pOnebyOne® 2A-Based Retroviral Expression Vectors

ME0013: pOnebyOne®I-Retroviral

ME0014: pOnebyOne®II-Retroviral

ME0015: pOnebyOne®III-Retroviral

ME0016: pOnebyOne®IV-Retroviral

Upon Receipt
Store Kits at -20ºC

PRODUCT MANUAL
Version 3.1
Last updated: August 2015

www.canvaxbiotech.com

Table of contents

Materials provided

Item	Quantity	Storage
pOnebyOne®Retroviral linearized vector (50ng/µL)	20 µL	-20ºC
Glue-Enzyme	40 µL	-20ºC
10x Glue-Enzyme Buffer	50 µL	-20ºC
Insert Control (30ng/µL)	10 µL	-20ºC

Storage temperature: -20ºC in a NON Frost-Free Freezer.

Expiration date: See on the kit label.

Additional material required

For cloning

- Proofreading DNA polymerase
- High efficiency competent cells (*Recommended* Cat. C0031 *from Canvax Biotech*)
- LB agar plates for ampicillin selection

For transformants analysis

- Taq DNA polymerase kit (*Recommended Red-Taq DNA polymerase kit (Cat. Nº P0027) from Canvax Biotech*)
- Forward primer for colony PCR
- Reverse primer for colony PCR
- Restriction enzymes
- Sequencing primer

For plasmid purification

- Plasmid Purification kit (Mini format) (*Recommended WideUSE Plasmid Purification Kit (Cat.Nº AN0068) from Canvax Biotech*)

For transfection

- Transfection Reagent (*Recommended Canfast transfection reagent (Cat.Nº T0082) from Canvax Biotech*)

For packaging and virus production

- Retroviral Packaging Vector Mix (*Recommended Cat.Nº* VP001 *from Canvax Biotech*)
- Polybrene
- HEK-293 cell line
- Culture media for HEK-293

For mammalian cell analysis and enrichment

- Anti-ΔNGFR antibody
- Anti immunoglobulin-FITC
- Anti-ΔNGFR magnetics beads
- Magnetic platform

1. INTRODUCTION

1.1 Description

The strategies for co-expressing at least two proteins involve the use of expression cassettes with different or bi-directional promoters, the employment of internal ribosome entry site (IRES) or the design of fused genes spaced by sequences of proteolytic recognition. There are some limitations with using these approaches including promoter interference, variability and imbalance in the expression or the size of recombinant vectors.

The pOnebyOne® family is designed for expression of two proteins in mammalian cells based in the 2A sequence. A strong promoter drives the expression cassette and 2A sequence allows the coexpression of the open reading frames included: one reading frame encoding the recombinant protein of interest and the other, a reporter protein. pOnebyOne® vector family is a versatile product that includes non-viral and viral expression vectors with different promoters and selectable markers. The kit includes linearized expression vector that permits plates without background and a robust enzyme to obtain recombinant clones efficiently.

pOnebyOne® retroviral expression vectors are an effective vehicle for delivering genetic material to almost any dividing mammalian cell with higher efficiency than using conventional transfection protocols. pOnebyOne® retroviral vectors combine an elevated retrovirus titer into a robust cloning system that allows the expression of two proteins simultaneously.

1.2 The family of pOnebyOne® retroviral expression vectors

The family of pOnebyOne® retroviral expression vectors comprise vectors that contain expression cassettes based in 2A sequence. 2A-like sequences are used by several families of viruses for producing multiple polypeptides. Unlike IRES based vectors where protein expression from the insert downstream of IRES is lower than the upstream insert, 2A based vectors allow both proteins to be produced in stoichiometric proportion. In addition, IRES is a relatively large sequence that must be packaged into virions and requires that the start codon of the second ORF to be fairly closed to the IRES, adding some difficulties to cloning.

2A short sequence allows multiple proteins to be encoded as polyproteins. It acts co-translationally, by preventing the formation of a normal peptide bond between the glycine and last proline of its sequence, resulting in the ribosome skipping to the next codon[1]. In the established model, the nascent 2A peptide interacts with the ribosome such that the C-terminal portion is sterically constrained within the peptidyl transferase sites. This blocks nucleophilic attack of ester linkage between 2A and tRNA-Gly by prolyl-tRNA in the ribosome acceptor site. Termination factors eRF1 and eRF3 are involved in the “cleavage” of

the polyprotein generating an upstream protein with a short 2A peptide C-terminal fusion, whereas the downstream protein includes a single proline residue on its N-terminus[2, 3].

2A-mediated cleavage is a universal phenomenon in all eukaryotic cells. 2A peptides have been used successfully to generate multiple proteins from a single promoter in some biological models: plants[4], zebrafish[5], transgenic mice[6] and human cell lines[7]. Many applications have been possible such as obtaining a sophisticated variety of plants (drought resistance, disease resistance and plants with engineered metabolic pathway) [4,8,9,10], reconstitution of T-cell receptor complex[11] and generation of pluripotent stem cells[12].

The pOnebyOne® family included non-viral and viral (retroviral and lentiviral) vectors with different reporter proteins and promoters. This versatility allows you to choose a vector based on your needs. Next table shows the available variants of pOnebyOne® retroviral vectors and general features of each one:

Catalogue Number	pOnebyOne® vector	Promoter	Reporter Protein	Size
ME0013	**I-Retroviral**	P_{CMV}	ΔNGFR	5811 bp
ME0014	**II-Retroviral**	P_{CMV}	eGFP	5696 bp
ME0015	**III-Retroviral**	$P_{EF1\alpha}$	ΔNGFR	6335 bp
ME0016	**IV-Retroviral**	$P_{EF1\alpha}$	eGFP	6220 bp

P_{CMV}, citomegalovirus promoter; $P_{EF1\alpha}$, elongation factor 1 alpha promoter; ΔNGFR, truncated nerve growth factor receptor; eGFP, enhanced green fluorescent protein

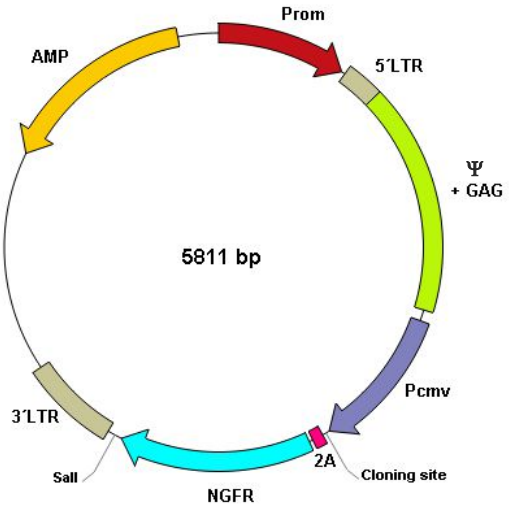
Both promoter employed in pOnebyOne® vectors are commonly used strong promoters. They constitutively express downstream genes. CMV promoter works perfectly in the most common cell lines but it shows poor expression in some stem cell lines and hematopoietic cell lines[13,14]. However, EF1 alpha promoter has been shown to work better than CMV promoter in hematopoietic cell lines, in primary cells and in some viral vectors.

Reporter markers used are a truncated version of nerve growth factor receptor (ΔNGFR) and an enhanced fluorescent protein from *Aequorea victoria* (eGFP). ΔNGFR is a complete solution to select positive clones, that can be visualized by cytometry using specific antibody labelled with FITC or similar and also, they could be enriched from negative clones with magnetic beads bearing anti-ΔNGFR antibody. While eGFP, optimized for brighter and higher expression in mammalian cells, could be visualized by cytometry or microscopy (Excitation wavelength maximum=488 nm/ Emission wavelength maximum=507 nm), positive cells can also be sorted.

1.3 Maps and sequences

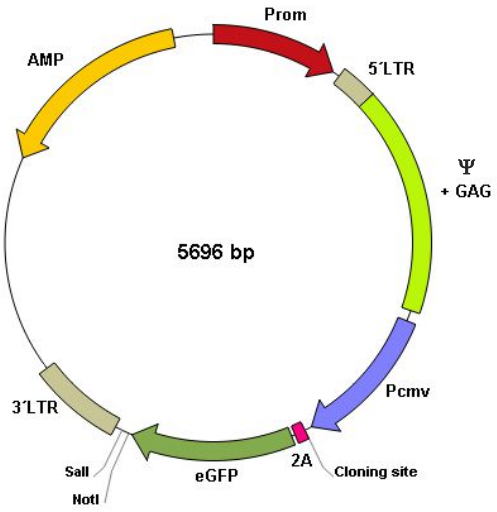
Vectors that constitute pOnebyOne® retroviral family are represented below. Indicated restriction sites correspond to unique restriction sites.

- **pOnebyOne®-I-Retroviral**



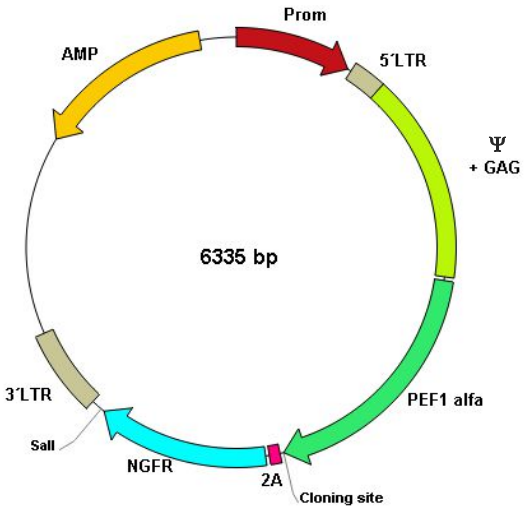
Features	
Promoter	8-567
5´LTR	574-748
Ψ+ packaging signal	749-1747
Citomegalovirus promoter	1780-2409
2A from equine rhinitis A virus	2442-2504
ΔNGFR	2511-3346
3´LTR syn	3415-3828
Ampicillin resistance gene (ORF) *(complementary strand)*	4782-5639

- **pOnebyOne®-II-Retroviral**



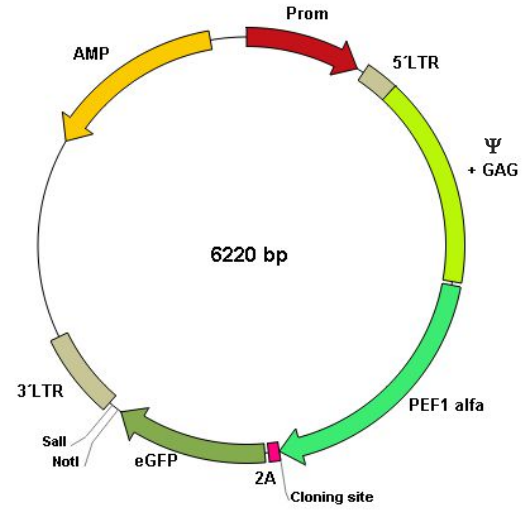
Features	
Promoter	8-567
5´LTR	574-748
Ψ+ packaging signal	749-1747
Citomegalovirus promoter	1780-2409
2A from equine rhinitis A virus	2442-2504
eGFP	2511-3227
3´LTR syn	3300-3713
Ampicillin resistance gene (ORF) *(complementary strand)*	4667-5524

- **pOnebyOne®-III-Retroviral**



Features	
Promoter	8-567
5´LTR	574-748
Ψ+ packaging signal	749-1747
Elongation factor 1 alpha promoter	1755-2956
2A from equine rhinitis A virus	2966-3028
ΔNGFR	3035-3870
3´LTR syn	3926-4352
Ampicillin resistance gene (ORF) *(complementary strand)*	5306-6162

- **pOnebyOne®-IV-Retroviral**



Features	
Promoter	8-567
5´LTR	574-748
Ψ+ packaging signal	749-1747
Elongation factor 1 alpha promoter	1755-2956
2A from equine rhinitis A virus	2966-3028
eGFP	3035-3751
3´LTR syn	3824-4237
Ampicillin resistance gene (ORF) *(complementary strand)*	5191-6048

2. PROCEDURES

2.1 Primers design

pOnebyOne® mammalian expression vectors allow directional cloning using specific sequence primers. These special considerations in the primers design will guarantee cloning your gene of interest in the correct orientation.

The **forward primer** (*5´PCR primer*) depends on the vector selected. The primer must include the next sequence

- For **pOnebyOne®-Retroviral-I and II**

5´ GTT TAA ACT TAA GCTG-**Kozak sequence*-ATG**-insert specific sequence 3´

- For **pOnebyOne®-Retroviral-III and IV**

5´ TAG GGA GAC CAA GCTG-**Kozak sequence*-ATG**-insert specific sequence 3´ **

* *The vertebrate consensus Kozak sequence is $G_{44}C_{39}C_{53}(A_{61}/G_{36})(C_{49}/A_{27})C_{55}$ but you can use the own Kozak sequence of your gene of interest (6 bp sequence just before ATG)*

** *This primer could be used to directional cloning into pOnebyOne®-I and II but the cloning efficiency is reduced 40-60 times.*

The **reverse primer** (*3´PCR primer*) is common to all vectors and it must include the next sequence:

5´ CTC TCC ACT GCC GAT insert specific sequence 3´

Do not include the stop codon of your gene at the 3´end of insert specific sequence of the primer –this would prevent the expression of the reporter protein gene.

Place the target sequence in frame with the 2A peptide (Figure 1)

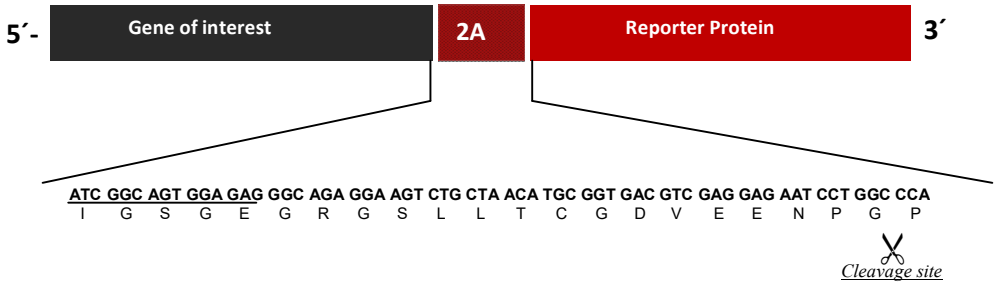


Figure 1. 2A sequence based vector. Both genes must be in frame and the nascent peptide is cleaving between the glycine and proline. After the cleavage, the short peptide IGSGEGRGSLLTCGDAEENPG (21 aminoacids) remains fused to the C-terminus of the protein of interest while the proline is added to the N-terminus of the reporter protein. 2A sequence used has high cleavage efficiency in some biological systems[17]. Essential reverse primer sequence for directional cloning is underlined.

Additional important tips to consider in primer design are:

1. The size of the specific annealing sequence to target a gene must be 18-30 bp for good target specificity.
2. Primers hairpins with a melting temperature higher than 46ºC should be avoided because of those structures decreasing PCR efficiency. Take care do not modify the specific sequence that guarantee directional cloning.

For PCR reaction, it is highly recommended to use proofreading enzymes or any other similar enzymes to generate high fidelity amplification products. Using non-proofreading enzyme reduces the cloning efficiency.

2.2 Vector consideration

In order to guarantee optimal cloning results, pOnebyOne® vectors are supplied linearized with a proved low background of non-recombinants. Positive clones are obtained with analysis of a minimal number of clones, with efficiency higher than 97%.

Selecting recombinant plasmids using pOnebyOne® linearized vectors are extremely easy. The pOnebyOne® vectors are design and prepared to prevent self-ligation without need phosphatases, special bacterial strains or other singular components.

pOnebyOne® Retroviral vectors are designed for maximal virus titer in 293 cells. Viral supernatants obtained three days after transfection allow for infection of dividing target cells. Keep in mind that the viral titer depends on the size of the insert. The highest titer has been obtained with 6 kb length between LTRs.

2.3 Cloning

1. Spin pOnebyOne® vector to collect content at the bottom of the tubes.

2. **On ice**, set up the reaction as described below. If you thawed all kits components out of ice, you must pre-chill all them before use during 10 minutes.

Match Reaction	Cloning Reaction	Control Reaction	Background Reaction
pOnebyOne® vector (50 ng/µL)	1 µL	1µL	1 µL
10x Glue-Enzyme Buffer	1.5 µL	1.5 µL	1.5 µL
PCR Product	X µL	-	-
Control Insert DNA	-	2 µL	-
Water (*Molecular Biology grade*)	up 13 µL	up 13 µL	up 13µL

3. Mix the reactions by pipetting.
4. Incubate 10 minutes on ice.
5. Add 2 µL Glue-Enzyme (10 U/µL) to each tube, mix gently and incubate 45 minutes on ice.
6. Proceed to transformation (see **Section 2.4**)

We strongly recommend:

Note

To prepare a match-reaction with the supplied control insert to check that match reaction and transformation process are working properly. This positive control MUST be prepared and transformed at the same time than your samples.

2.4 Transformation using chemical competent cells

Use competent cells with a competence of at least $1x10^7$ colonies/ µg DNA following the protocol provided with the competent cells. Standard and fast protocol for transformation can be found below. The fast transformation protocol should not be less than 4-5 fold of the number of colonies obtained using the classical transformation protocol. Thus, for the most demanding cloning task the classical protocol is recommended but for routine cloning the fast transformation protocol is suggested as it saves 1.5 hours.

2.4.1 Standard protocol for transformation

1. Prepare one LB ampicillin plate for each match reaction, plus one plate for determining transformation efficiency and one plate for control transformation (vector without insert). Equilibrate the plates to room temperature prior to plating (**Step 8**).

2. Centrifuge the tubes containing the reactions to collect content at the bottom of the tube. Add 15 µL of each reaction to a sterile 1.5 mL microcentrifuge tube on ice. Set up another tube on ice with 50 pg uncut plasmid (*non supplied*) for determination of the transformation efficiency of the competent cells.

3. Remove one tube of frozen Competent Cells (*non supplied*) from storage at -80°C and place it in an ice bath until just thawed (about 10 to 15 minutes). Mix the cells by **gently** flicking the tube with your fingertips.

IMPORTANT: *Do not thaw competent cells with your hands. Keeping competent cells out of an ice bath even for extremely short times strongly affects the transformation efficiency of cells. Also avoid excessive pipetting, as the competent cells are extremely fragile and thus mixing of DNA with competent cells should be made by gently flicking and not by pipetting.*

4. **Carefully** transfer 50 µL of cells into each tube prepared in **Step 2**.

5. **Gently** flick the tubes to mix and place them on ice for 30 minutes.

6. Heat-shock the cells for exactly 45 seconds in a water bath at exactly 42°C (***Do not shake nor heat shock more than 45 seconds***).

7. Immediately return the tubes to ice for 2 minutes and plate all transformation mix onto pre-warmed LB ampicillin plates

8. Incubate the plates overnight (12–16 hours) at 37°C.

2.4.2 Fast transformation protocol

An alternative transformation protocol of only 5 minutes is recommended. The main difference between this protocol and the standard protocol is that this fast protocol avoids the heat shock step and instead this essential step occurs directly on the plate. If you feel unfamiliar with this protocol, please use the standard transformation protocol.

EXTREMELY IMPORTANT: *Before starting this protocol you must pre-warm LB Agar-antibiotic-X-Gal-IPTG plates at 37 ^{0}C for at least 1 hour. Heat shock occurs on the pre-warmed plates when using this protocol, thus it is essential that plates are pre-warmed before transformation and withdrawn from incubator to laminar flow cabinet only just before transformation.*

1. Prepare one LB ampicillin plate for each match reaction, plus one plate for determining transformation efficiency and one plate for control transformation (vector without insert). Pre-warm plates at 37^{o}C in the incubator for at least one hour before transformation.

2. Centrifuge the tubes containing the match reactions and the control reaction to collect content at the bottom of the tube. Add 10 µL of each reaction to a sterile 1.5 mL microcentrifuge tube on ice. Set up another tube on ice with 25 pg uncut plasmid (*non supplied*) for determination of the transformation efficiency of the competent cells.

3. Remove a tube of frozen Competent Cells (*non supplied*) from storage at -80^{o}C and place it in an ice bath until just thawed (about 10 to 15 minutes). Mix the cells by **gently** flicking the tube with your fingertips.

IMPORTANT: *Do not thaw competent cells with your hands. Keeping competent cells out of an ice bath even for extremely short times strongly affects the transformation efficiency of cells. Also avoid excessive pipetting, as the competent cells are extremely fragile and thus mixing of DNA with competent cells should be made by gently flicking and not by pipetting.*

4. **Carefully** transfer 50 µL of cells into each tube prepared in **Step 2**. For determination of transformation efficiency add 50 µL of competent cells to the tube prepared in **Step 2** containing 25 pg of uncut plasmid DNA.

5. **Gently** flick the tubes to mix and place them on ice for 5 minutes.

6. Plate all transformation mix onto pre-warmed LB ampicillin plates

7. Incubate the plates overnight (12–16 hours) at 37°C.

2.4.3 Analysis of transformants

Colony PCR is a suitable approach for screening colonies before isolation of plasmid DNA. The principle behind colony PCR is the lysis of plasmid bearing bacteria (after saving a portion of the bacterial colony since the sample is destroyed by colony PCR) and PCR using as template the crude unpurified plasmid

DNA released from bacteria. The most common method for bacterial lysis is boiling at 100°C for 10 minutes. Some protocols use the initial DNA denaturation step of PCR as the bacterial lysis step and thus in this protocol the bacterial colony is added directly to a master mix of polymerase, buffer, dNTPs and primers.

The protocol below is one we have tested at Canvax but other protocols are also suitable.

Protocol for colony PCR

Previously, check the material required not supplied with kit.

1. For each bacterial colony to be screened prepare a 1.5 mL microcentrifuge tube with 30 µL of water.
2. Pick one colony with a sterile toothpick or a sterile pipet tip and resuspend the colony in the 1.5 mL microcentrifuge tube with water.
3. Streak the toothpick from **Step 2** in either a plate with antibiotic or liquid media with antibiotic (e.g. LB with antibiotic) for growing positive colonies. Discard the toothpick and repeat **Steps 2** and **3** for each colony to be screened.
4. Boil the tubes of **Step 2** in a water bath at 100°C for 10 minutes to lyse the cells and inactivate nucleases. Please make sure that the tubes are tightly closed because by boiling the lids can pop open.
5. While tubes are boiling, prepare a PCR master mix follow the guidelines of your Taq polymerase supplier for a 50 µL final volume reaction. Prepare a reaction master mix for at least one more sample than the total number of colonies to be screened. Distribute 30 µL of the master mix into sterile PCR tubes.
6. Spin boiled tubes from **Step 4** at 14500 rpm during 5 minutes in a microcentrifuge.
7. Add 20 µL of cleared lysate from **Step 6** to each PCR tube with 30 µL of master mix prepared in **Step 5**.
8. Run your specific PCR program in a thermocycler.
9. Load the tube contents on agarose gel and visualize it.
10. Choose positive clones and grow it in an appropriate amount of LB-Amp Broth, and purify the construct


Note

Note: *You could analyse the recombinant plasmids by restriction analysis and by sequencing.*

2.4.4 Sequencing

Confirm identity of your insert by sequence analysis of the construct. You may use one of the following sequencing primers which are located upstream of the cloning site:

Vectors with CMV promoter: 5'-CTA ACT AGA GAA CCC ACT GC-3'

Vectors with EF1α promoter: 5'-TGG TTC ATT CTC AAG CCT CAG-3'

3. TROUBLESHOOTING

For questions not addressed here, please contact us at www.canvaxbiotech.com or alternatively contact your local Distributor.

PROBLEM	CAUSE	SOLUTION
No colonies (even in Control Insert Reaction)	Any component is missing in the match reaction	Repeat match reaction and transformation and don´t forget to include controls
	Competent cells are damaged or with very low efficiency	Check the transformation efficiency of *E. coli* competent cells. A transformation efficiency lower than $1x10^7$ cfu/µg is not recommended
Colonies only in the Control Insert Reaction	Any component is missing in the sample reaction	Repeat match reaction and transformation and don´t forget to include controls
	PCR insert is degraded or damaged	Check quality of insert by gel electrophoresis
	A very low amount or no PCR insert has been used for the reaction. Alternatively, a very high amount of insert was used	Check by gel electrophoresis the yield of PCR or agarose purification. Purified products can also be quantified by Abs at 260/280 nm (eg. by Nanodrop™). If needed increase/decrease amount of insert in a new match reaction.
	Salts and/or ethanol present in the purified PCR insert	Repeat PCR and purification from agarose for a new reaction and transformation.
	The reaction is not optimal	Optimise the reaction by trying other insert to vector ratios
	The PCR insert product has multiple bands and is used unpurified directly for cloning	- Gel purified your PCR insert or screen more colonies by colony PCR. - Also is possible that the template complexity is high (eg. genomic DNA), then is preferably to synthetize two couple of primers: One, to obtain the gene of interest and the other pair, to clone in the expression vector. This fact avoids cloning artefacts due to the presence of additional long sequences for orientated cloning.
	PCR primers have a bad design	Check your primers have been designed accordingly to our recommendations. If the primers don´t anneal with the vector ends, no colonies will be obtained.
	There is a negative correlation between the size of the fragments and the number of colonies after transformation (fewer colonies with increasing size insert)	Adjust the reaction in order to have a molar ratio vector: insert 1:5

4. REFERENCES

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