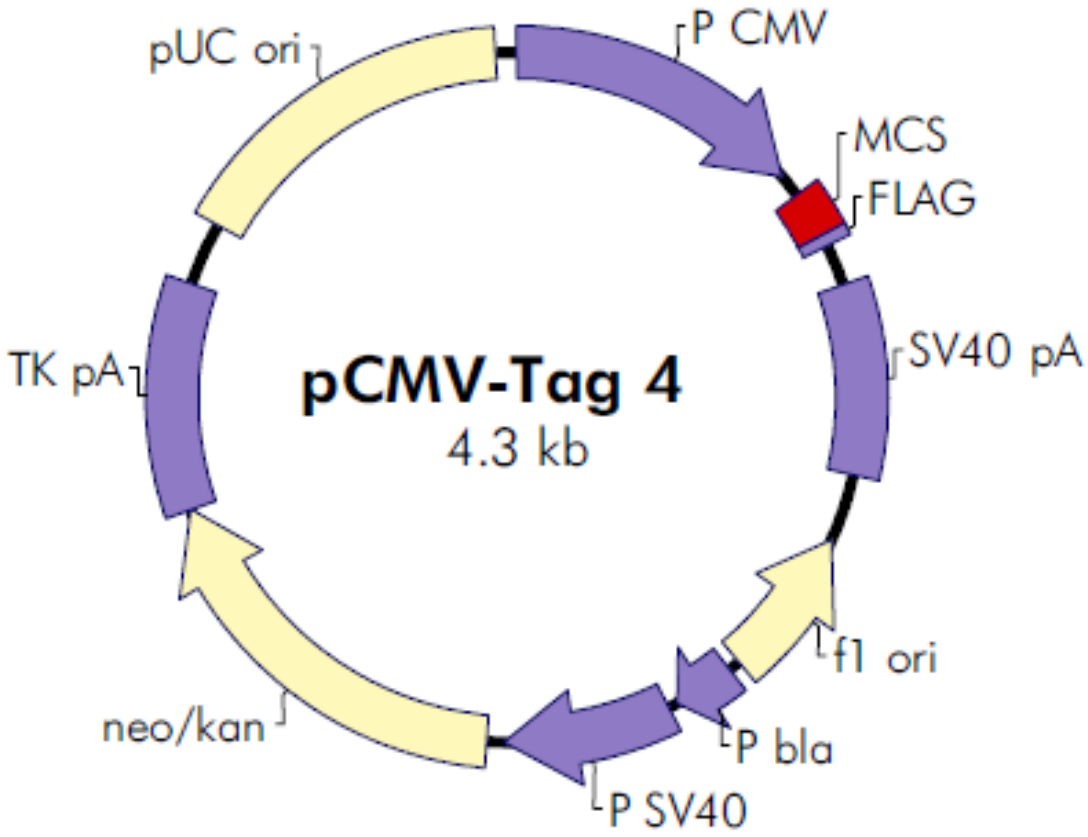




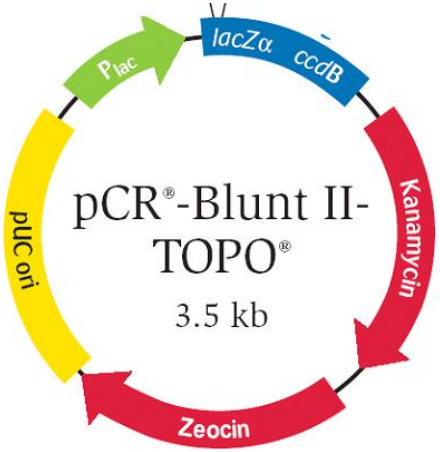


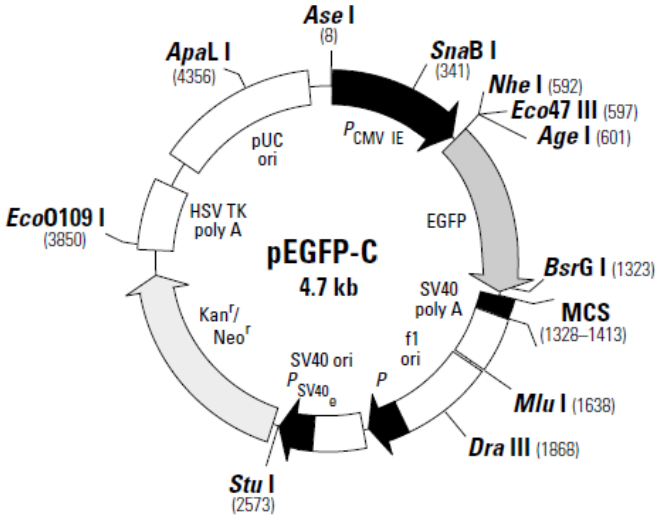


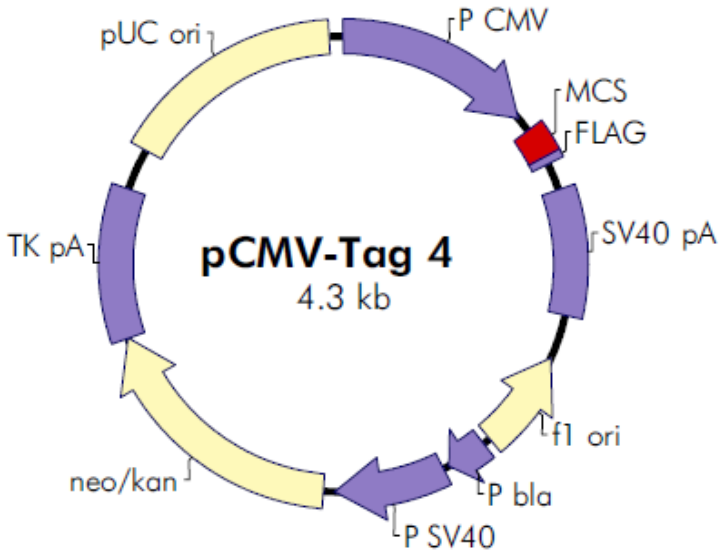
2-Subcloning into the expression vector **pCMV-Tag4**

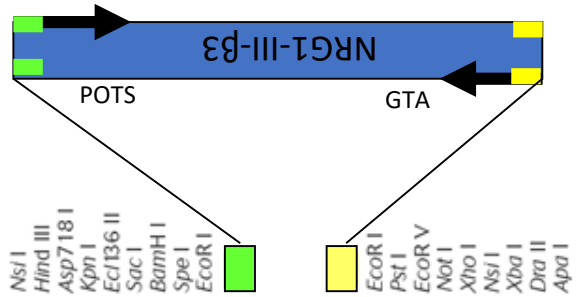


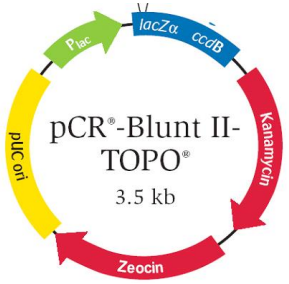
Subcloning into the expression vector **pCMV-Tag4**

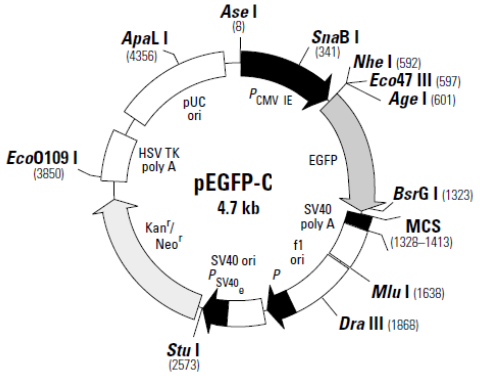


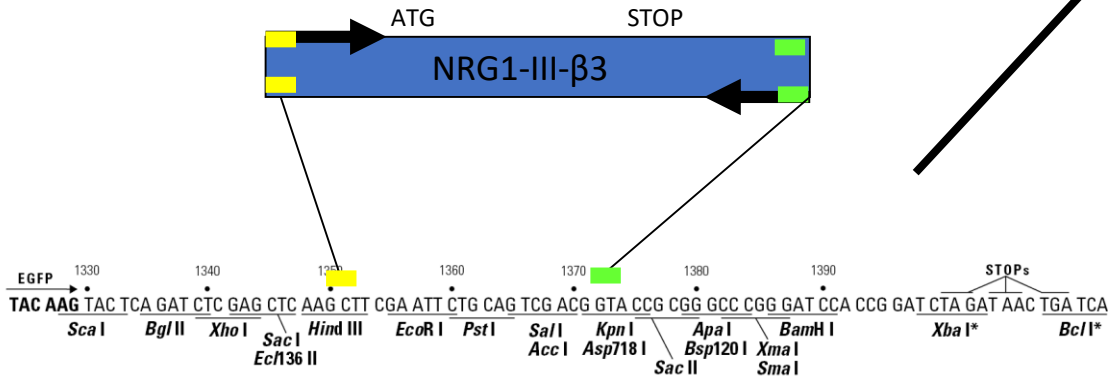


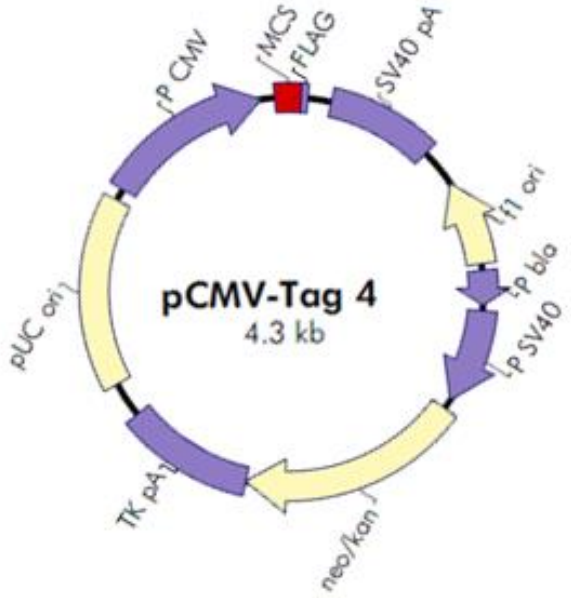



NRG1-III-β3
POTS
GTA
Nsi I
Hind III
Asp718 I
Kpn I
Ecl136 II
Sac I
BamH I
Spe I
EcoR I
EcoR I
Pst I
EcoR V
Not I
Xho I
Nsi I
Xba I
Dra II
Apa I

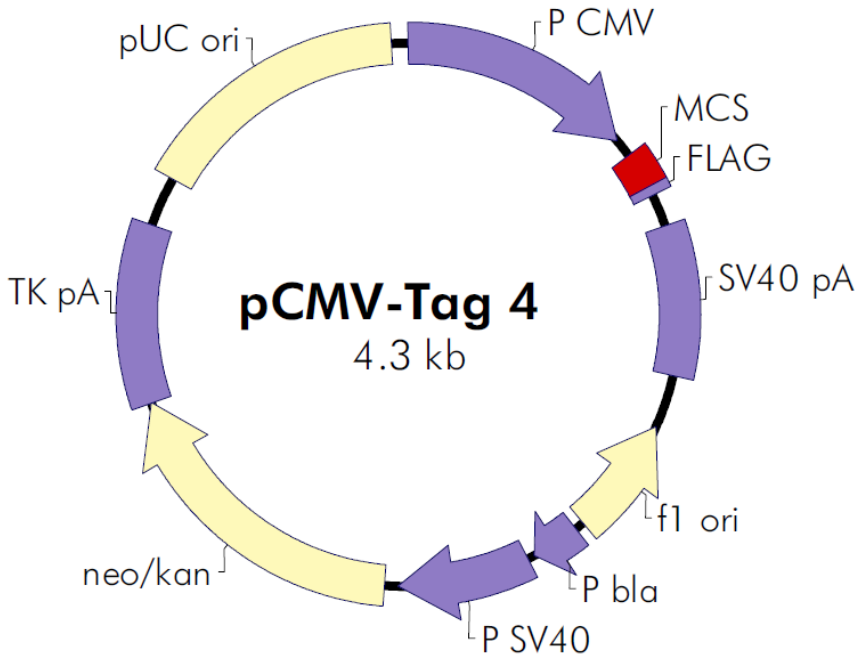

pCR®-Blunt II-TOPO®
3.5 kb
lacZα
ccdB
Kanamycin
Zeocin
pUC ori


pEGFP-C
4.7 kb
Ase I
(8)
ApaL I
(4356)
SnaB I
(341)
Nhe I (592)
Eco47 III (597)
Age I (601)
pUC ori
P CMV IE
EGFP
HSV TK poly A
Eco0109 I
(3850)
BsrG I (1323)
SV40 poly A
MCS
(1328–1413)
Kanr/ Neor
f1 ori
SV40 ori
Mlu I (1638)
Dra III (1868)
Stu I
(2573)


ATG
STOP
NRG1-III-β3
EGFP
TAC AAG TAC TCA GAT CTC GAG CTC AAG CTT CGA ATT CTG CAG TCG ACG GTA CCG CGG GCC CGG GAT CCA CCG GAT CTA GAT AAC TGA TCA
Sca I
Bgl II
Xho I
Sac I
Ecl136 II
Hind III
EcoR I
Pst I
Sal I
Acc I
Kpn I
Asp718 I
Sac II
Apa I
Bsp120 I
Xma I
Sma I
BamH I
Xba I*
Bcl I*
STOPs


pCMV-Tag 4
4.3 kb
P CMV
MCS
FLAG
SV40 pA
f1 ori
P bla
P SV40
neo/kan
TK pA
pUC ori

pCMV-Tag 4A



pCMV-Tag 4 Multiple Cloning Site Region
(sequence shown 620–839)

T3 promoter

Sac I BstX I Sac II Not I

A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT A...

Srf I BamH I Pst I EcoR I EcoR V Hind III Acc I/Sal I

...GC CCG GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC* *

Xho I FLAG tag

D Y K D D D D K

...CTC GAG GAT TAC AAG GAT GAC GAC GAT AAG TAG GGCCCGGTACCT...

STOP

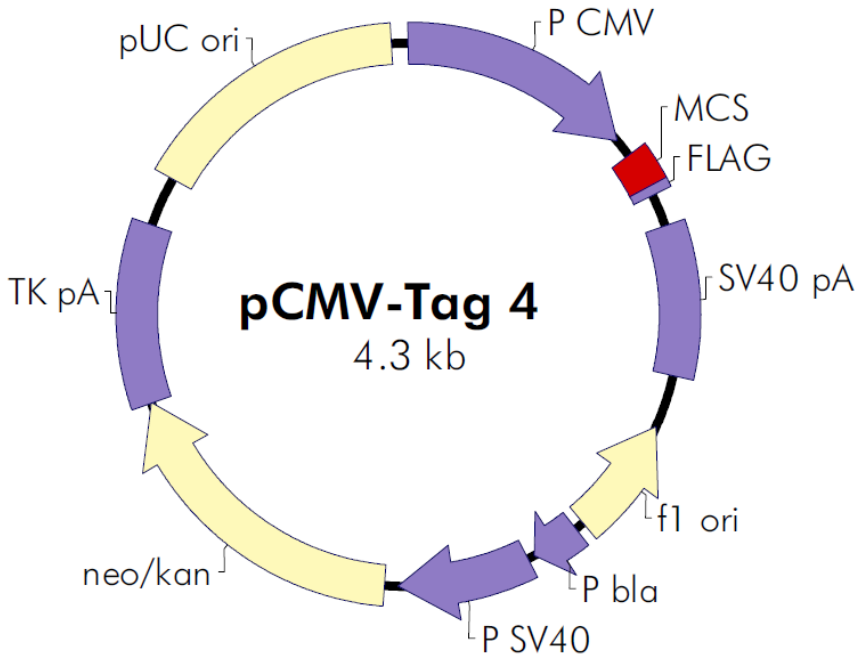
T7 promoter

...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTA

MULTIPLE STOP CODONS

* In pCMV-Tag 4A, no bases inserted; in pCMV-Tag 4B, A inserted; in pCMV-Tag 4C, AA inserted

pCMV-Tag 4B



pCMV-Tag 4 Multiple Cloning Site Region
(sequence shown 620–839)

T3 promoter

Sac I BstX I Sac II Not I

A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT A...

Srf I BamH I Pst I EcoR I EcoR V Hind III Acc I/Sal I

...GC CCG GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC* A

Xho I FLAG tag

D Y K D D D D K

...CTC GAG GAT TAC AAG GAT GAC GAC GAT AAG TAG GGCCCGGTACCT...

STOP

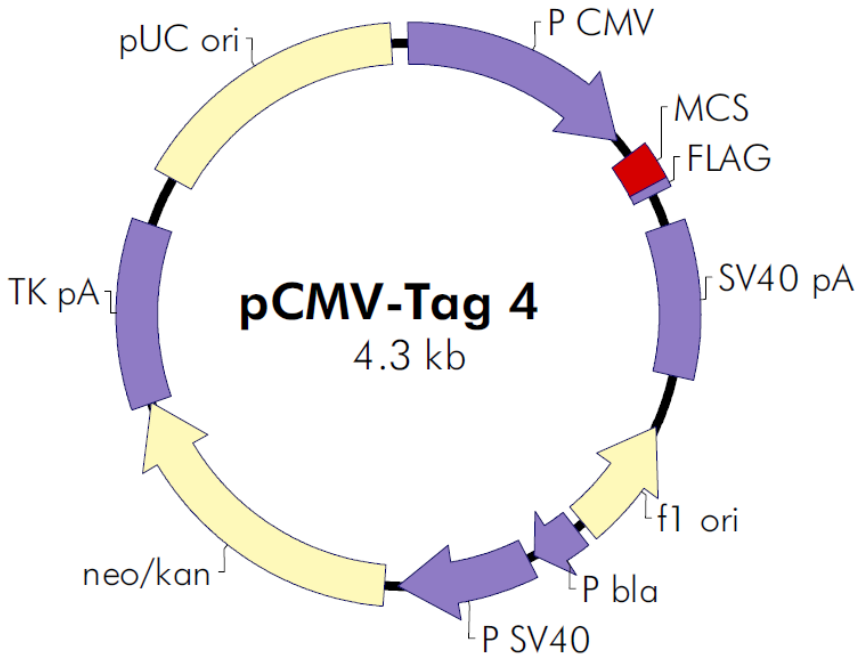
T7 promoter

...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTA

MULTIPLE STOP CODONS

* In pCMV-Tag 4A, no bases inserted; in pCMV-Tag 4B, A inserted; in pCMV-Tag 4C, AA inserted

pCMV-Tag 4C



pCMV-Tag 4 Multiple Cloning Site Region
(sequence shown 620–839)

T3 promoter

Sac I BstX I Sac II Not I

A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT A...

Srf I BamH I Pst I EcoR I EcoR V Hind III Acc I/Sal I

...GC CCG GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC* AA

Xho I FLAG tag

D Y K D D D D K

...CTC GAG GAT TAC AAG GAT GAC GAC GAT AAG TAG GGCCCGGTACCT...

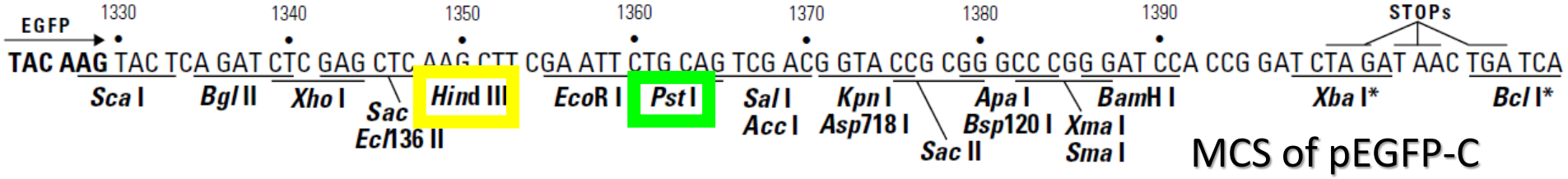
STOP

T7 promoter

...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTA

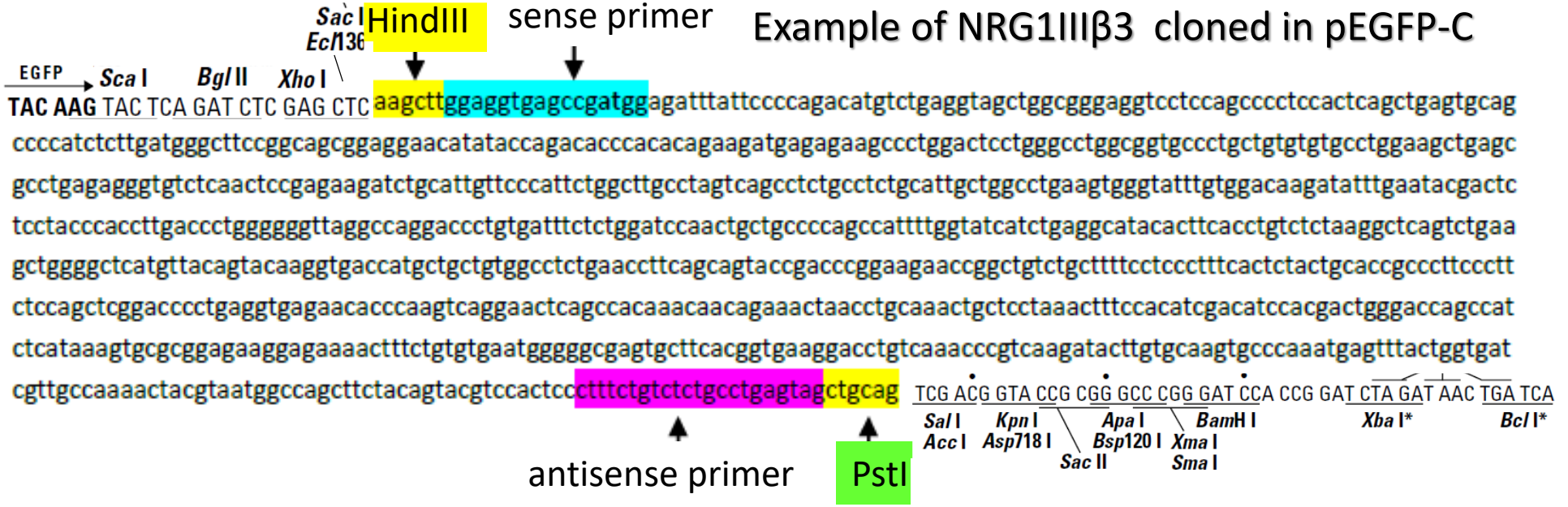
MULTIPLE STOP CODONS

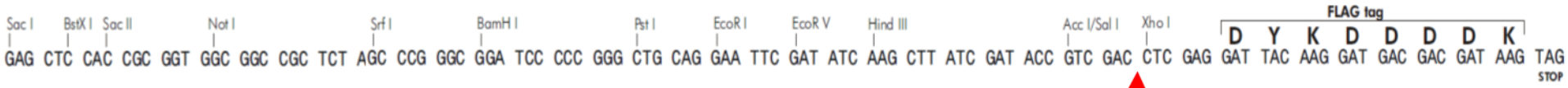
* In pCMV-Tag 4A, no bases inserted; in pCMV-Tag 4B, A inserted; in pCMV-Tag 4C, AA inserted



MCS of pEGFP-C

Example of NRG1IIIβ3 cloned in pEGFP-C





MCS of pCMV-Tag4

* A AA

pCMV-Tag 4A Multiple Cloning Site Region

(sequence shown 620–839)

```
ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC
GGC CGC TCT AGC CCG GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC
AAG CTT ATC GAT ACC GTC GAC*CTC GAG GAT TAC AAG GAT GAC GAC GAT
AAG TAG GGC CCG GTA CCT TAA TTA ATT AAG GTA CCA GGT AAG TGT ACC
CAA TTC GCC CTA TAG TGA GTC GTA TTA
```

EcoRI	PstI	SacII
EcoRV	BamHI	
HindIII	SacI	FLAG

pCMV-Tag 4B Multiple Cloning Site Region

(sequence shown 621–840)

```
TTA ACC CTC ACT AAA GGG AAC AAA AGC TGG AGC TCC ACC GCG GTG GCG
GCC GCT CTA GCC CGG GCG GAT CCC CCG GGC TGC AGG AAT TCG ATA TCA
AGC TTA TCG ATA CCG TCG ACA CTC GAG GAT TAC AAG GAT GAC GAC GAT
AAG TAG GGC CCG GTA CCT TAA TTA ATT AAG GTA CCA GGT AAG TGT ACC
CAA TTC GCC CTA TAG TGA GTC GTA TTA
```

pCMV-Tag 4C Multiple Cloning Site Region

(sequence shown 622–841)

```
TAA CCC TCA CTA AAG GGA ACA AAA GCT GGA GCT CCA CCG CGG TGG CGG
CCG CTC TAG CCC GGG CGG ATC CCC GGG GCT GCA GGA ATT CGA TAT CAA
GCT TAT CGA TAC CGT CGA CAA CTC GAG GAT TAC AAG GAT GAC GAC GAT
AAG TAG GGC CCG GTA CCT TAA TTA ATT AAG GTA CCA GGT AAG TGT ACC
CAA TTC GCC CTA TAG TGA GTC GTA TTA
```

II Exercise

• find a cloning strategy to subclone the NRG1-III beta3 from the vector **pCR-bluntII-topo** **OR** from the vector **pEGFP-C** into the vector **pCMV-Tag4**

• identify which restriction sites can be used to recover the insert from one vector and which can be used to clone the insert

• identify which of the three plasmids (4A, 4B or 4C) do you have to use to maintain the frame between the NRG1 and the FLAG

-> Translate the protein that you obtain after the cloning:
if you have the correct frame you will obtain the NRG1 followed by the **FLAG: DYKDDDDK**

• if using plasmid 4A protein NRG1 is NOT in frame with the FLAG,
try the other two plasmids: 4B and 4C,
(you have to add A to obtain plasmid 4B or AA to obtain plasmid 4C to the multiple cloning site where there is an asterisk *)

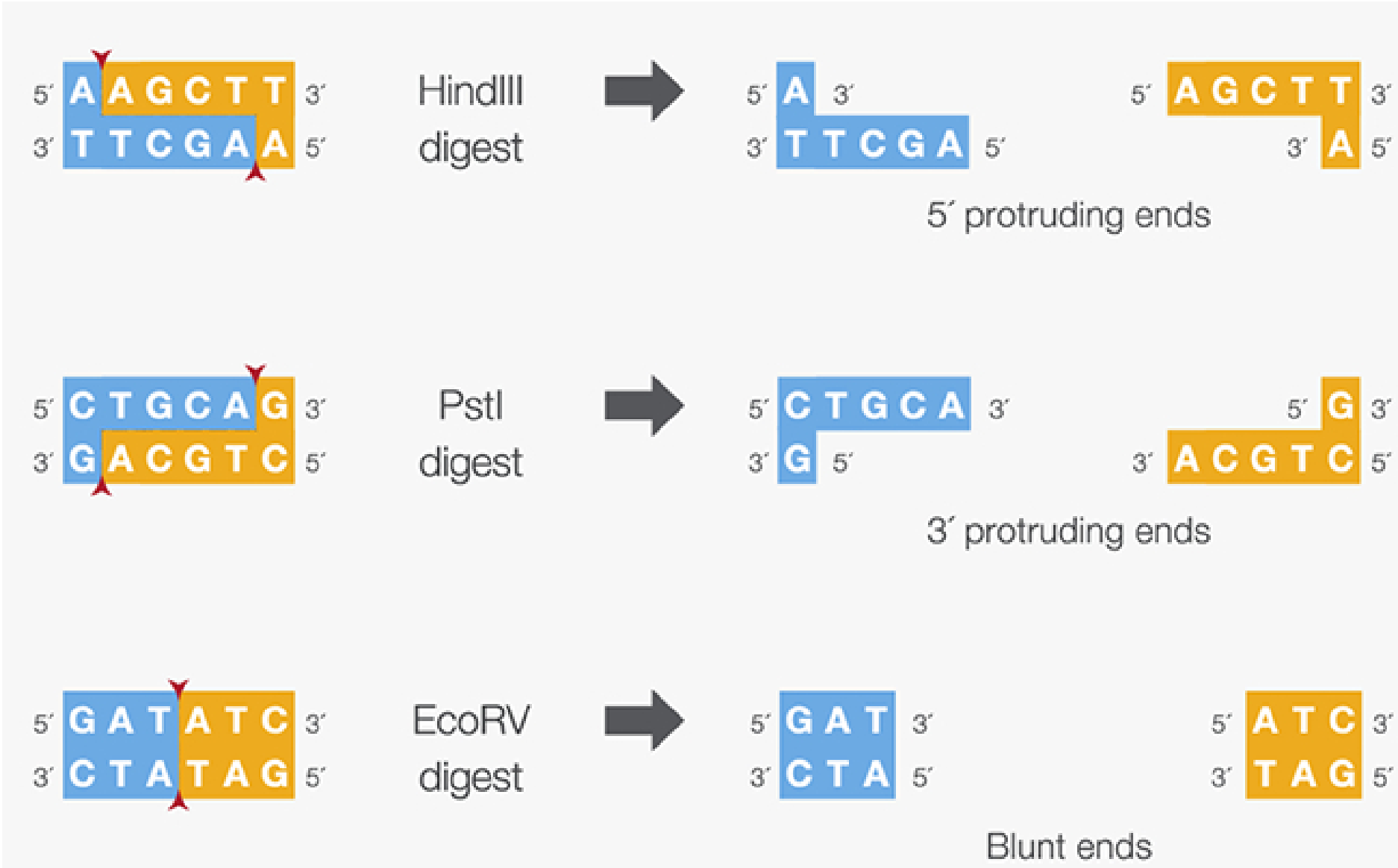
• prepare a sequence containing the multiple cloning site of the vector and NRG1 and, using neb-cutter, translate it to verify that NRG1 is in frame with the FLAG

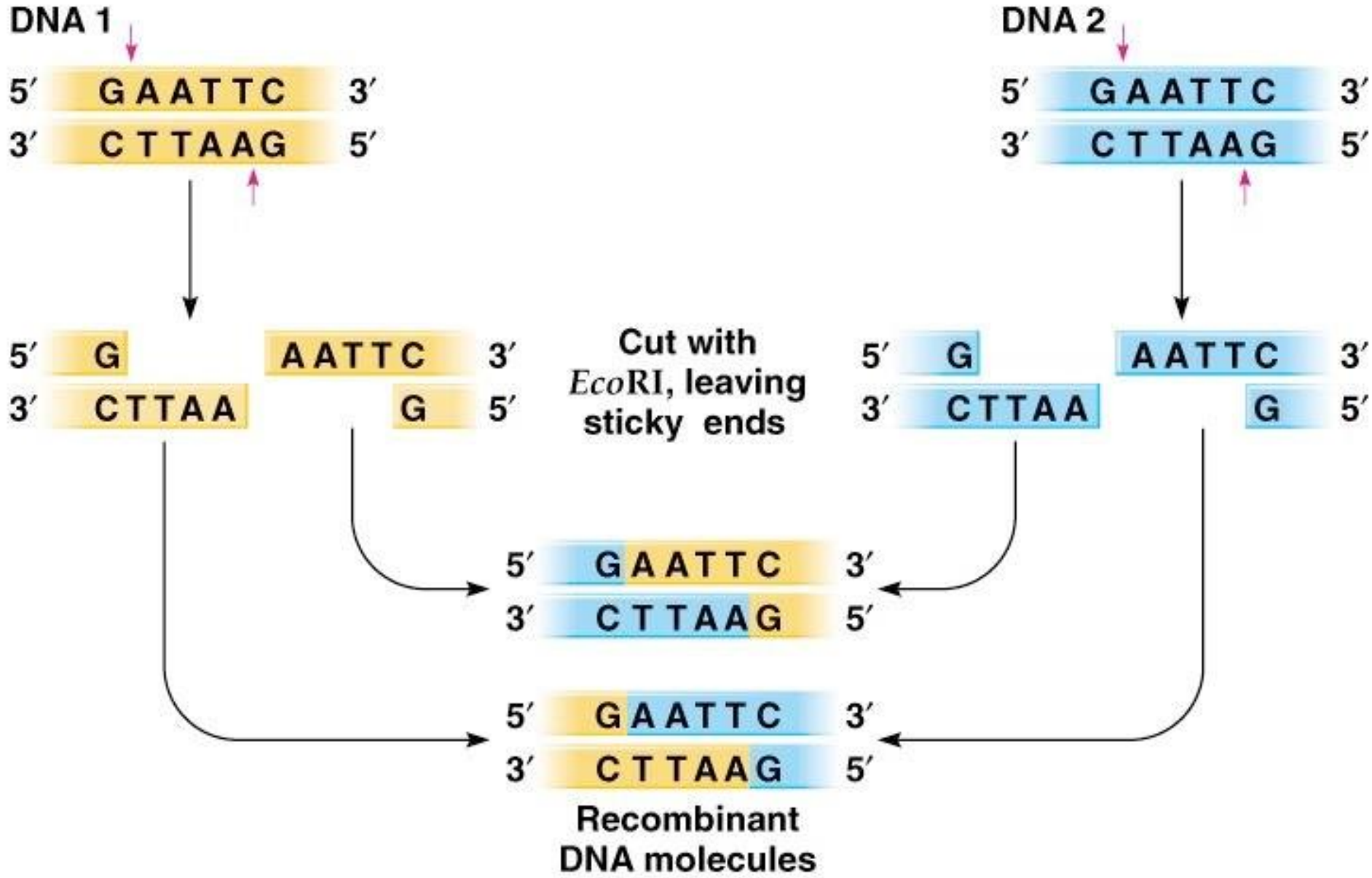
• in a slide show the translation of NRG1 + flag (DYKDDDDK), with the information about the restriction enzymes you used to clone it

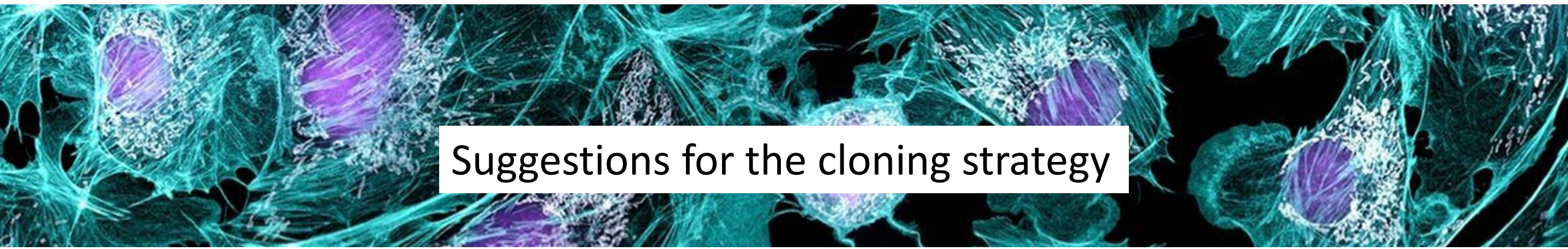
• if your insert can enter either sense or antisense, **please find** a restriction enzyme digestion to determine the insert orientation and write in the slide the name of the enzyme and the length of the fragments that you obtain when the insert is sense or antisense

• you can up-load the slide with map and cloning information on moodle
• **Please, up-load a single slide with all information!**

Ends after restriction enzyme digestion





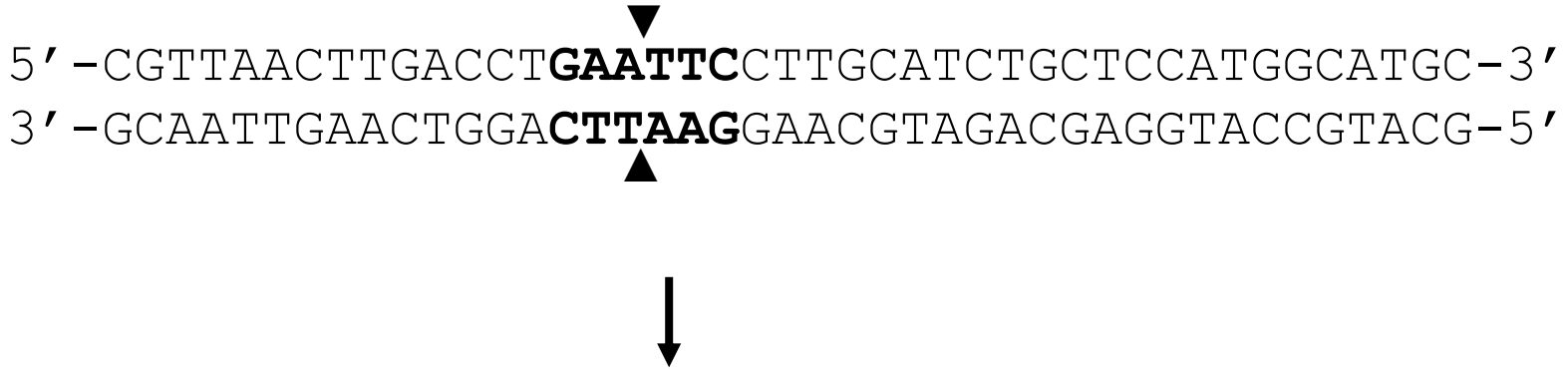


Suggestions for the cloning strategy

If the ends are both blunt you can ligate them,
even if you used different restriction enzymes

5′-CGTTAACTTGACCT**GAATTC**CTTGCATCTGCTCCATGGCATGC-3′
3′-GCAATTGAACTGGA**CTTAAG**GAACGTAGACGAGGTACCGTACG-5′

If the ends are both blunt you can ligate them,
even if you used different restriction enzymes



• if you do not find suitable enzymes, you can blunt the ends obtained after restriction enzyme digestion with different enzymes, so they will become compatible

5′-CGTTAACTTGACCT**GAATTC**CTTGCATCTGCTCCATGGCATGC-3′
3′-GCAATTGAACTGGA**CTTAAG**GAACGTAGACGAGGTACCGTACG-5′

TYPE I

5′-CGTTAACTTGACCT**G**-3′
3′-GCAATTGAACTGGA**CTTAA**-5′

5′-**AATTC**CTTGCATCTGCTCCATGGCATGC-3′
3′-**G**GAACGTAGACGAGGTACCGTACG-5′

?

BLUNTING TYPE I (5’ PROTRUDING)

5’-CGTTAACTTGACCT**GAATTC**CTTGCATCTGCTCCATGGCATGC-3’
3’-GCAATTGAACTGGA**CTTAAG**GAACGTAGACGAGGTACCGTACG-5’

5’-CGTTAACTTGACCT**G**-3’
3’-GCAATTGAACTGGA**CTTAA**-5’

5’-**AATTC**CTTGCATCTGCTCCATGGCATGC-3’
3’-**G**GAACGTAGACGAGGTACCGTACG-5’

DNA polimerase (klenow)

5’-CGTTAACTTGACCT**GAATT**-3’
3’-GCAATTGAACTGGA**CTTAA**-5’

5’-**AATTC**CTTGCATCTGCTCCATGGCATGC-3’
3’-**TTAAG**GAACGTAGACGAGGTACCGTACG-5’

• if you do not find suitable enzymes, you can blunt the ends obtained after restriction enzyme digestion with different enzymes, so they will become compatible

5′-CGTTAACTTGACCT**GAATTC**CTTGCATCTGCTCCATGGCATGC-3′
3′-GCAATTGAACTGGA**CTTAAG**GAACGTAGACGAGGTACCGTACG-5′

TYPE II

5′-CGTTAACTTGACCT**GAATT**-3′
3′-GCAATTGAACTGGA**C**-5′

5′-**C**CTTGCATCTGCTCCATGGCATGC-3′
3′-**TTAAG**GAACGTAGACGAGGTACCGTACG-5′

?

BLUNTING TYPE II (3’ PROTRUDING)

5’-CGTTAACTTGACCT**GAATTC**CTTGCATCTGCTCCATGGCATGC-3’
3’-GCAATTGAACTGGA**CTTAAG**GAACGTAGACGAGGTACCGTACG-5’

↓

5’-CGTTAACTTGACCT**GAATT**-3’
3’-GCAATTGAACTGGA**C**-5’

5’-**C**CTTGCATCTGCTCCATGGCATGC-3’
3’-**TTAAG**GAACGTAGACGAGGTACCGTACG-5’

↓

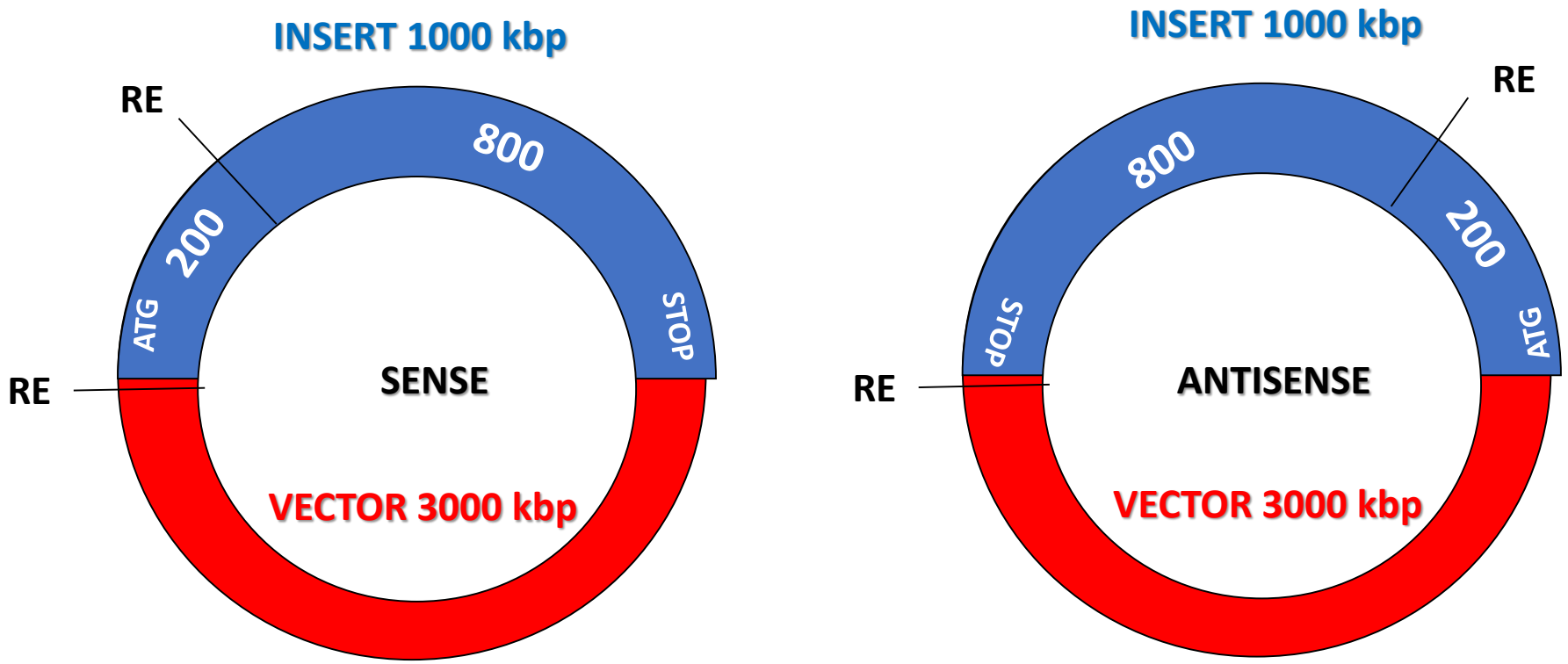
3’-DNA exonuclease

5’-CGTTAACTTGACCT**G**-3’
3’-GCAATTGAACTGGA**C**-5’

5’-**C**CTTGCATCTGCTCCATGGCATGC-3’
3’-**G**GAACGTAGACGAGGTACCGTACG-5’

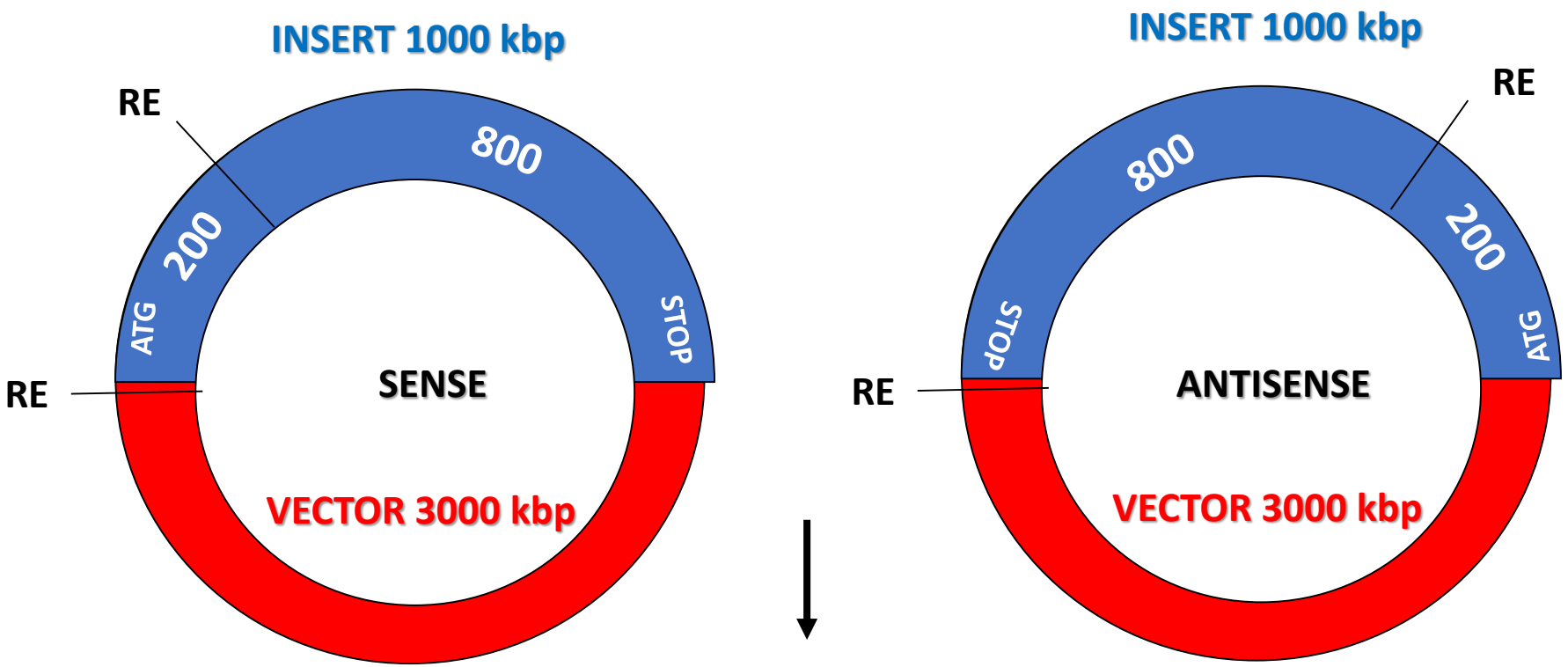
• if you do not find suitable enzymes, you can blunt the ends obtained after restriction enzyme digestion with different enzymes, so they will become compatible

• in this case, your insert can be cloned sense or antisense, and then you will have to design a digestion to verify the orientation of the insert



• if you design a sub-cloning using blunt ends at both ends of the insert or the same restriction enzyme at both ends of the insert, the insert can be sub-cloned sense or antisense.

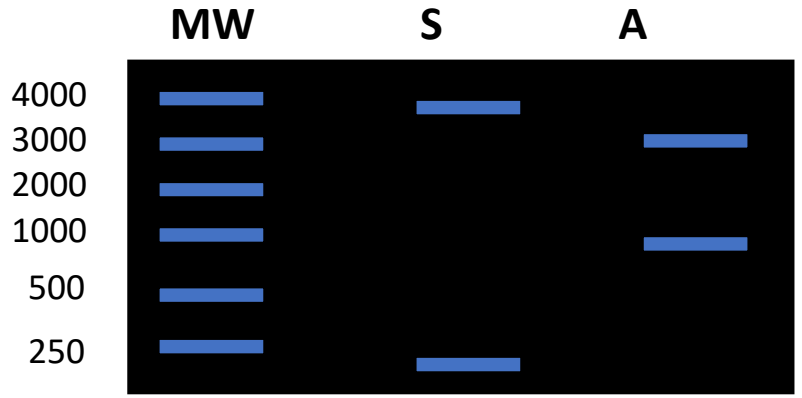
• therefore you have to identify a **restriction enzyme** (**RE**) that will enable you to verify the correct orientation of the insert into the new vector.



restriction enzyme (RE) digestion
electrophoretic run

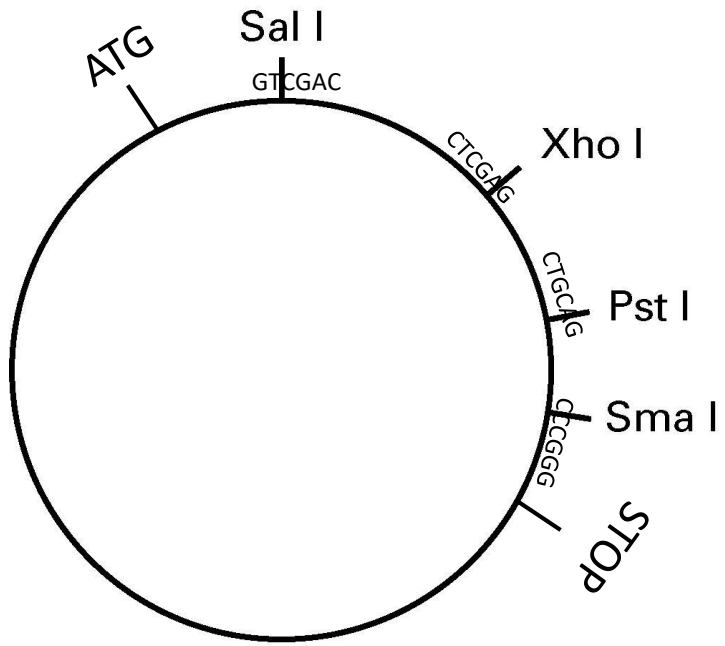
SENSE

3800 + 200



ANTISENSE

3200 + 800



Sal I

5' G T C G A C 3'
3' C A G C T G 5'

Xho I

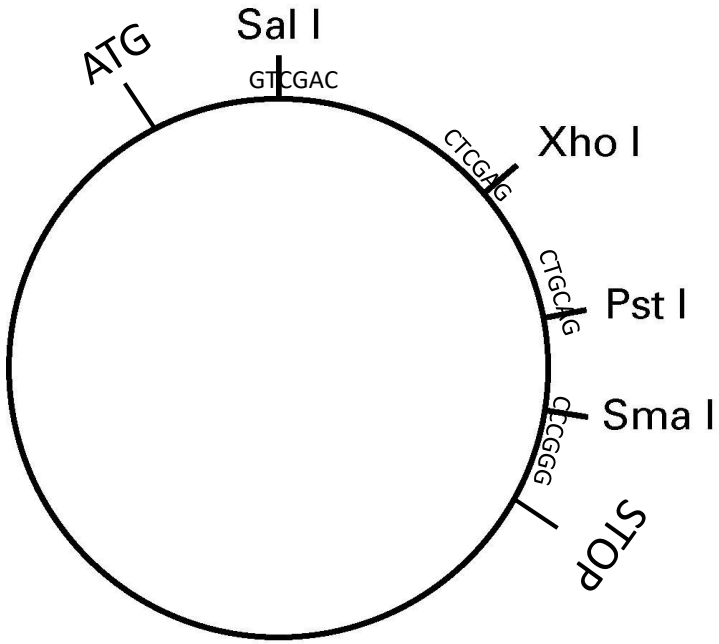
5' C T C G A G 3'
3' G A G C T C 5'

Pst I

5' C T G C A G 3'
3' G A C G T C 5'

Sma I

5' C C C G G G 3'
3' G G G C C C 5'



Sal I

5' G T C G A C 3'
3' C A G C T G 5'

Xho I

5' C T C G A G 3'
3' G A G C T C 5'

Pst I

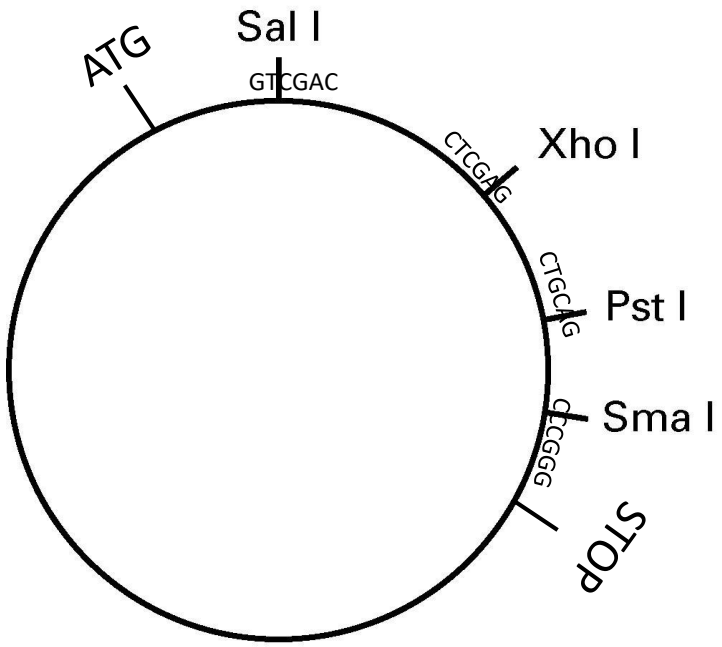
5' C T G C A G 3'
3' G A C G T C 5'

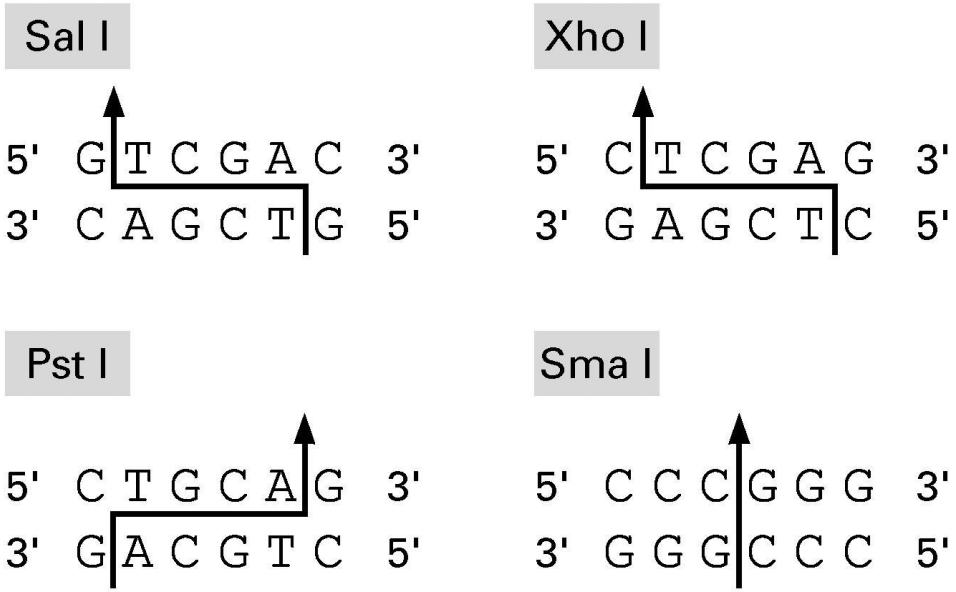
Sma I

5' C C C G G G 3'
3' G G G C C C 5'

Exercise:

-How can you ligate **SalI** with **XhoI**?





Exercise:

-How can you ligate **SalI** with **XhoI**? 5'-**GTCGAG**-3' (compatible)
5'-**GTCGATCGAG**-3' (blunted)

-How can you ligate **SalI** with **PstI**? 5'-.....-3'
-How can you ligate **SalI** with **SmaI**? 5'-**.....**-3'
-How can you ligate **XhoI** with **PstI**? 5'-**.....**-3'
-How can you ligate **XhoI** with **SmaI**? 5'-**.....**-3'
-How can you ligate **PstI** with **SmaI**? 5'-**.....**-3'