canvax

pOnebyOne® 2A-Based Mammalian Expression Vectors

ME001-N: pOnebyOne®I-Neo

ME002-N: pOnebyOne®II-Neo

ME003-N: pOnebyOne®III-Neo

ME004-N: pOnebyOne®IV-Neo

ME005-N: pOnebyOne®V-Neo

ME006-N: pOnebyOne®VI-Neo

ME001-P: pOnebyOne®I-Puro

ME002-P: pOnebyOne®II- Puro

ME003-P: pOnebyOne®III- Puro

ME004-P: pOnebyOne®IV- Puro

ME005-P: pOnebyOne®V-Puro

ME006-P: pOnebyOne®VI-Puro

ME001-H: pOnebyOne®I-Hygro

ME002-H: pOnebyOne®II- Hygro

ME003-H: pOnebyOne®III- Hygro

ME004-H: pOnebyOne®IV- Hygro

ME005-H: pOnebyOne®V-Hygro

ME006-H: pOnebyOne®VI-Hygro

Upon Receipt
Store Kits at -20ºC

PRODUCT MANUAL
Version 3.2
Last updated: May 2015

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Materials provided

Item	Quantity	Storage
pOnebyOne® linearized vector (50 ng/µL)	20 µL	-20ºC
Glue-Enzyme	40 µL	-20ºC
10x Glue-Enzyme Buffer	50 µL	-20ºC
Insert Control (30 ng/µL)	10 µL	-20ºC
pOnebyOne® circular control vector	5 µL	-20ºC

Storage temperature: -20ºC.

Expiration date: See on the label kit.

Additional material required

For cloning

- Proofreading DNA polymerase
- High efficiency competent cells
- 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*)
- Primer forward for colony PCR
- Primer reverse for colony PCR
- Restriction enzymes
- Sequencing primer

For plasmid purification

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

For transfection

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

For mammalian cell analysis and enrichment

- Anti-ΔNGFR antibody
- Anti inmunoglobulin-FITC
- Anti-ΔNGFR magnetics beads
- Magnetic platform
- Neomycin, Puromycin or Hygromycin

1. INTRODUCTION

1.1 Description

The strategies for co-expressing at least two proteins involve the use of two expression cassettes or bi-directional promoters, the employment of internal ribosome entry site (IRES) or the design of fusion of 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 are a mammalian expression vectors designed for expression of two proteins in mammalian cells based in 2A sequence. A strong promoter drives the expression cassette and 2A sequence allows the co-expression of the open reading frames included: one reading frame encoding the recombinant protein of interest and the other, a reporter protein. The use of pOnebyOne® family vectors significantly facilitates the selection of positive cells expressing the recombinant gene of interest. In transient, the cells could be selected using the reporter marker and the creation of stable cell lines is also possible because of the vector has an antibiotic resistant cassette (NEO^R, $PURO^R$ or HYG^R).

pOnebyOne® mammalian expression kits include ready to use optimized mammalian expression vectors without cloning background and a robust enzyme to obtain recombinant clones efficiently. Mammalian expression vectors are linearized and ready to clone into your gene of interest.

1.2 The family of pOnebyOne® mammalian expression vectors

The family of pOnebyOne® mammalian 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 IRES is lower than of the upstream insert, 2A based vectors allow both proteins are produced in stoichiometric proportion. In addition, IRES is relatively large sequence that requires that the start codon of the second ORF is fairly closed to the IRES, adding some difficulties to cloning.

2A 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 since to obtain 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 vectors with different reporter proteins, promoters and resistance markers. This versatility permits to choose a vector in base your needs. Next table shows the available variants of pOnebyOne® vectors and general features of each one:

Catalogue Nº	pOnebyOne®	Promoter	Reporter Protein	Resistance	Size
ME001-N	I-NEO	P_{CMV}	ΔNGFR	NEOMYCIN	6258 bp
ME002-N	II-NEO	P_{CMV}	eGFP		6141 bp
ME003-N	III-NEO	$P_{EF1\alpha}$	ΔNGFR		6568 bp
ME004-N	IV-NEO	$P_{EF1\alpha}$	eGFP		6451 bp
ME005-N	V-NEO	P_{CMV}	LUC		7072 bp
ME006-N	VI-NEO	$P_{EF1\alpha}$	LUC		7382 bp
ME001-P	I-PURO	P_{CMV}	ΔNGFR	PUROMYCIN	5926 bp
ME002-P	II-PURO	P_{CMV}	eGFP		5808 bp
ME003-P	III-PURO	$P_{EF1\alpha}$	ΔNGFR		6236 bp
ME004-P	IV-PURO	$P_{EF1\alpha}$	eGFP		6119 bp
ME005-P	V-PURO	P_{CMV}	LUC		6740 bp
ME006-P	VI-PURO	$P_{EF1\alpha}$	LUC		7050 bp
ME001-H	I-HYG	P_{CMV}	ΔNGFR	HYGROMYCIN	6314 bp
ME002-H	II-HYG	P_{CMV}	eGFP		6306 bp
ME003-H	III-HYG	$P_{EF1\alpha}$	ΔNGFR		6624 bp
ME004-H	IV-HYG	$P_{EF1\alpha}$	eGFP		6615 bp
ME005-H	V-HYG	P_{CMV}	LUC		7128 bp
ME006-H	VI-HYG	$P_{EF1\alpha}$	LUC		7438 bp

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

Both promoter employed in pOnebyOne® vectors are common 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), an enhanced fluorescent protein from *Aequorea victoria* (eGFP) and the firefly luciferase from *Photinus pyralis*[15]. ΔNGFR is a complete solution to selected positive clones. They could 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 could be sorted by sorter cytometer. Luciferase is a sensitive enzymatic reporter that can be assayed by standard luciferase activity reaction.

1.3 Maps and sequences

You can see on the Canvax´s website the Maps and sequences of vectors that constitute pOnebyOne® family.

2. PROCEDURES

2.1 Primers design

pOnebyOne® mammalian expression vectors permit 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®-I, II and V**

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

- For **pOnebyOne®-III, IV and VI**

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, II and V 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 ᔓ insert specific sequence 3´

ᔓ *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.*

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

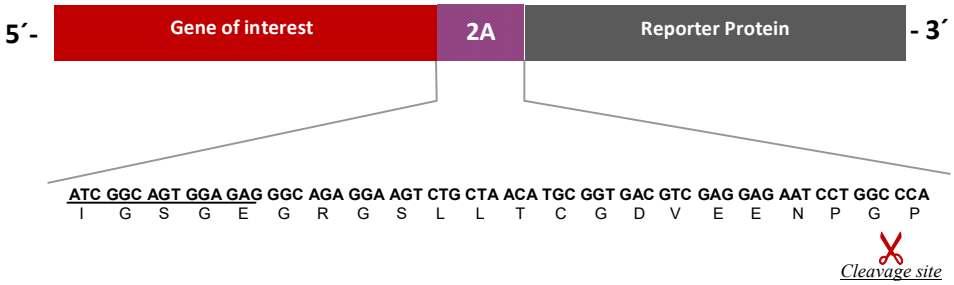

Figure 1. 2A sequence based vector. Both genes must be in frame and the nascent peptide is cleaving between the glycine and proline. After the cleavage, the short peptide IGSGEGRGSLLTCGDAEENPG (21 aminoacids) remains fused to the C-terminus of the protein of interest while the proline is added to the N-terminus of the reporter protein. 2A sequence used has a 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 specific annealing sequence to target 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 like 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 checked low background of non-recombinants. With the analysis of a minimal number of clones are obtained positive clones, for 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.

2.3 Cloning

1. Spin pOnebyOne® vector to collect content at the bottom of the tubes.
2. **On ice**, set up 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 13 µL

** *Relation vector: insert 1:5 is recommended*

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. In our hands the fast transformation protocol gives no 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 (*not supplied*) for determination of the transformation efficiency of the competent cells.

3. Remove a tube of frozen Competent Cells (*not supplied*) from storage at -80ºC and place 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***).
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 37ºC 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 37ºC 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 (*not supplied*) for determination of the transformation efficiency of the competent cells.
3. Remove a tube of frozen Competent Cells (*not supplied*) from storage at -80ºC and place 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 100ºC 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 100ºC 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 at least one reaction master mix more 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. Running PCR program at thermocycler machine.
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

You could analyze 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 have 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 synthetized 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 designed according our recommendations. If the primers don´t aneal with the vector ends, no colonies are 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

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