Advanced Cell Biology & Biotechnology

Biotechnology Project Lab

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& Isabella Tarulli

The lecture of December 13th 2021 is about to begin….

Advanced Biotechnology

- Summary
- https://www.ensembl.org/index.html
- https://en.vectorbuilder.com/
- Welcome test & goodbye test
- Exercise: Transwell assay - correction
- Exercize: Ingredients western blot – correction
- Exercize: proliferation assay
- Exercize: real time PCR for p75
- Exercize & Correction: How many molecules/µl of plasmid DNA
- How to modify a buffer to another buffer
- Last subcloning

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- identification of specific exons belonging to different NRG1 isoforms
- identification of exons and introns using BLAST and Ensemble
- use of Annhyb to design primers and to organize and edit sequences
- design primers for isoform specific expression analysis and for full length cDNA cloning
- design primers for quantitative gene expression analysis
- design primers for full length cDNA cloning (with additional restriction enzyme sites to facilitate subcloning)
- plan a reverse transcriptase (RT) reaction with positive and negative controls
- plan a polymerase chain reaction (PCR) with positive and negative controls
- use of Chromas to read an electropherogram
- plan cloning and subcloning from a vector to another vector
- restriction enzyme analysis and preparation of plasmid maps (with neb-cutter or other apps)
- blunting sticky ends
- project hybrid proteins fused with GFP
- project proteins with a FLAG tag
- solve some typical problems you can encounter in the laboratory like:
- analyze relative/absolute quantitative real time PCR data
- analyze protein quantification data
- calculate the amount of cells you have to plate for a specific experiment
- calculate the amount of ingredients in a reaction or to prepare a solution

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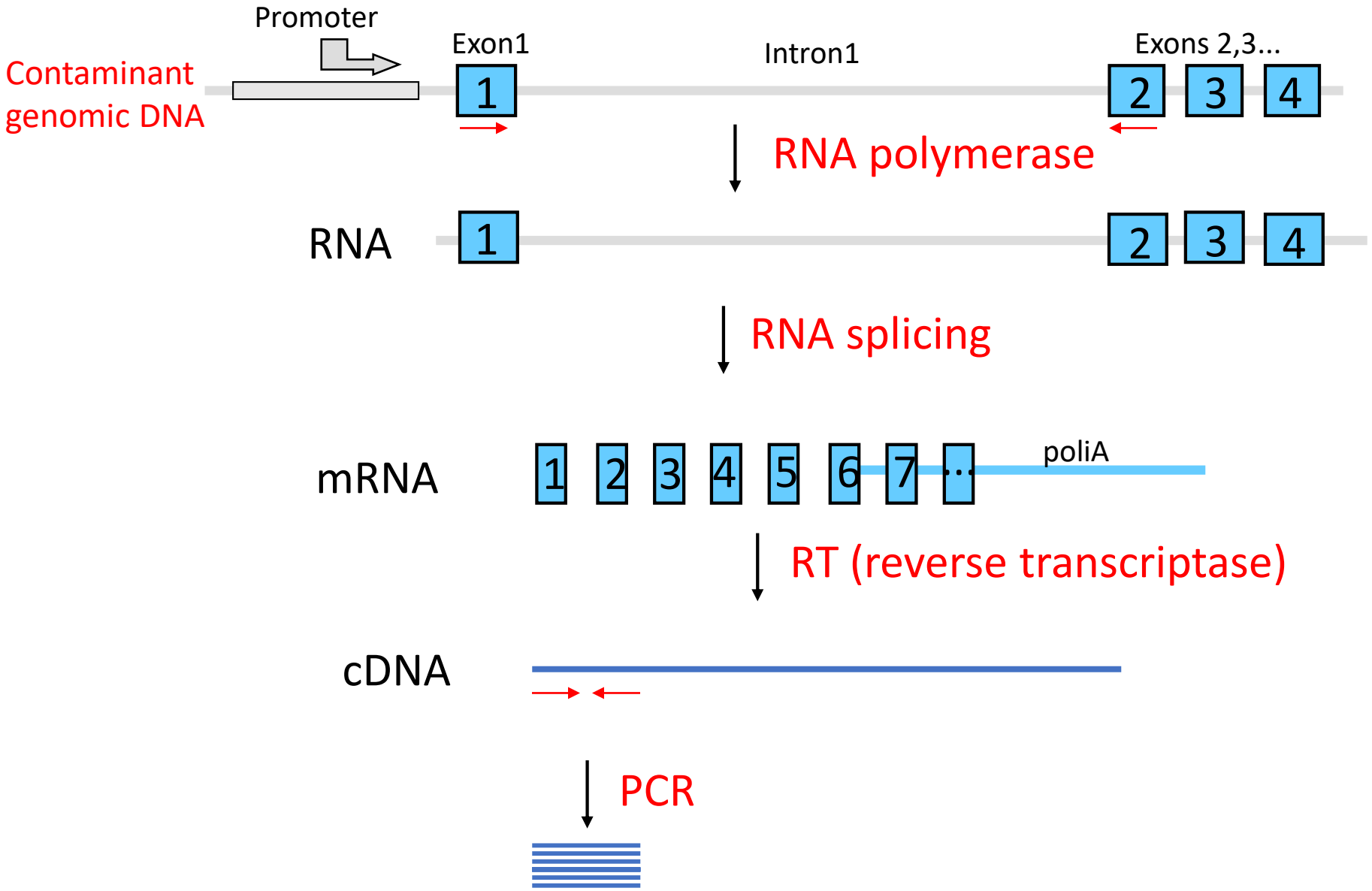
design primers on different exons separated by a big intron (≥1000 bp)



https://www.ensembl.org/Rattus_norvegicus/Gene/Splice?db=core;g=ENSRNOG00000008292;r=6:96810907-9685605

Gene: Hif1a

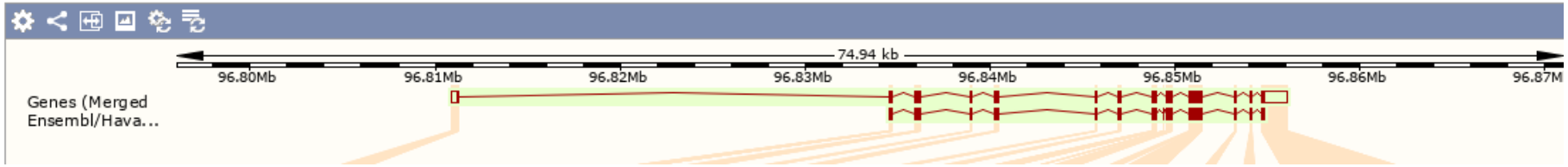
Gene: Hif1a ENSRNOG00000008292

Description	hypoxia inducible factor 1 subunit alpha [Source:RGD Symbol;Acc:61928]
Gene Synonyms	MOP1
Location	Chromosome 6: 96,810,907-96,856,052 forward strand. Rnor_6.0:CM000077.5
About this gene	This gene has 2 transcripts (splice variants), 308 orthologues, 6 paralogues, is a member of 1 Ensembl protein family and is associated with 104 phenotypes.
Transcripts	Hide transcript table

Show/hide columns (1 hidden) Filter

Name	Transcript ID	bp	Protein	Biotype	UniProt	Flags
Hif1a-201	ENSRNOT00000049725.3	3977	825aa	Protein coding	D4A8P8	APPRIS P1
Hif1a-202	ENSRNOT00000077935.1	2451	816aa	Protein coding	A0A0G2JX87	-

Splice variants



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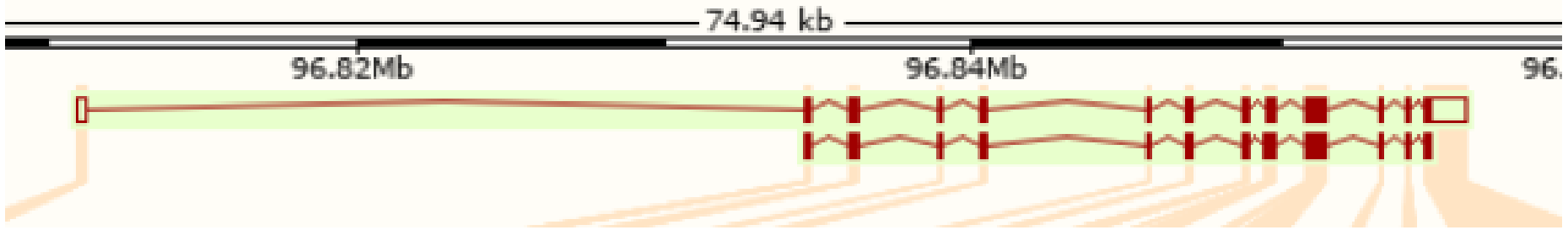

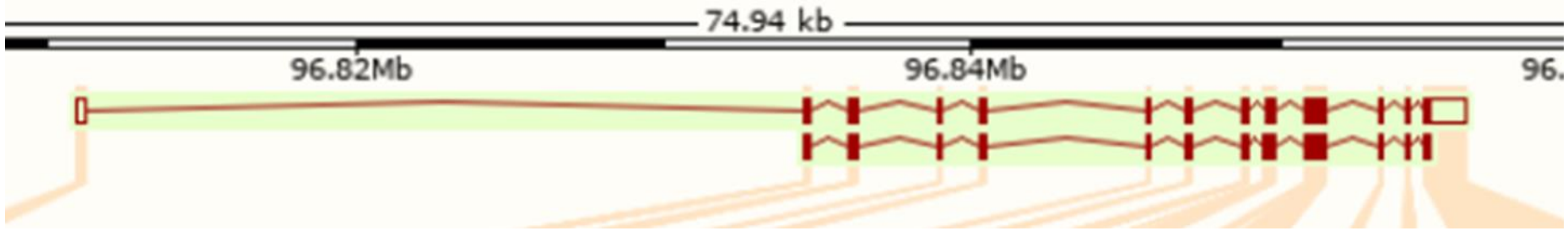

Show All entries | Show/hide columns | Filter

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
	5' upstream sequence						..........tcgccgcgcgcccgagcgcgcctccgccttgcccgcccctgccgctgc
1	ENSRNOE00000344821	96,810,907	96,811,242	-	2	336	CTCAGCGCCTCAGTGCACAGAGGCTCCTCGTCTGAGGGGACGCGAGGACTGTCCTCGCTA CCGTCGCGAGCAGTGTCTGGCCAGGCCTTGACAAGCCAGCCGGAGGAGCAACTAGGAACC CGAACCAGAGCCCGGCGAGCGCAGCCTGCAGCTCCCGCCTCGCCGTCCCGGGGGGGTGTC CCGCCTCACACCCCGCCTCTGGACTTGCCCCTTTCTCTGCGCGTGAGGACAGAGCCGGCG CTTAGGCCGGAGCGAGCCCAGGGGCTGCCGGCCGGGAAGACACCGCGGGCACCGATTCGC CATGGAGGGCGCCGGCGGCGAGAACGAGAAGAAAAA
	Intron 1-2	96,811,243	96,834,526			23,284	gtaagccgggccgccgcgttctgtg.....ccctcccccccccctcattaagtag
2	ENSRNOE00000291372	96,834,527	96,834,717	2	1	191	GATGAGTTCCGAACGTCGAAAAGAAAAGTCTAGGGATGCAGCACGATCTCGGCGAAGCAA AGAGTCTGAAGTTTTTTATGAGCTTGCTCATCAGTTGCCACTTCCCCACAACGTGAGCTC CCATCTTGATAAAGCTTCTGTTATGAGGCTCACCATCAGTTACTTACGTGTGAGGAAACT TCTAGATGCTG
	Intron 2-3	96,834,718	96,835,963			1,246	gtgagttctgctaaagcgtaagaga.....tttcttcccacatgtgcccttacag
3	ENSRNOE00000079173	96,835,964	96,836,109	1	0	146	GTGATCTTGACATTGAAGATGAAATGAAAGCACAGATGAACTGCTTTTATCTGAAAGCCC TGGATGGCTTTGTTATGGTGCTAACAGATGATGGTGACATGATTTACATTTCTGATAACG TGAACAAATACATGGGGTTGACTCAG
	Intron 3-4	96,836,110	96,836,191			82	gtaaagtcaccacatatgaagagca.....tctaatttttttaatgtgtttgcag
4	ENSRNOE00000078038	96,836,192	96,836,276	0	1	85	TTTGAACTAACTGGACACAGTGTGTTTGATTTTACCCATCCATGTGACCATGAGGAAATG AGAGAAATGCTTACACACAGAAATG
	Intron 4-5	96,836,277	96,838,916			2,640	gtgagaaaagtctattgtttgattt.....aaactttgtttcttgctttcattag

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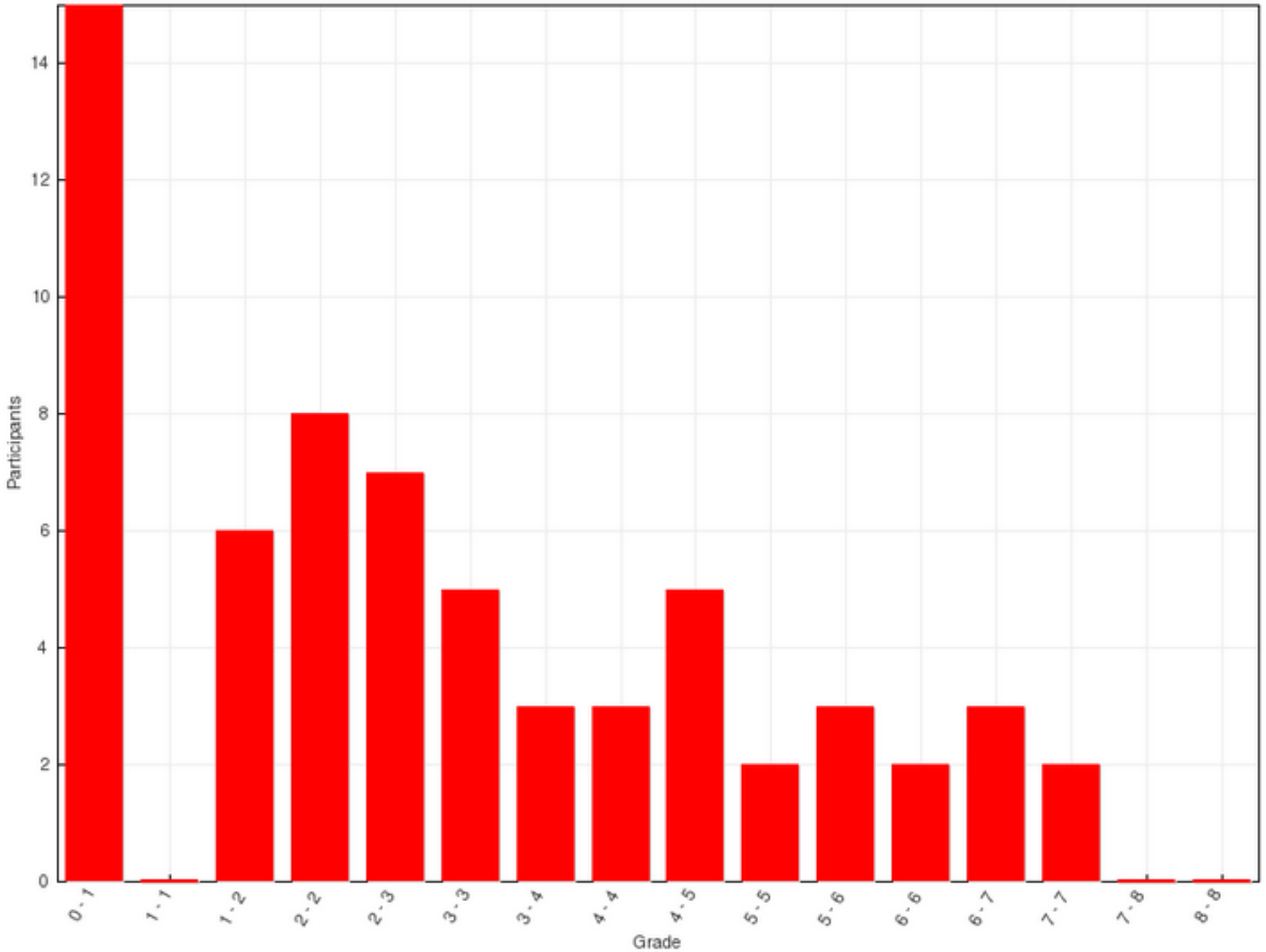
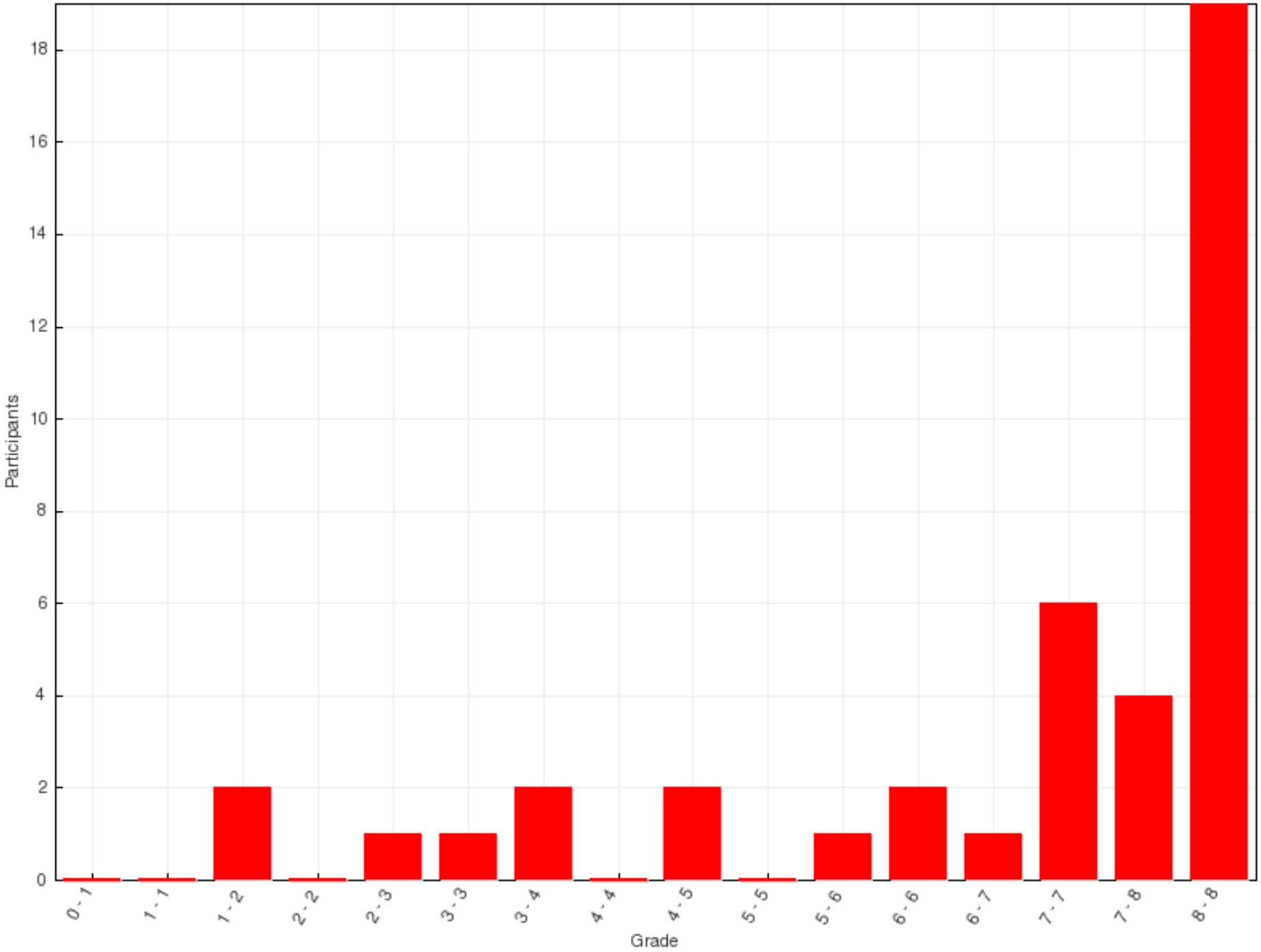


Welcome test 18-10-2021 (64 students)



Goodbye test
13-12-2021
(41 students)

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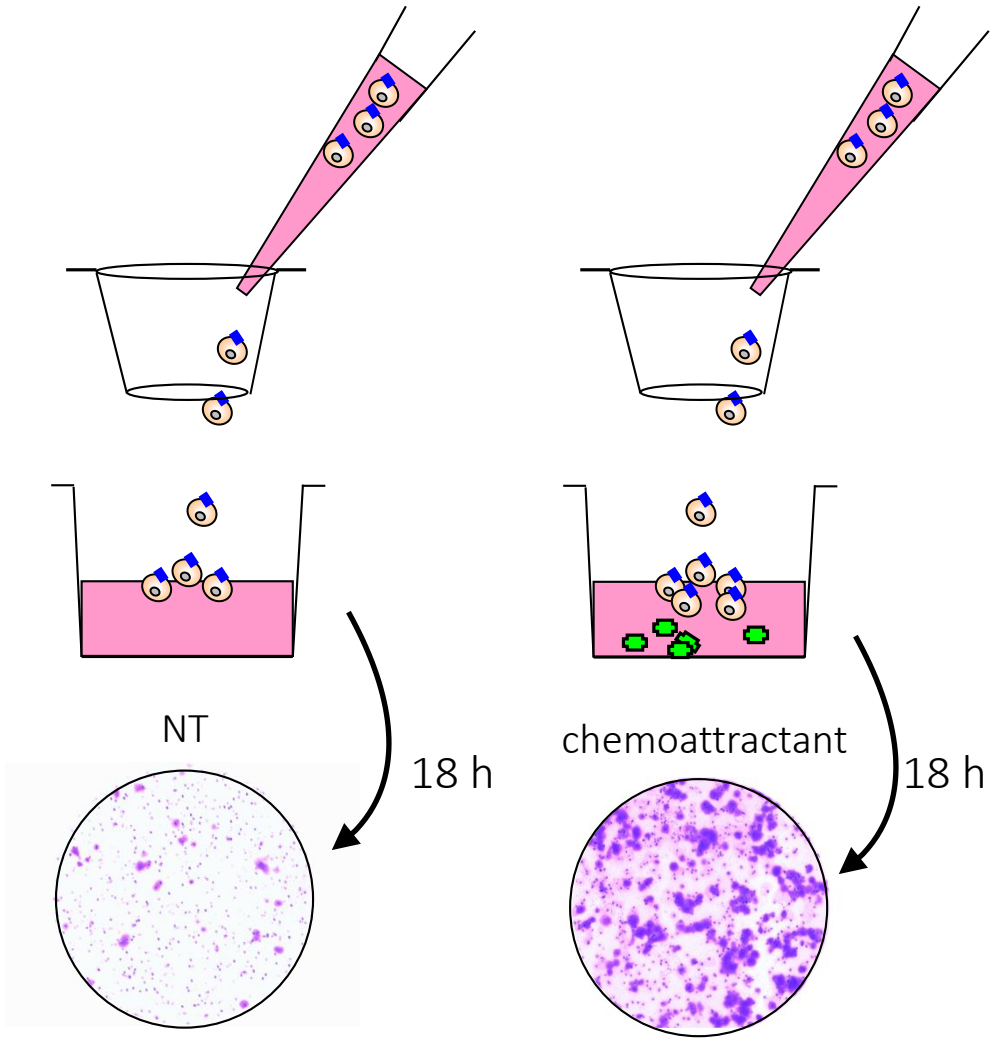
Exercise – Transwell assay

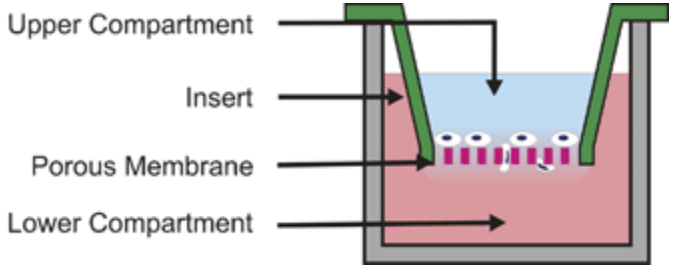
Migration Assay

The Transwell migration assay was used to measure three-dimensional movement. Cells (**10^5**) resuspended in **200 µl** of DMEM containing 2% FBS were seeded in the upper chamber of a Transwell (cell culture insert, no. 353097, BD Biosciences) on a porous transparent polyethylene terephthalate membrane (8.0-µm pore size, 1 X 10^5 pores/cm^2). The lower chamber (a 24-well plate well) was filled with 800 µl DMEM containing 2% FBS with or without 5 nM recombinant NRG1β1. The 24-well plates containing cell culture inserts were incubated at 33 °C in a 5% CO_2 atmosphere saturated with H_2O. After 18 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed with 2% glutaraldehyde in PBS for 15 min at room temperature, washed five times with water, stained with 0.1% crystal violet and 20% methanol for 20 min at room temperature, washed five times with water, air-dried, and photographed using an Olympus IX50 inverted microscope equipped with a Cool SNAP-Pro CCD camera; images were edited with Image Pro-Plus software.

TRANSWELL ASSAY

http://www.youtube.com/watch?v=6SON7VAA5-k





Exercise – Transwell assay

- grow cells until confluence in a 10cm diameter dish
- aspirate medium
- wash with 5 ml PBS
- aspirate PBS
- add 1,5 ml trypsin, incubate 2 min at 37°C
- add 6,5 ml medium containing 10% FBS (foetal bovine serum)
- resuspend well the cells pipetting up and down
- take a drop to count the cell number with the Bürker chamber
- spin cells in the centrifuge 5 min, 800 rpm, room temperature
- discard supernatant
- resuspend the pellet in **XX** ml of 2% FBS DMEM in order to have a suitable concentration;

-> Indeed, you want to pipet **200 µl** containing **10^5** cells in different transwells

- Add 200 µl containing 10^5 cells to different transwell and put them in a multiwell containing 800 µl 2% FBS DEMEM with or without ligands.

Exercise – Transwell assay

Question:

If you count **35 51 45 31 46** cells in five squares of the Bürker chamber (0,1mm^3 each)

- How many cells do you have? …..
- in which volume of 2% FBS DMEM do you have to resuspend the cells to have 100000 cells in 200 µl? ….ml (use 2 decimal numbers)

To be more precise, you count again your cells to be sure that you really put 10^5 cells/transwell.

You count again and you find: **46 44 42 50 34**

How many microliters of cells do you have to put in the transwell to have 10^5 cells? (no decimal numbers)

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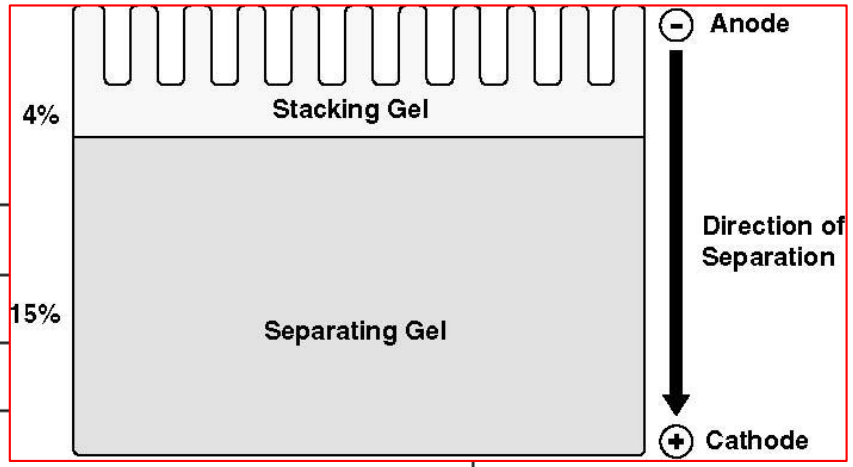
Exercise - Western blot



- **For a 5 ml stacking gel:**

H_2O	2.975 ml
→ 0.5 M Tris-HCl, pH 6.8	1.25 ml
→ 10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

- **For a 10ml separating gel:**

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
→ 1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
→ 10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

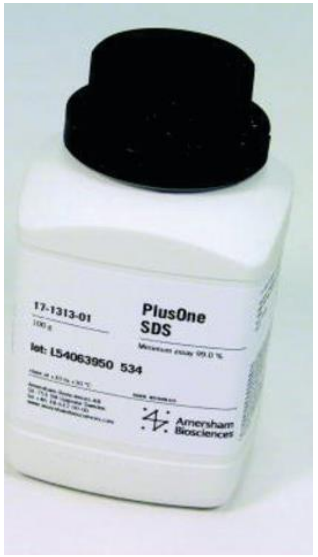
Exercise – Reagents for western blot

TRIS Base

Molecular Weight 121,14

1,5 M Tris pH 8.8 - How many grams for 250 ml?

0,5 M Tris pH 6,8 - How many grams for 250 ml?



Sodium dodecyl sulfate (SDS)

Molecular Weight 288.38

10% SDS - How many grams for 250 ml?

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Exercise - Proliferation assay

- take a 10 cm diameter plate containing confluent cells
- aspirate medium
- wash with 5 ml PBS
- aspirate PBS
- add 1,5 ml trypsin, incubate 2 min at 37°C
- add 6,5 ml medium containing 10% FBS (foetal bovine serum) to inactivate trypsin
- resuspend well the cells pipetting up and down
- take a drop to count the cell number with the Bürker chamber
- you count for example: 15 27 32 26 20

- how many cells do you have in 1 ml?
- how many µl do you have to use if you want to plate 10^5 cells?

- if you want to do a proliferation assay in a 96 well plate, you have to use 10^3 cells/well
- how many µl of cells do you need if you want to plate 1000 cells in a well?
- if you prepare 4 plates 96 wells for a time course assay (control=time 0, 1 day, 3 days, 5 days) you can prepare a solution containing all the cells and all the medium necessary for the entire experiment.
- If you add 100µl/ well and 1000 cells/well, how do you prepare your cell mix solution?
- how many cells? Number and µl?
- how much medium? ml?

- in the lab usually we prepare a solution more abundant in order to be sure to have enough material, but now we plan to prepare the precise volume

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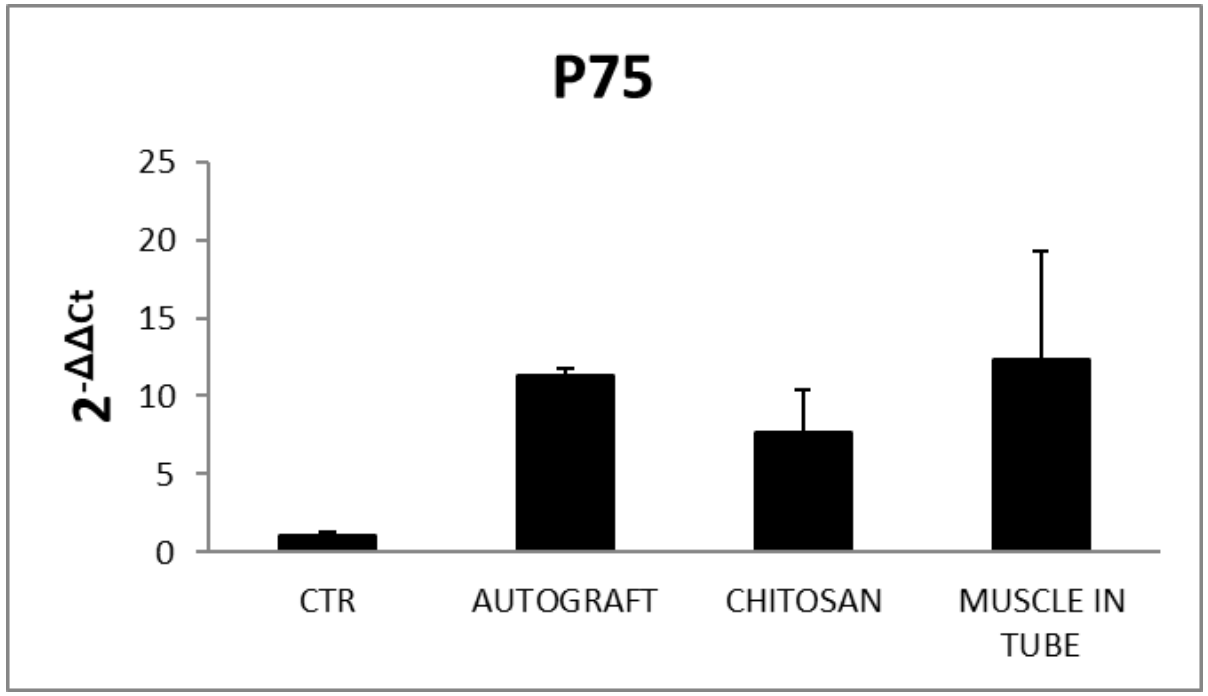
		CT p75	CT HKG
CTRL NERVE	53,86	25,97	26,61
	53,87	25,919	26,712
	53,88	26,323	27,007
	53,89	27,136	27,1
AUTOGRAFT	72,46	23,974	27,998
	72,70	23,727	27,806
	72,76	24,149	28,115
CHITOSAN TUBE	72,56	24,904	27,692
	72,66	23,83	27,664
	72,68	24,992	28,091
	72,74	25,193	29,04
MUSCLE IN TUBE	72,44	24,828	28,164
	72,48	23,572	28,584
	72,50	25,146	28,29
	72,60	23,837	26,873
	72,62	23,441	27,17
	72,64	23,541	28,26
	72,72	22,113	26,766



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How many molecules/µl of plasmid DNA do you have in your tube?

- you cloned NRG1-typeIIIβ3 (900 bp) in a vector long 3000 bp.
- you carried out a DNA miniprep
- you read 2 µl of your DNA diluted in 1ml H_2O at the spectrofotometer and you obtain an absorbance at 260nm = **0.05 OD**
  Conversion factor: **1OD = 50 µg DNA/ml**

1-How much DNA (**µg/µl**) do you have in your original tube? **µg/µl**

2-How many **molecules/µl** do you have in your tube? …… x 10 to the power ……..**molecules/µl**

Suggestion:

• You have to calculate the molecular weight of your construct, considering that each nucleotide is about 330 Dalton:…….

• Then you have to calculate how many moles you have in 1 µl ……

How many molecules/µl of plasmid DNA do you have in your tube?

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  Conversion factor: **1OD = 50 µg DNA/ml**

- 1-How much DNA (**µg/µl**) do you have in your original tube?

- 1 OD : 50µg DNA/ml = 0.05 OD : X µg DNA/ml

- X µg DNA/ml = (0.05 OD x 50µg DNA/ml) / 1 OD = 2.5 µg/ml in the cuvette used to read your sample at the spectrofotometer.

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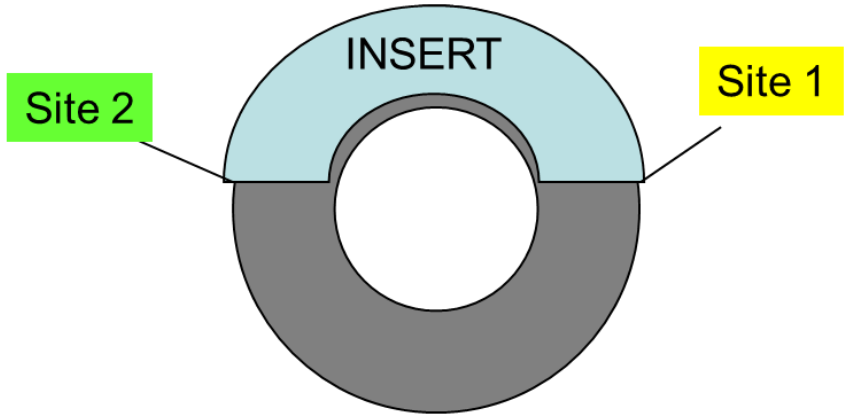
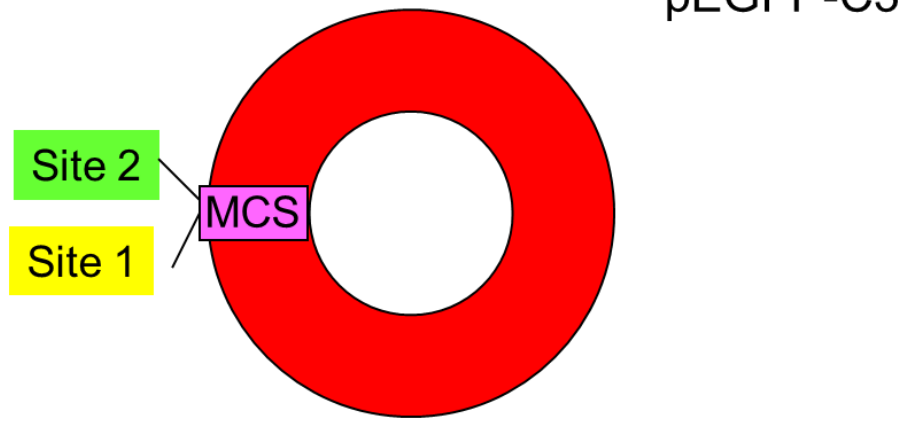
First case:

- the two enzymes cut in the same buffer



Second case:

- the two enzymes cut in very different buffers



Third case:

- the two enzymes cut in very similar buffers

1X NEBuffer 1:
10 mM Bis-Tris-Propane-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.0 @ 25°C

1X NEBuffer 3:
100 mM NaCl
50 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

1X NEBuffer 2:
50 mM NaCl
10 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

1X NEBuffer 4:
20 mM Tris-acetate
50 mM potassium acetate
10 mM Magnesium Acetate
1 mM Dithiothreitol
pH 7.9 @ 25°C

HindIII

1X NEBuffer 2:
50 mM NaCl
10 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

PstI

1X NEBuffer 3:
100 mM NaCl
50 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

HindIII

1X NEBuffer 2:
50 mM NaCl
10 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

PstI

1X NEBuffer 3:
100 mM NaCl
50 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

HindIII

1X NEBuffer 2:
50 mM NaCl
10 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

PstI

1X NEBuffer 3:
100 mM NaCl
50 mM Tris-HCl
10 mM MgCl2
1 mM Dithiothreitol
pH 7.9 @ 25°C

Digest DNA first with enzyme HindIII, which cut in buffer 2

DNA	20 ul
Buffer 2 10x	5 ul
BSA 10x	5 ul
HindIII 20u/ul	1 ul
Water	21 ul
Tot	50 ul

Verify that HindIII completely digested DNA, running 5 ul on agarose gel

Digest the remaining 45 µl of DNA with the second enzyme PstI which cuts in buffer 3.

Do not load DNA on a column to purify it, but increase the volume of the reaction to 100 µl and add buffer*, NaCl and Tris buffer to convert the buffer 2 into buffer 3.

*Try two approaches:

1-add buffer 2
2-add buffer 3

?

A) ADD BUFFER 2

DNA digested in buffer 2	45 µl
Buffer 2 10x	µl
NaCl 1M	µl
Tris HCl 1M	µl
water	µl
BSA 10x	µl
Enzyme PstI 20u/ul µl	
Tot	100 µl

B) ADD BUFFER 3

DNA digested in buffer 2	45 µl
Buffer 3 10x	µl
NaCl 1M	µl
Tris HCl 1M	µl
water	µl
BSA 10x	µl
Enzyme PstI 20u/ul µl	
Tot	100 µl

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PROJECT OVERVIEW

Genebank sequence analysis

↓

primer design

↓

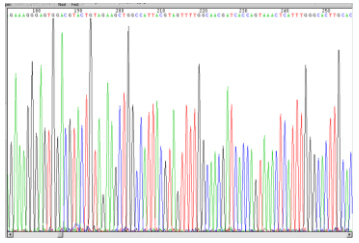
RT-PCR

↓

cloning in the vector pCRII-blunt

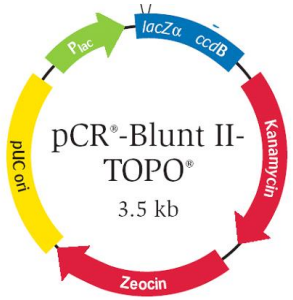
↓

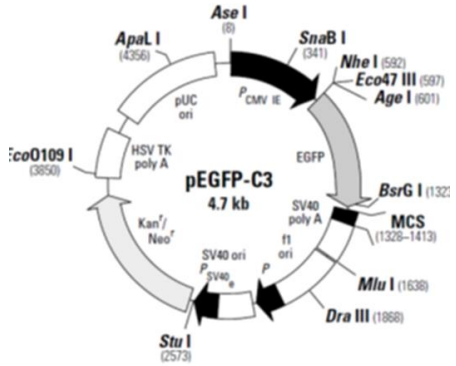
sequence analysis

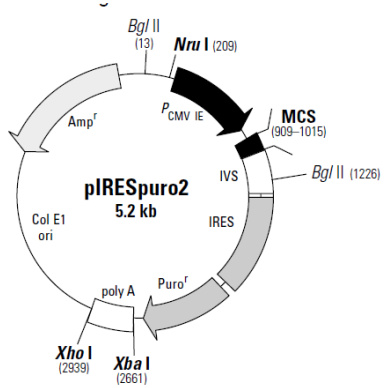


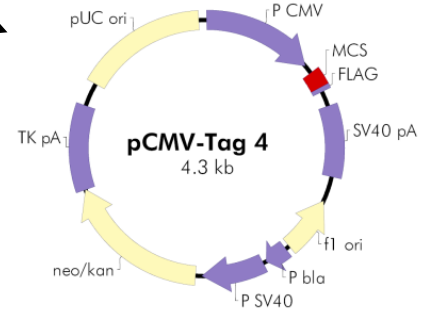
- real time PCR
- protein quantification

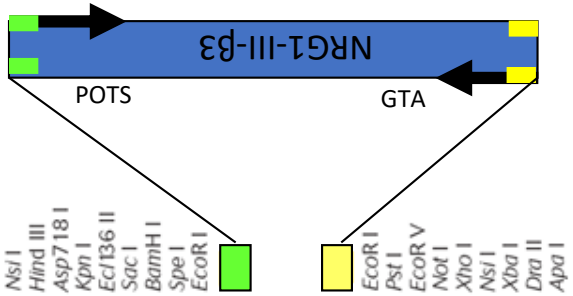
subcloning in different expression vectors

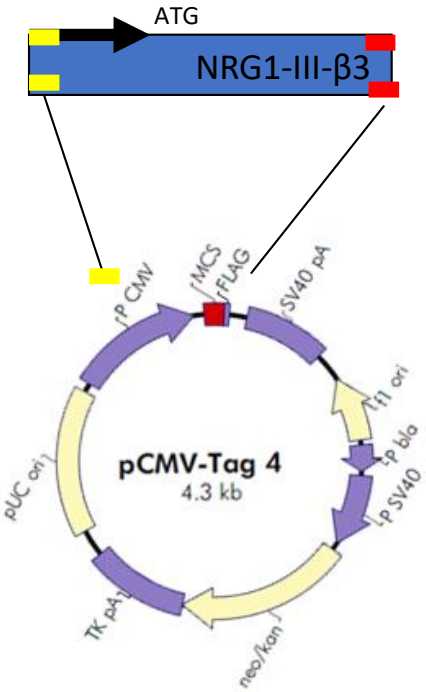


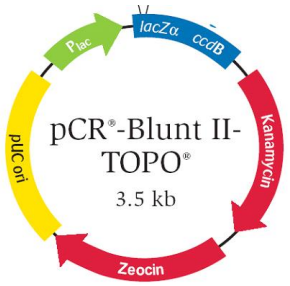


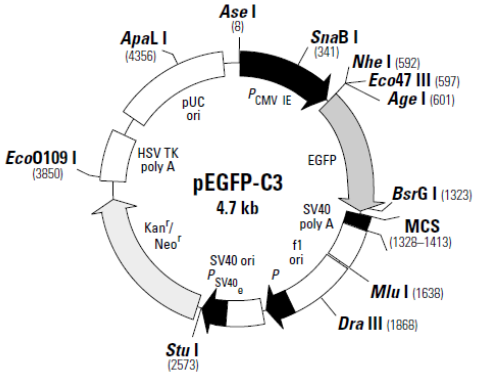


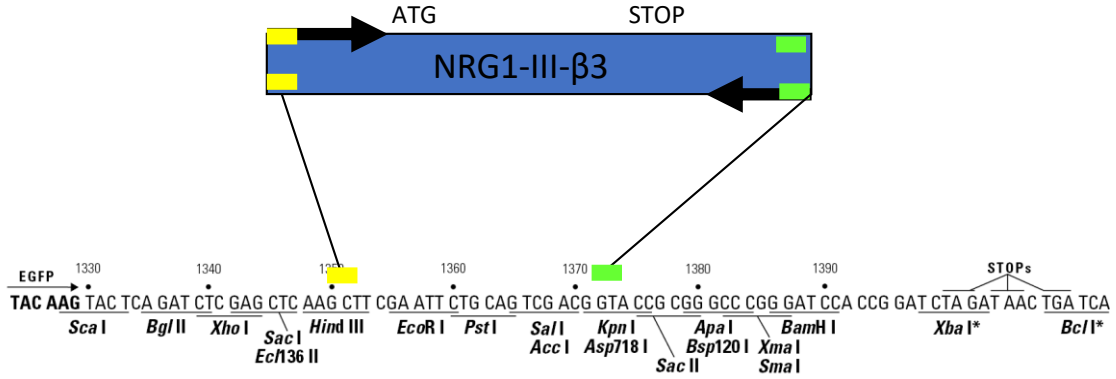


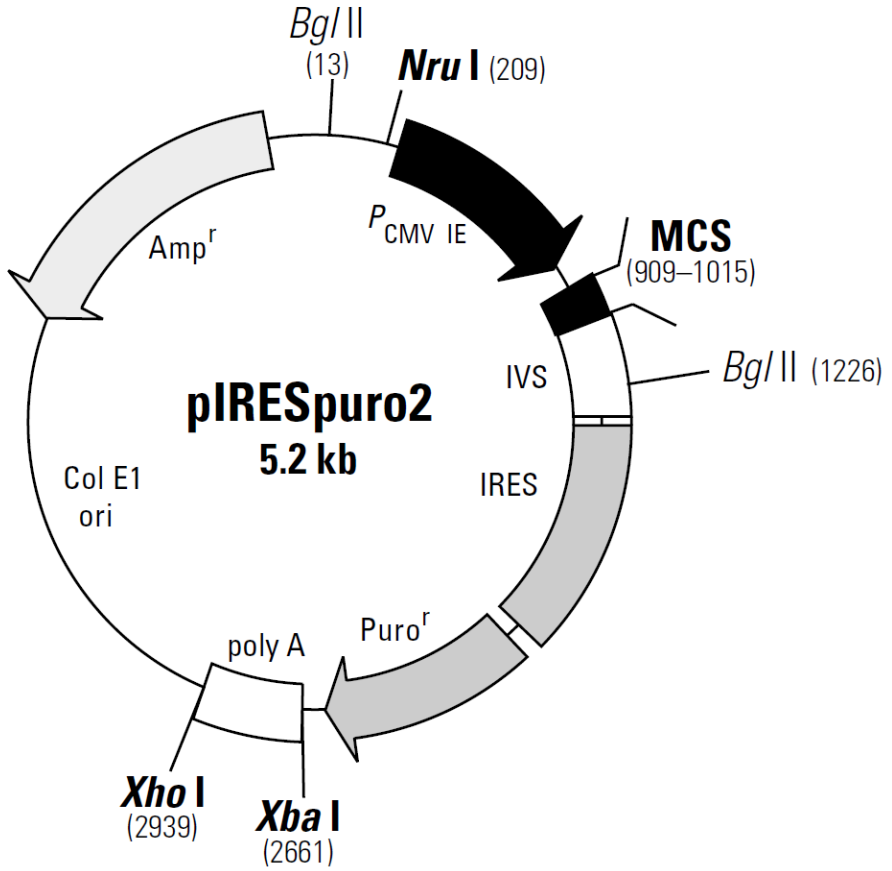

NRG1-III-β3
POTS
GTA
Nsi I
Hind III
Asp718 I
Kpn I
Ecl136 II
Sac I
BamH I
Spe I
EcoR I
EcoR I
Pst I
EcoR V
Not I
Xho I
Nsi I
Xba I
Dra II
Apa I

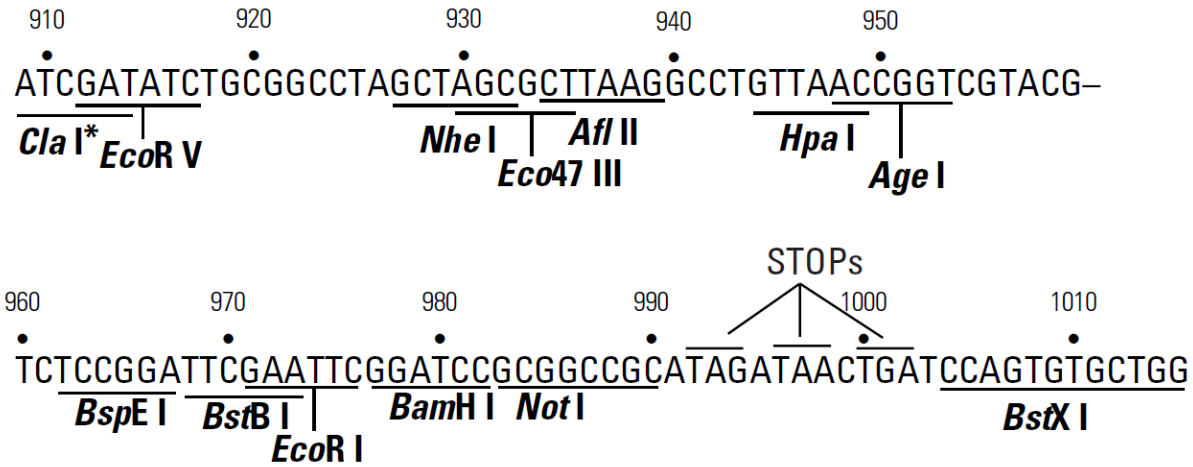

ATG
NRG1-III-β3
pCMV-Tag 4
4.3 kb
MCS
FLAG
SV40 pA
f1 ori
bla
SV40
neo/kan
TK pA
pUC ori


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


pEGFP-C3
4.7 kb
Ase I (8)
SnaB I (341)
Nhe I (592)
Eco47 III (597)
Age I (601)
ApaL I (4356)
pUC ori
HSV TK poly A
Eco0109 I (3850)
EGFP
BsrG I (1323)
SV40 poly A
MCS (1328–1413)
f1 ori
Mlu I (1638)
Dra III (1868)
SV40 ori
Kanr/ Neor
Stu I (2573)


ATG
STOP
NRG1-III-β3
EGFP
1330
1340
1360
1370
1380
1390
STOPs
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*


Bgl II
(13)
Nru I (209)
MCS
(909–1015)
Bgl II (1226)
IVS
IRES
Puro^r
poly A
Xho I
(2939)
Xba I
(2661)
Col E1
ori
Amp^r
P CMV IE
pIRESpuro2
5.2 kb


910
920
930
940
950
ATCGATATCTGCGGCCTAGCTAGCGCTTAAGGCCTGTTAACCGGTCGTACG–
Cla I* EcoR V
Nhe I
Afl II
Eco47 III
Hpa I
Age I
STOPs
960
970
980
990
1000
1010
TCTCCGGATTCGAATTCGGATCCGCGGCCGCATAGATAACTGATCCAGTGTGCTGG
BspE I
BstB I
EcoR I
BamH I
Not I
BstX I