

# **pSpark<sup>®</sup> DNA cloning system**

Manual for cat. n<sup>o</sup> :

C0001 (pSpark<sup>®</sup> I)

C0002 (pSpark<sup>®</sup> II)

C0003 (pSpark<sup>®</sup> III)

C0004 (pSpark<sup>®</sup> IV)

C0005 (pSpark<sup>®</sup> V)

C0006 (pSpark<sup>®</sup> Done)

**Upon Receipt  
Store Kits at -20°C**

**PRODUCT MANUAL**

Version 3.0

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## MATERIALS PROVIDED, KIT STORAGE AND EXPIRATION DATE

pSpark® DNA Cloning Vector (20 ng/μL) 20 rxn (20 μL)						
Item	I	II	III	IV	V	Done
T4 DNA Ligase (5 Weiss Units/μL)	100 U (20 μL)					
5x T4 DNA Ligase Buffer	100 μL					
10x PEG 6000	50 μL					
Control Insert (1 kb)	5 μL					

See the kit label and vector vial label for more information

**Storage temperature: -20 °C in a NON frost-free freezer.**

**Expiration date: See on the kit label**



**IMPORTANT:** pSpark® DNA cloning systems are one of the most stable cloning kits available on the market. **However kits MUST be stored at -20 °C in a non-frost free freezer since temperature rises above 0 °C daily in frost-free freezers. If properly stored, kits are guaranteed for 9 months from the date of purchase.**



Note

**ADVICE:** All pSpark® DNA cloning vectors are stable for at least 1 month at 4 °C and even at 20-25 °C for up to 2 days although storage temperatures above -20 °C are not recommended. , In case of incident, such as a power failure, stored vectors should be tested with the supplied control insert before considering discarding it. However, please note that T4 DNA ligase is extremely temperature-sensitive and storage temperatures above -20 °C inactivates the enzyme.

# 1. INTRODUCTION

## 1.1 Principle and advantages

The pSpark® DNA cloning system is based on a novel technology (patent pending) for cloning of blunt-ended DNA, for example DNA amplified by PCR with proofreading or high-fidelity DNA polymerases. The vector is prepared by digestion of pSpark® DNA cloning vectors at EcoRV site before treating both ends to prevent vector self-ligation. This treatment results in a vector with a cloning efficiency about 50 times higher than T/A based vectors, that is, up to 4 more colonies are obtained using 12.5 times less DNA.

**Table 1. Main advantages of pSpark® DNA cloning systems**

Feature	pSpark® DNA cloning systems
<b>Efficient</b>	<ol style="list-style-type: none"> <li>1. Up to <b>50x more cloning efficiency</b> than T/A based kits</li> <li>2. Just <b>6,7 ng per kb</b> of insert needed for optimal ligation</li> <li>3. Over <b>2500 positive colonies</b> expected under optimal conditions</li> </ol>
<b>Fast&amp;Easy</b>	<ol style="list-style-type: none"> <li>1. <b>No</b> steps after PCR</li> <li>2. Just <b>20-25 minutes</b> from PCR end <b>to plating</b></li> <li>3. Use of <b>PCR product directly</b> for cloning is possible</li> </ol>
<b>Flexible</b>	<ol style="list-style-type: none"> <li>1. <b>No</b> special competent cells needed</li> <li>2. <b>No</b> special primer design needed</li> <li>3. Use of <b>competent cells</b> with <b>subcloning efficiency</b> possible</li> <li>4. <b>No</b> primer phosphorylation required</li> <li>5. Size of insert form less than <b>0.1 kb to up to 14 kb</b></li> <li>6. Ligation time from <b>10 minutes to overnight</b></li> <li>7. Any proofreading polymerase could be used</li> </ol>
<b>Robust</b>	<ol style="list-style-type: none"> <li>1. <b>No cloning bias</b> due to <b>transcription</b> of toxic genes</li> <li>2. <b>No cloning pitfalls</b> due to variable Taq overhanging efficiency</li> <li>3. <b>High vector stability</b></li> <li>4. Very low <b>background</b> of <b>less than 1%</b> (2% to pSpark® II DNA Cloning vector).</li> <li>5. Cloning possible with <b>less than 1 ng/kb of insert</b></li> </ol>

pSpark® DNA cloning system, in combination with the recent development of highly robust high-fidelity DNA polymerases like Phusion®, iProof™, KAPAHiFi™ and PfuUltra™ makes DNA cloning an extremely easy task.

With as little as 1 ng of a 1 kb insert hundreds positive colonies could be readily obtained. As the system is not based on toxic genes to eliminate background there is no cloning bias due to cloning of sequences that for example behave as promoters in *E. coli* or due to cloning of Open Reading Frames (ORF).

## 1.2 The family of pSpark® DNA cloning vectors

All pSpark® DNA cloning vectors comprise vectors that contain both T7 and SP6 RNA polymerase promoters flanking the multiple cloning region (MCS) for *in vitro* transcription of cloned DNA using either T7 or SP6 RNA polymerases. Also, all vectors belonging to pSpark® DNA cloning vectors family have the origin of replication of the filamentous phage f1. Synthesis of single-stranded DNA requires phage-encoded gene II, X and V and is initiated at f1 ori. Also, all pSpark® DNA cloning vectors have binding sites for pUC/M13 forward and reverse primers and thus the cloned insert can be amplified or sequenced with those primers.

Two versions of the MCS have been developed for pSpark® DNA cloning system: one *classic MCS (cMCS)* with only one nucleotide difference from the popular MCS derived from pGEM® vector from Promega, and one *advanced MCS (aMCS)* with blunt restriction enzymes at each side of the cloned insert, 8bp rare cutters at each side of cloned insert, inexpensive restriction enzymes recognition sites at each side of cloned insert, enzymes that generate ends compatible each other at each side of cloned insert and enzymes with activity in several buffers, for fast and inexpensive analysis of recombinants. In several pSpark® DNA cloning vectors the MCS has been properly inserted within the alpha-peptide coding region of the enzyme beta-galactosidase for insertional inactivation of the alpha-peptide by recombinant clones, thus allowing positive clones to be directly identified by blue/white screening on X-Gal plates.

The MCS of the **pSpark®-Done Vector** contains sequences on either side of the insert that are recognized by the restriction enzymes Not I and EcoR I. This allows the insert DNA to be removed with a single restriction digest using either of these enzymes.

Background of pSpark® vector self-ligation in a ligation reaction without insert produces less than 1% of background of blue colonies when compared with a ligation containing both insert and vector, and thus blue/white screening is not required. For example, under optimal conditions a ligation of 30 ng of a 1 kb insert and transformation into cells with a transformation efficiency of  $4 \times 10^7$  cfu/ $\mu$ g produces over 2500 white colonies and less than 20 blue colonies.

Some pSpark® DNA cloning vectors exploit the very low background feature of the vector for the expression of toxic genes under transcription-free conditions. In those variants, the lac promoter has been eliminated and therefore blue/white screening is not allowed, but those vectors are useful for cloning genes that produce toxic polypeptides by transcription/translation. For those transcription-free pSpark® DNA cloning vectors, a background of less than 1% white colonies should be expected under optimal conditions. No more than 30 white colonies are obtained in a ligation without insert when using cells with a transformation efficiency of  $2 \times 10^7$  cfu/ $\mu$ g.

As a help for cloning of inserts with about 8-14 kb, a variant of pSpark® DNA cloning vector with low copy number has been developed. This low copy variant of pSpark® DNA cloning vector is also transcription-free, for the most demanding cloning tasks. Finally, there are variants of pSpark® DNA cloning vector with either ampicillin resistance or with combined ampicillin and kanamycin resistance. The vector with both ampicillin and kanamycin resistance

is useful for cloning PCR products amplified from any plasmid vector without the need to gel-purify bands to eliminate the background due to the template vector used for PCR. **Table 2** shows the available variants of pSpark® DNA cloning kits and features of each one.

**Table 2. Family of pSpark® vectors, main characteristics and applications**

Vector	OriC (copy number)	MCS	Blue/white feature	Antibiotic	Size (bp)	Application
pSpark® I	pUC (High)	Advanced	Yes	Amp	3013	General cloning
pSpark® II	pUC (High)	Classical	Yes	Amp	3001	General cloning
pSpark® III	pUC (High)	Advanced	Yes	Amp/Kan	3980	Unpurified PCR cloning
pSpark® IV	pUC (High)	Advanced	No (transcription free)	Amp	2811	Toxic genes cloning
pSpark® V	pBR322 (Low)	Advanced	No (transcription free)	Amp	3369	Unstable and toxic genes cloning
pSpark® Done	pUC (High)	Classical	Yes	Amp	3016	General cloning

**Note:** All pSpark® DNA cloning vectors have f1 ori, pUC/M13 forward and reverse primers binding sites and T7/SP6 RNA polymerases binding sites.

For MCS sequences for the various vectors, please refer to Appendix I.

### 1.3 pSpark® DNA cloning vector maps

See Appendix I and II. The maps in Appendix I shows the features of pSpark® DNA cloning vectors and the sequence surrounding the advanced Multiple Cloning Site (aMCS) in Appendix II. Unique restriction sites in the aMCS are indicated. The arrows indicate the start of transcription for the T7 and SP6 polymerases.

The complete sequence of each pSpark® DNA cloning system can be found on our web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)) and are also deposited in the Genbank with the following accession numbers:

<u>Vector</u>	<u>GenBank accession number</u>
pSpark® I	GQ489184
pSpark® II	GQ489185
pSpark® III	GQ489186
pSpark® IV	GQ489187
pSpark® V	GQ489188

**Note:** Inserts can be sequenced using the SP6 Promoter Primer, T7 promoter primer, pUC/M13 forward primer, or pUC/M13 reverse primer.

Sequences and melting temperatures of pUC/M13 primers are the following:

Primer	Sequence	Melting temperature
pUC/M13 Forward	5'-GTAAAACGACGGCCAGT-3'	52 °C
pUC/M13 Reverse	5'-AGGAAACAGCTATGACCATG-3'	58 °C

## 1.4 Specialized applications of the pSpark® DNA cloning systems

- Cloning of high fidelity PCR amplified products.
- Production of ssDNA.
- Blue/white screening for recombinants (NOT available in transcription-free variants)
- In vitro transcription from T7/SP6 dual-opposed promoters.
- Cloning of toxic genes (ONLY transcription-free vector variants)
- Cloning of unstable genes, for example genes with repeated sequences (ONLY transcription-free and low copy vector variants)
- Cloning directly from PCR using plasmid cloned genes as template (SPECIALLY vectors with dual antibiotic resistance to avoid background due to template)

## 1.5 Additional materials required (but NOT supplied with kits unless otherwise stated)

1. **A proofreading DNA polymerase and ultrapure dNTPs.** Any thermostable DNA polymerase that produces at least a fraction of molecules with both blunt ends might be used to amplify by PCR the DNA to be cloned using pSpark® DNA cloning systems. (see **Appendix III** and **Section 2.2.2.1**)

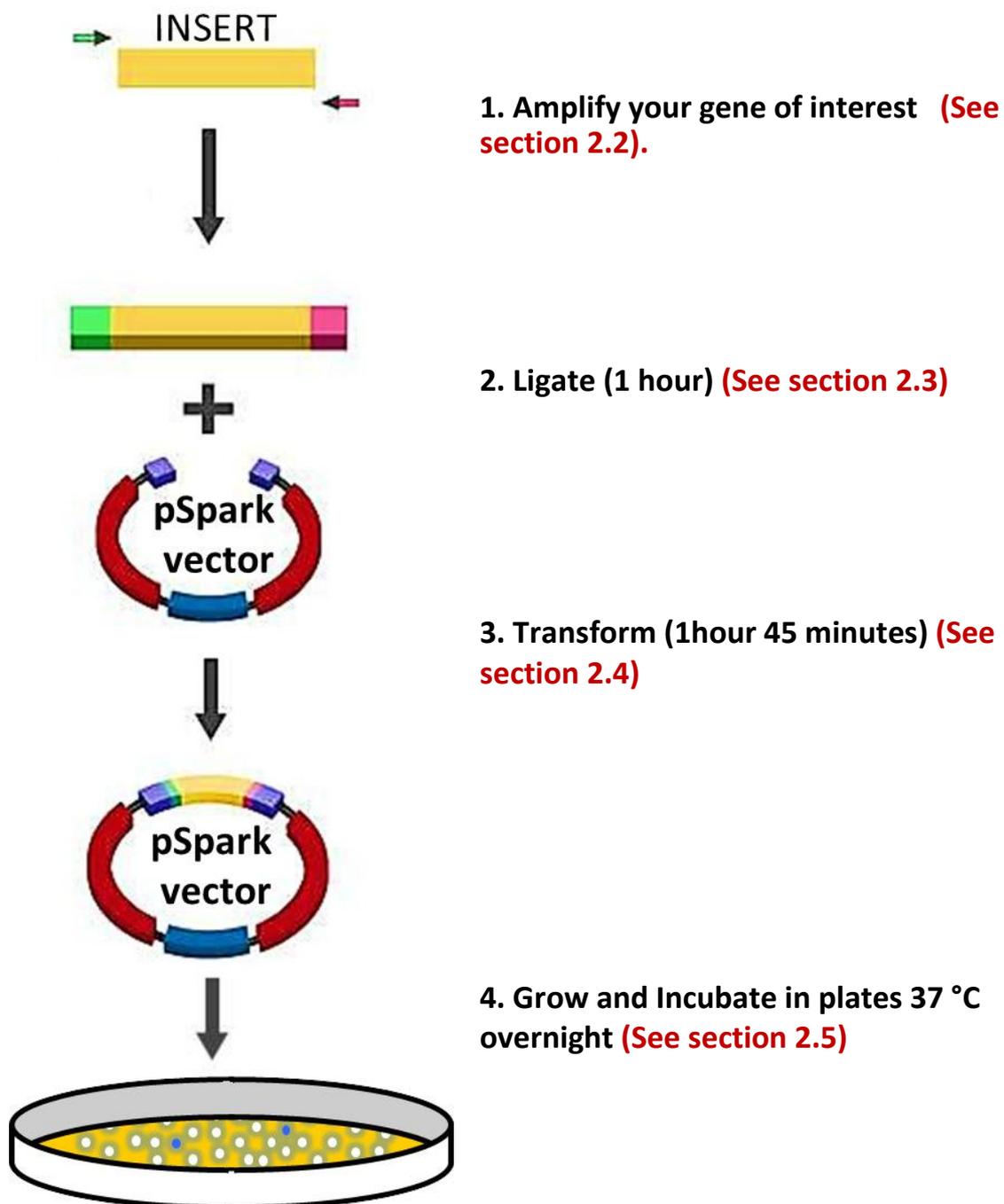


**EXTREMELY IMPORTANT:** *Please do not use Taq or Tth DNA polymerases for amplification. Low fidelity amplified DNA has single base overhangs at each 3'-end and thus this DNA CAN NOT be cloned into pSpark® DNA cloning systems, unless an additional DNA blunting step is performed.*

2. Thermocycler.
3. Gel electrophoresis equipment.
4. Microcentrifuge
5. Agarose DNA purification Kit.
6. Competent *E. coli* cells
7. Material required for transformation and transformants selection:
  - Autoclave for media sterilization.
  - LB-agar plates with an antibiotic such as Ampicillin optionally containing both X-Gal and IPTG. (See **Appendix III** for preparation of Media and Reagents).
  - Incubator at 37 °C.
  - LB Ampicillin.
  - Orbital shaker at 37 °C and 230-260 rpm for plasmid purification
  - Water bath with regulated temperature exactly at 42 °C
  - Ice bath
  - SOC medium (Optional)
  - Timer
  - Taq DNA polymerase and dNTPs (if colonies are screened by colony PCR)
  - Sterile tubes (both 0.2 mL tubes for PCR and 1.5 mL microcentrifuge tubes for ligation, transformation and colony PCR)
  - Sterile pipet tips (and optional sterile toothpick to use in protocol for colony PCR)

## 2. DETAILED PROTOCOL

### 2.1 Experimental outline



## 2.2 PCR

### 2.2.1 PCR Primers design

One of the main advantages of pSpark® DNA cloning systems is that any primer could be used for cloning. This includes, but it is not limited to, unphosphorylated and phosphorylated primers, primers purified by any technique such as desalted only, reverse cartridge-purified, HPLC or PAGE-purified, primers with modified bases and primers with any sequence at their 5'-ends. Expensive primers such as 5'-phosphorylated are not needed for a successful cloning with pSpark® DNA cloning systems. Also, no special sequences at the 5'-ends of the primers are needed, thus avoiding cloning artefacts due to the presence on long sequences in the primers, specially when using complex template DNA such as genomic DNA.

Some important tips to consider in primer design are:

1. The size of the primers is generally between 18-30 bp for good target specificity. This also depends on the template complexity: complex templates such as genomic DNA are more difficult to be amplified with high specificity with short primers, while short primers are acceptable for low complexity templates such as plasmids or lambda DNA. As a general rule, both short and extremely long primers favour unspecific binding.
2. Primer hairpins with a melting temperature higher than 46-48 °C should be avoided. Such hairpins should remain undissociated at annealing temperatures thus decreasing PCR efficiency.
3. Primer dimers should be avoided. Such dimers decrease PCR efficiency.
4. The melting temperature of the primer pair used for PCR should be about the same with no more than 4-6 °C difference. As the melting temperature of the PCR should be selected according to the primer with the lower melting temperature, if there is a high difference in  $T_m$  between the primer pair used for PCR then the primer with the higher melting temperature should bind to unspecific sites, resulting in at least several bands in the PCR product.
5. Avoid unspecific binding sites of primers of at least 6-7 bp on template DNA mostly at the 3'-ends of the primers.
6. Use a primer-design software and if not familiar with primer design, seek advice.

### 2.2.2 PCR Amplification

**Polymerase Selection for PCR:** The pSpark® DNA cloning systems are designed for the cloning of blunt PCR products amplified by proofreading or high-fidelity DNA polymerases. Those polymerases include first-generation thermostable DNA polymerases like Pfu, Pwo, KOD HiFi and Platinum® Pfx DNA polymerases, polymerase blends such as Expand™ High Fidelity PCR System, Platinum® Taq High Fidelity and AccuTaq® DNA polymerase and second generation thermostable high fidelity DNA polymerases like Phusion®, iProof™, KAPAHiFi™

and PfuUltra™ DNA polymerases. In particular second-generation thermostable DNA polymerases like Phusion®, iProof™ and KAPAHiFi™ are recommended, but any proofreading thermostable DNA polymerase that produces at least a fraction of molecules with both blunt ends is compatible with the pSpark® DNA cloning systems. An extensive albeit not complete list of polymerases suitable for cloning into pSpark® DNA cloning systems is listed on **Appendix III**.



**IMPORTANT:** *Please do not use Taq or Tth DNA polymerase for amplification. Low fidelity amplified DNA has single base overhangs at each 3'-end and thus this DNA can not be cloned into pSpark® DNA cloning systems, unless an additional DNA blunting step is performed.*

While Taq polymerase is a highly robust enzyme and is a familiar product in every molecular biology laboratory, this enzyme introduces mutations in a large percentage of amplified molecules and thus it is not recommended for cloning DNA. Instead high fidelity DNA polymerases have been considered for a long time enzymes that produce a high percentage of reaction failures, produce low yields and need long amplification times. The second generation of high-fidelity DNA polymerases that comprises Phusion®, iProof™, KAPAHiFi™ and PfuUltra™ addresses such issues. Internal R&D at Canvax has used Phusion®, iProof™ and also KAPAHiFi™ DNA polymerases in over 1000 different amplifications over the last two years and we have found they are as robust and easy to use as Taq polymerase, but in over 800.000 bases sequenced to date we have found no mutations. When using both Taq polymerase and blends of polymerases, we have found that about 10% of sequenced clones had mutations.

We strongly recommend reading and following protocols of polymerases manufacturers for using their enzymes.

**PCR Products:** PCR amplified DNA should be analysed on an agarose gel before use in the ligation reaction to verify both the quality and quantity of your PCR product. A PCR that contains one single homogeneous band with only primers-dimers can be purified by any high quality PCR clean-up kit for cloning into pSpark® DNA cloning systems. In several cases crude unpurified PCR products can be used directly for ligation into pSpark® DNA cloning systems, but even in those cases we recommend to run an aliquot of PCR amplified DNA by gel electrophoresis to check both quality and quantity.



**IMPORTANT:** *If unpurified PCR is used directly for DNA cloning into pSpark® DNA cloning system and a plasmid template DNA has been used for PCR, please check that plasmid template has a different antibiotic resistance than pSpark® DNA cloning vector to be used for PCR cloning. If both plasmids share the same antibiotic resistance, many colonies in the transformation will be the original plasmid vector used as template for PCR. Alternatively, use the pSpark® DNA cloning system with both Kanamycin and Ampicillin antibiotics resistance and select on plates with either these two antibiotics or the antibiotic different from that of plasmid template (see Section 1.2).*

On the other hand, PCR products often contain numerous spurious bands that must be purified away from the correct product. Agarose gels electrophoresis is highly recommended to size-fractionate the desired fragments, or example, if smearing of the PCR product or inappropriate banding is observed on the gel, or if the plasmid template shares the same antibiotic resistance than the pSpark® DNA cloning system to be used. Use only high-quality Agarose DNA Purification Kits.



**IMPORTANT:** DNA resolved on agarose gels is generally stained with ethidium bromide and visualized by illumination with UV light. Exposure to short wavelength UV light (e.g., 254, 302, or 312 nm) for 2 minutes reduces the cloning efficiency of DNA up to 10.000 times due to formation of pyrimidine dimers. We strongly recommend the use of a long wavelength lamp (e.g. 360 nm) and the shortest exposure times when isolating DNA from agarose gels for cloning. Even better and safer is the use of stains such as GelGreen™ Nucleic Acid Gel Stain (Biotium, Cat. No.41004) and a Dark Reader® (Clare Chemical, Cat. No. DR46B) for visualizing DNA with visible blue light that is safe for humans and does not affects cloning efficiency. As alternative you can use FlashGel® System from Lonza.

## 2.3 Ligation

Due to the extremely high cloning efficiency of pSpark® DNA cloning systems, ligations can be performed in as little as 10 minutes with the supplied protocol. When only 10 minutes of ligation are used, the number of colonies obtained is about the same than a T/A cloning reaction made in parallel under optimal conditions (60 minutes to overnight ligation at 16°C). Generally, incubation at 22°C for 60 minutes produces about 5 times more transformants than a ligation for 10 minutes. For example, using 1µL of the supplied Control Insert for a ligation in 10 minutes, a standard transformation protocol (see **Section 2.4.3**) and competent cells with a transformation efficiency of  $4 \times 10^7$  cfu/µg about 400 to 500 white colonies are obtained. About 900 white colonies are obtained when ligation time is increased to 30 minutes. Overnight incubation does not improve nor is detrimental for transformation. Visit the web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)) for additional information about ligation into pSpark® DNA cloning systems.

### 2.3.1 Amount of insert needed for ligation into pSpark® DNA cloning systems.

A highly relevant feature of pSpark® DNA cloning systems is that they use about 5-10 times less DNA than any other cloning kit available on the market.

pSpark® DNA Cloning Systems have been optimized using an insert to vector ratio of 5:1. Optimal ratio of insert to vector is from 3:1 to 5:1. However, ratios of 0.2:1 to 50:1 have been used successfully. See some results on the web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)).



**IMPORTANT:** Please follow recommended protocol. pSpark® DNA cloning systems use much less DNA for cloning than any other kit available on the market.

If initial experiments with your PCR product are suboptimal, ratio optimization may be necessary. Ratios of insert to vector from 5:1 to 1:1 provide good initial parameters.



Note

**ADVICE:** Our R&D scientists have found that for large inserts (more than 7 kb) insert-to-vector ratios of 3:1 to 1:1 produce more colonies than insert to vector ratios from 5:1 to 3:1 that are optimal for inserts of 0.1 kb to 4 kb. This initial finding awaits confirmation for other large inserts, but for large inserts (>7 kb) an insert-to-vector ratio of 3:1 represents a good starting point for cloning into pSpark® DNA cloning systems. See some results on the web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com))

The concentration of PCR product should be estimated either by comparison to quantitative DNA mass standards on an agarose gel, by absorbance at 260 nm/280 nm quantification using for example a Nanodrop™ spectrophotometer or by using a fluorescent assay. The pSpark® DNA cloning vectors I, II and IV have about 3.0 kb and are supplied at 20 ng/μL; the pSpark® DNA cloning vector III has about 4.0 kb and is supplied at 20 ng/μL and the pSpark® DNA cloning vector V has about 3.4 kb and is supplied at 20 ng/μL. In all cases only 1 μL is used for a cloning reaction. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation:

$$\frac{1}{5} = \frac{\text{ng vector} \times \text{pb insert}}{\text{pb vector} \times \text{ng insert}} \quad \text{then} \quad \text{ng insert} = \frac{\text{pb insert} \times \text{ng vector} \times 5}{\text{pb vector} \times 1}$$

**Example of amount of insert calculation:**

How much 1 kb PCR product should be added to a ligation in which 20 ng of pSpark® DNA vector (3.0 kb) will be used if a 5:1 insert to vector ratio is used?

$$\frac{20 \text{ ng vector} \times 1000 \text{ pb insert} \times 5}{3000 \text{ pb vector} \times 1} = \mathbf{33,3 \text{ ng of insert}}$$

**ng of insert= 30 ng \* insert size (in kb) (Note: this equation is valid for a 5:1 insert:vector ratio with all pSpark® DNA cloning systems)**



Note

**RULE OF THUMB:**

1. use **30 ng** of insert per kilobase for a ligation of **purified PCR** product.
2. use **1-2 μL** of **unpurified PCR** product for a ligation.

### 2.3.2 Protocol for ligation using the pSpark® DNA cloning systems

1. Briefly centrifuge the pSpark® DNA cloning vector and control insert DNA tubes to collect contents at the bottom of the tubes.
2. Vortex the 5x T4 DNA ligase buffer vigorously before each use.
3. Set up ligation reactions as described below.

#### Ligation reaction

Reagent	Cloning Reaction	Control Reaction	Background Reaction
pSpark® DNA cloning vector	1 µL	1 µL	1 µL
5x T4 DNA ligase buffer	2 µL	2 µL	2 µL
PCR product	X µL*	--	--
Control insert DNA (1000 bp)	--	1 µL	--
T4 DNA ligase (5 Weiss units/µL)	1 µL	1 µL	1 µL
10x PEG 6000	1 µL	1 µL	1 µL
Molecular biology grade water to	10 µL	10 µL	10 µL
*See <b>Section 2.3.1</b> for insert-to-vector ratio			

4. Mix the reactions by pipetting slowly. Incubate the reactions from 10 minutes to 60 minutes at 22 °C in either a thermoblock, a thermocycler, a water bath or at bench if room temperature is between 20-24 °C.

The suggested optimal incubation time is of 60 minutes. Longer incubation times have no benefit nor are detrimental to the cloning efficiency. Incubation overnight at 4 °C will produce about the same number of transformants than incubation for 60 minutes at 22 °C. Incubation times from 10 minutes to overnight could be used albeit 10 minutes produces about 5 times less colonies than a 60 minutes ligation. For example, using 1 µL of the supplied Control Insert for a ligation in 10 minutes, a standard transformation protocol (see **Section 2.4.2**) and competent cells with a transformation efficiency of  $4 \times 10^7$  cfu/µg about 400 to 500 white colonies are obtained. Increasing the ligation time to 30 minutes about 900 white colonies are obtained, while a 60 minutes ligation gives 2500 white colonies. Overnight incubation does not improve nor is detrimental for transformation.



**IMPORTANT:** (a) Do not heat-inactivate a ligation with PEG as heat inactivation of ligase dramatically reduces transformation efficiency of DNA heated in the presence of PEG.

(b) The supplied 5X T4 DNA ligase buffer contains ATP, which degrades during temperature fluctuations. It is frequent to see a white precipitate on 5X T4 DNA Ligase Buffer. This precipitated buffer can be used without loss of performance. It is strongly advised to make aliquots. Do not try to heat to dissolve the precipitate as the ATP will be degraded.

5. Proceed to transformation (see **Section 2.4**)



Note

**ADVICE.** We strongly recommend:

- a) To prepare a ligation with the supplied control insert to check that ligation and transformation process are working properly. This positive control **MUST** be prepared and transformed at the same time than your ligation.
- b) The use of supplied T4 DNA ligase to perform pSpark® DNA cloning vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may contribute to a high background in cloning. At Canvax we have tested T4 DNA ligases from several major suppliers and found they are all suitable for cloning into pSpark® DNA cloning system, albeit the number of positive colonies is about 2 to 4 fold lower and the number of blue colonies are 10-20 fold higher. See in our web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)) for details.
- c) If your template DNA for PCR is a plasmid that shares the same antibiotic resistance than the pSpark® DNA cloning system we advice to set up a control ligation without pSpark® DNA cloning vector but containing insert, T4 DNA ligase, 5x T4 DNA ligase buffer, PEG 6000 and water to check if insert produces colonies.

### 2.3.3 Tips for cloning of long or problematic PCR products.

The pSpark® I DNA cloning system is designed and tested for routine cloning of PCR products up to 9 kb in length with high efficiency. If a gene of more than 9 kb is to be cloned, we strongly recommend the use of the transcription-free pSpark® DNA cloning vector with low copy number to help in insert stabilization (pSpark V).



Note

**ADVICE:** pSpark® DNA cloning systems are compatible with any E. coli strain. Several E. coli strains have been developed to help stabilization of unstable DNA. For example, SURE® E. coli cells from Stratagene or Clean Genome® E. coli cells from Scarabgenomics LLC are claimed to stabilize inverted repeated sequences and plasmid rearrangements during propagation. Although we have not tested them for cloning unstable DNA, they may be an alternative if cloning such problematic DNA. Also, XL10-Gold® from Stratagene has been designed for transformation with very large plasmids.

The cloning efficiency varies significantly according to the size and sequence of the PCR product.

When cloning long PCR products, it is especially important to analyze the PCR products on a gel prior to performing the ligation reaction. If gel analysis reveals inefficient production of the desired PCR product or reveals the presence of non-specific products, it is strongly recommended to gel-purify the PCR product of interest and to quantify it in order to prepare an optimal cloning reaction. This reduces the number of white colonies containing inserts other than the desired PCR product. The use of competent cells with a transformation

efficiency of more than  $4 \times 10^7$  cfu/ $\mu$ g is strongly recommended for cloning of long PCR products.



Note

**ADVICE:** Our R&D scientists have found that for large inserts (more than 7 kb) insert-to-vector ratios of 3:1 to 1:1 produce more colonies than insert to vector ratios from 5:1 to 3:1 that are optimal for inserts of 0.1 kb to 4 kb. This initial finding awaits confirmation for other large inserts, but for large inserts (>7 kb) an insert to vector ratio of 3:1 represents a good starting point for cloning into pSpark® DNA cloning systems. See some results on the web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)).

## 2.4 Transformation

### 2.4.1 General considerations about transformation into *E. coli*

As blunt end ligations into pSpark® DNA cloning systems are far more efficient than ligations of single base overhangs, for example T/A-based ligations, the use high-efficiency competent cells (those with more than  $1 \times 10^8$  cfu/ $\mu$ g DNA) for transformations is less important in pSpark® DNA cloning system than in T/A based cloning vectors. In general, cells with 10 times lower transformation efficiency could be used with pSpark® DNA-based cloning systems when compared with other kits available on the market. Moreover, due to the very high efficiency of pSpark® DNA cloning systems a fast transformation protocol of only 5 minutes could be used (see expected results on **Section 2.4.5**). In fact, pSpark® DNA cloning vectors have been optimized with this protocol. Anyway, the use of cells with more than  $1 \times 10^7$  cfu/ $\mu$ g is recommended for a successful transformation. Any *E. coli* strain could be used for transformation but they must be compatible with standard ampicillin selection (Ampicillin/Kanamycin in pSpark III) and also blue/white screening compatibility is recommended albeit not essential (except pSpark IV and V) If a blue/white incompatible strain is used or transformation is plated in media without X-Gal then a background of less than 20 white colonies (less than 1% of total colonies) is expected when using competent cells with  $4 \times 10^7$  cfu/ $\mu$ g DNA. Selection for transformants should be on LB/Antibiotic/IPTG/X-Gal plates (see **Appendix II**). For best results, do not use plates that are more than 1 month old.

Transformation of chemically competent *E. coli* cells is based in an empirical classical protocol where DNA is pre-incubated on ice with competent cells and then such mix is heat-shocked at exactly 42 °C for a critical time period and then returned to ice before plating. But at Canvax we have found that pSpark® DNA cloning vectors cloning efficiency is so high that an alternative short transformation protocol could be routinely employed and the classical protocol is recommended only for the most demanding cloning experiments such as cloning of very long (>8 kb) genes. In our hands the 5-minutes transformation protocol gives no less than 4-5-fold of the number of colonies obtained using the classical transformation protocol including 1 hour recovery of cells is SOC media. 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.

Also, pSpark® DNA cloning systems could be used with any *E. coli* chemical transformation protocol, and we supply one classical protocol that have been functionally tested with pSpark® DNA cloning systems. In the fast transformation protocol DNA is pre-incubated on ice with competent cells for 5 minutes and the mix is directly plated into pre-warmed at 37 °C LB/antibiotic plates or liquid LB pre-warmed at 37 °C. Using cells with  $4 \times 10^7$  cfu/ $\mu$ g and 30 ng of a 1000 bp insert amplified by PCR (the same supplied as control insert) about 2500 positive colonies are obtained using the standard transformation protocol.

### 2.4.2 Standard protocol for transformation

Please see **Section 1.6** for Additional material required

1. Prepare one **LB/antibiotic/IPTG/X-Gal** (*IPTG/X-Gal not necessary for pSpark IV and V*) plate for each ligation reaction, plus one plate for determining transformation efficiency and one plate for control insert transformation (see **Section 2.4.5**). Equilibrate the plates to room temperature prior to plating (**Step 8**).
2. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 10  $\mu$ L of each ligation reaction to a sterile 1.5 mL microcentrifuge tube on ice. Set up another tube on ice with 100 pg uncut plasmid for determination of the transformation efficiency of the competent cells.
3. Remove a tube of frozen Competent Cells from storage at -80 °C 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  $\mu$ L of cells into each tube prepared in **Step 2**. For determination of transformation efficiency add 50  $\mu$ L of competent cells to the tube prepared in **Step 2** containing 100 pg of uncut plasmid DNA. We recommend adding competent cells to ice-cooled microcentrifuge tubes containing ligated DNA. If you prefer to add ligation to competent cells please make SURE your pipette tip goes all the way down in to the cells, so that you are adding DNA to the cells.
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.

8. If the selection antibiotic is Amp, plate 60  $\mu\text{L}$  of each transformation culture onto LB/antibiotic/IPTG/X-Gal\* plates. If the selection antibiotic is other than ampicillin (e.g. kanamycin) then add to the cells 950  $\mu\text{L}$  of SOC liquid medium (WITHOUT antibiotics) pre-warmed at 37 °C and incubate for 1-1.5 hours at 37 °C with shaking (~150 rpm) before plating into LB/antibiotic/IPTG/X-Gal\* plates. After incubation, centrifuge cells at 10000 g for 2 minutes and resuspend in 50-100  $\mu\text{L}$  of liquid media for plating.

\*IPTG/X-Gal is not necessary for pSpark IV and V.



**ADVICE:** (a) If cells transformed with ligation reactions from **Step 7** are incubated before plating for 1 hour at 37 °C with shaking (~150 rpm) in 950  $\mu\text{L}$  of SOC medium the number of transformants is about two-fold higher. This step is not needed in routine cloning into ampicillin pSpark® DNA cloning systems because of their very high cloning efficiency (see **Section 2.4.5**).

Note

(b) For the transformation control if expected transformation efficiency of cells is higher than  $4 \times 10^7$  cfu/ $\mu\text{g}$  then at least a 1:10 dilution with SOC medium is recommended for plating. For example, competent cells with  $2 \times 10^8$  cfu/ $\mu\text{g}$  will produce up to 20,000 colonies under optimal conditions, thus at least a 1:10 dilution is recommended for plating.

9. Incubate the plates overnight (12–16 hours) at 37 °C. In our experience, if 10  $\mu\text{L}$  of ligation are transformed into 50  $\mu\text{L}$  of competent cells and the resulting 60  $\mu\text{L}$  are directly plated, up to 2500 colonies per plate are routinely seen when using competent cells with a transformation efficiency of  $4 \times 10^7$  cfu/ $\mu\text{g}$  DNA and inserts of less than 2500 bp. If a 60 minutes recovery step of cells in SOC media is included, then the number of transformants is about 2-fold higher, that is, about 3500 positive colonies are obtained.

### 2.4.3 Fast transformation protocol (Recommended alternative)

An alternative transformation protocol of only 5 minutes is recommended. pSpark® DNA cloning systems have been optimized using this protocol. 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. When using this protocol you need only 25 minutes from PCR end to plating transformed cells provided you use unpurified PCR for transformation, a ligation of only 10 minutes and this fast transformation protocol.



**EXTREMELY IMPORTANT:** Before starting this protocol you must pre-warm LB agar-antibiotic-X-Gal-IPTG\* plates at 37 °C for at least 1 hour. (See **Appendix II: Preparation of media and reagents**). 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/antibiotic/IPTG/X-Gal plate for each ligation reaction, plus one plate for determining transformation efficiency and one plate for control insert transformation

efficiency (see **Section 2.4.5**). Pre-warm plates at 37 °C in the incubator for at least one hour before transformation.

2. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 10 µL of each ligation reaction to a sterile 1.5 mL microcentrifuge tube on ice. Set up another tube on ice with 100 pg uncut plasmid for determination of the transformation efficiency of the competent cells.
3. Remove a tube of frozen competent cells from storage at -80°C 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 the transformation efficiency, add 50 µL of competent cells to the tube prepared in **Step 2** containing 100 pg of uncut plasmid DNA.
5. **Gently** flick the tubes to mix and place them on ice for 5 minutes.
6. If the selection antibiotic is ampicillin, plate 60 µL of each transformation culture onto pre-warmed LB/ampicillin/IPTG/X-Gal\* plates **withdrawn from the incubator at 37°C just before plating**. If the selection antibiotic is other than ampicillin (e.g. kanamycin) then add to the cells 950 µL of SOC liquid medium (WITHOUT Antibiotics) **pre-warmed at 37°C** and incubate for 1-1.5 hours at 37°C with shaking (~150 rpm) before plating into LB/antibiotic/IPTG/X-Gal plates. After incubation, centrifuge cells at 2500 g for 10 minutes and resuspend in 50-100 µL of liquid media for plating.

*\*IPTG/X-Gal is not necessary for pSpark IV and V.*



Note

**ADVICE:** *(a) If cells transformed with ligation reactions from **Step 6** are incubated before plating for 1 hour at 37 °C with shaking (~150 rpm) in 950 µL of SOC medium pre-warmed at 37 °C the number of transformants is about 1.5-2 times more than that obtained if the 1 hour incubation step is omitted. Thus, this step is not needed in routine cloning into ampicillin pSpark® DNA cloning vectors, but it is essential if selection is made with other antibiotics, eg, kanamycin, or chloramphenicol.*

*(b) If more than 5 transformations are to be made at the same time, withdraw pre-warmed plates from the incubator in groups of no more than 5 plates. Depending on the temperature inside the laminar flow cabinet, more than 5 plates could be withdrawn from the incubator at the same time.*

(c) For the transformation of supplied control insert, if expected transformation efficiency of cells is higher than  $4 \times 10^7$  cfu/ $\mu$ g, then a 1:10 dilution with SOC medium is recommended for plating. For example, competent cells with  $2 \times 10^8$  cfu/ $\mu$ g will produce up to 20,000 colonies, thus at least a 1:10 dilution is recommended for plating.

7. Incubate the plates overnight (12–16 hours) at 37 °C. In our experience, if 10  $\mu$ L of ligation are transformed into 50  $\mu$ L of competent cells and the resulting 60  $\mu$ L are directly plated up to 1250 colonies per plate are routinely seen when using competent cells that are  $4 \times 10^7$  cfu/ $\mu$ g DNA and inserts of less than 1000 bp.

#### 2.4.4 Transformation by electroporation protocol

Electroporation was introduced in 1988 for bacterial transformation and it has become the method of choice when being dependent on obtaining extremely high efficiencies either for the construction of cDNA libraries or “difficult” ligations with minimal amounts of the desired plasmid. Due to the electric field of about 12–18 kV/cm in a 0.1 cm electrode gap cuvette, the conductivity of the sample must be very low to prevent arcing. Only tiny and often insufficient amounts of a standard ligation mixture can be added to 20–40  $\mu$ L of electrocompetent cells before arcing begins to occur. Thus, recovery and purification of ligated plasmid DNA is a critical and limiting step in modern molecular cloning techniques prior to electroporation in *E. coli*. Several methods have been developed for desalting and purification of plasmid DNA: silica based methods of DNA purification using chaotropic salts; ethanol precipitation; phenol/chloroform extraction plus ethanol precipitation; Sephadex™ Gel-50 gel filtration; tRNA assisted precipitation and desalting using Montage™ PCR centrifugal filter devices from Millipore. pSpark® DNA cloning systems use PEG to improve the efficiency of ligation but PEG must be eliminated before electroporation. Precipitation based methods have in general low recovery and thus we recommend a spin column purification method.



**IMPORTANT:** Do not heat-inactivate a ligation with PEG as heat inactivation of ligase dramatically reduces transformation efficiency of DNA heated in the presence of PEG.



Note

**ADVICE:** Because pSpark® DNA cloning systems use very low amounts of DNA for ligation, recovery of ligated DNA is about 10–15% even when using recommended spin columns purification methods. Thus, when using electroporation the total number of colonies obtained per ligation is only higher if electroporation competent cells have a transformation efficiency at least ten times higher than chemically competent cells. Alternatively, using chemically competent cells with a transformation efficiency of  $2 \times 10^9$  cfu/ $\mu$ g you can get  $1 \times 10^5$  colonies using just 20 ng of vector and about 30 ng/kb of insert.

Protocols for *E. coli* electrotransformation have been developed for both 1-mm gap and 2-mm gap electroporation cuvettes but the electroporation parameters are different. Follow any electroporation protocol suggested by major electroporator suppliers such as Bio-Rad Laboratories ([www.bio-rad.com](http://www.bio-rad.com)), BTX ([www.btxonline.com](http://www.btxonline.com)) or Eppendorf AG ([www.eppendorf.com](http://www.eppendorf.com)).

## 2.5 Selection of recombinants

The need to identify the cells that contain the desired insert at the appropriate and right orientation and isolate these from those not successfully transformed is of utmost importance to researchers. pSpark cloning vectors include selectable markers (antibiotic resistance markers) that allow only cells in which the vector, but not necessarily the insert, has been transformed to grow. Additionally, the cloning vectors contain color-selection markers which provide **blue/white screening** (via  $\alpha$ -factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells. Further investigation of the resulting colonies is required to confirm that cloning was successful. This may be accomplished by means of PCR, restriction fragment analysis and/or DNA sequencing.



Note

**REMEMBER:** In pSpark IV and V not is necessary the blue/white screening.

\*pSpark IV vector have the  $\alpha$ -peptide but don't have the Lac promoter.

\*The pSpark V vector have a truncated, not functional  $\alpha$ -peptide, and lacking the Lac promoter.

### 2.5.1 Expected results

The background of any pSpark® vector self-ligation in a ligation reaction without insert produces less than 1% of background of blue colonies than a ligation with both insert and vector and thus Blue/White screening is not required. For example, under optimal conditions a ligation of 30 ng of a 1 kb insert and transformation into cells with a transformation efficiency of  $4 \times 10^7$  cfu/ $\mu$ g produces over 2500 white colonies and less than 20 blue colonies. But if ligation is carried out under non-optimal conditions, for example if the ratio of insert to vector is less than 3:1 or the size of the insert is larger than 7 kb then background could be higher.

The suggested optimal incubation time is of 60 minutes. Incubation times from 10 minutes to overnight at 22°C could be used albeit 10 minutes produces about 5 times less colonies than a 60 minutes ligation. For example, using 1  $\mu$ L of the supplied Control Insert for a ligation in 10 minutes, a standard transformation protocol (see **Section 2.4.2**) and competent cells with a transformation efficiency of  $4 \times 10^7$  cfu/ $\mu$ g about 400 to 500 white colonies are obtained. Increasing the ligation time to 30 minutes about 900 white colonies are obtained while a 60 minutes ligation gives about 2500 white colonies. Incubation overnight at 4 °C will produce about the same number of transformants than incubation for 60 minutes at 22°C. See some results on the web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)).

White colonies will generally contain insert but about 0,5 percent of pSpark® DNA cloning systems colonies are white colonies without insert probably due to traces of nucleases present during purification of DNA or contaminating the EcoRV enzyme used for digestion of DNA. Alternatively, all restriction enzymes have a variable level of star activity, that is, under certain condition restriction endonucleases cleave DNA at other sites different from their recognition site. About 8 every 10 of such white colonies without insert obtained with pSpark® DNA cloning systems will not produce amplification with pUC/M13 forward and reverse primer indicating they have lost at least part of the Multiple Cloning Site.

Also, if crude (unpurified) PCR is used directly for ligation into pSpark® DNA cloning systems, unspecific bands of PCR and also primers-dimers will also be cloned. In such situation, white colonies should be screened by colony PCR before inoculation for plasmid purification. Colony PCR is in general a good screening technique but is less useful for bands larger than 2000 bp or in cases where yield of PCR is not high.

Blue colonies are generally negative, that is, they generally do not contain insert. But many inserts should contain an open reading frame and thus colonies in at least one of two the orientations will produce the alpha-peptide and there will be blue color due to complementation. One common situation where blue colonies with insert are obtained is when short inserts are cloned into vectors with blue/white screening feature. pSpark® DNA cloning systems could be even used for cloning of very short sequences like blunt-ended annealed oligonucleotides.

Blue colonies are also obtained if the cloned insert behave as a promoter in *E. coli*. It is almost impossible to predict all the short sequences within an insert that should behave as promoters in *E. coli*. Contrary to positive selection vectors, pSpark® DNA cloning systems do not use transcription to reduce background and thus pSpark® DNA cloning systems are among the DNA cloning systems available on the market with a lower bias in DNA cloning.

Still another common cause of blue colonies with insert is the extraordinary high frequency of frameshifting present in *E. coli*. Many sequences in *E. coli* produce a change from one reading frame to another and thus, even when no ORF is identified in the cloned insert, it is difficult to be sure that a fusion peptide comprising the alpha-peptide is not produced. Also several stop codons are leaky in *E. coli*, that is, translation is not terminated but an amino acid is inserted into the polypeptide growing chain instead of termination and thus the presence of a codon stop within a sequence will not always terminate polypeptide synthesis. Again, as pSpark® DNA cloning systems do not use transcription to reduce background, this pSpark® DNA cloning systems are among the cloning systems with a lower bias and artefacts in DNA cloning.

However, in most situations blue colonies are negative that is they are the results of self-vector recircularization. At Canvax we have observed that most T4 DNA ligases available on the market even those from several major suppliers contain traces of an exonuclease activity that eliminates bases from the 3'-ends of the treated vector. Surprisingly, we have even found that single base mispaired DNA is efficiently ligated by T4 DNA ligase and thus, the reading frame of pSpark® DNA cloning vector has been adapted to consider this fact. As a result, if the vector is ligated with the T4 DNA ligase supplied, less than 0,5 percent of colonies will be empty blue colonies. In about 9 of every 10 of such colonies, the original EcoRV site is not restored. If other T4 DNA ligases with traces of exonuclease activity are used for ligation, then the background could be very high, even with ligases purchased from major suppliers. See on our web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)) for information about T4 DNA ligases from other suppliers tested for use with pSpark® DNA cloning systems.



**EXTREMELY IMPORTANT:** Use only the supplied T4 DNA Ligase to perform ligations into pSpark® DNA cloning systems, which has been tested for almost undetectable contaminating exonuclease activity. Other commercial preparations of T4 DNA ligase

*may contain exonuclease activities that may contribute to a high background in cloning. At Canvax we have tested T4 DNA Ligases from several major suppliers and found that only some of them are suitable for cloning into pSpark® DNA cloning systems, albeit the number of positive colonies is about 2- to 4-fold lower and the number of negative colonies is 10-20-fold higher. Alternatively, you can use your regular T4 DNA ligase if you find it is suitable for cloning into pSpark® DNA cloning systems.*

**Expected results for cloning of amplified DNA:** The number of colonies obtained using pSpark® DNA cloning systems and the cloning efficiency depend upon the size, amount, sequence, proofreading polymerase used for amplification (either a blend, a first generation or a second generation proofreading polymerase) and purity, quality and integrity of the PCR product used for ligation. Generally, high yield PCR amplifications will produce also a good yield after purification from agarose gels. As a consequence, the most common result obtained using pSpark® DNA cloning systems is several hundreds or even thousand white positive colonies . As these vectors use about 10-fold less DNA than any other kit available on the market, cloning is still possible with suboptimal conditions, such as a very low yield of either PCR or gel purification. At Canvax, our scientists have found that 1 µL of a very low yield PCR could be transformed directly without purification, and most of the times generate colonies with the desired insert but cloning of crude PCR products should be checked by colony PCR because white colonies could be due to cloning of contaminating bands and even primers dimers.

**Expected result for the DNA control transformation:** If transformation of a DNA control plasmid (not supplied) is performed with 25 pg of DNA and about 250 colonies are observed, then the transformation efficiency is  $1 \times 10^7$  cfu/µg. Transformation efficiency of competent cells means colony number obtained per microgram of circular DNA added to transformation. The transformation efficiency of the cells is calculated as in the following example

Example of transformation efficiency calculation:

50 µL competent cells are transformed with 25 pg uncut plasmid DNA. If 250 colonies are obtained, which is the transformation efficiency?

$$\begin{aligned}
 &25 \text{ pg} = 0.025 \text{ ng DNA} \text{-----} 250 \text{ colonies (cfu)} \\
 &1000 \text{ ng} = 1 \text{ µg DNA} \text{-----} X \text{ (transformation efficiency)} \\
 &\text{Transformation efficiency} = \frac{250 \text{ cfu}}{0.025 \text{ ng}} \times 1000 \text{ ng/µg} = 1 \times 10^7 \text{ cfu/µg DNA}
 \end{aligned}$$

**Expected result for the control insert transformation:** After plating 60 µL of the Control Insert transformation reaction, about 2500 white colonies are expected if using cells with a transformation efficiency of about  $4 \times 10^7$  cfu/µg. Less than 20 blue colonies are expected if cells are plated on agar plates containing X-Gal/IPTG. The presence of the Control Insert is easily verified by either colony PCR, with pUC/M13 forward and reverse primers that should give an insert of about 1100 bp, while empty colonies should give an amplification of about 200 bp, or

by digestion of purified plasmid purified with *Pvu* II restriction enzyme. For example, in pSpark® I DNA cloning vector, DNA fragments expected from *Pvu* II digestion of plasmids containing the Control Insert are 2564 pb and 1449 pb, while digestion of self-ligated pSpark® I DNA cloning vector gives a band of 2564 bp and of 449 bp.

## 2.6 Analysis of transformants

After overnight incubation colonies obtained in transformation should be screened to find positive colonies. If pale blue colonies are obtained please consider a longer incubation of the plates at 37°C that favour blue color development but also favour the appearance of satellites, that is, colonies without insert that grow around positive colonies due to beta-lactamase spill out into media by positive colonies that degrades the ampicillin around positive colonies. Alternatively, short-term storage (about 2 hours) of plates at 4 °C should be a good method to facilitate blue color development.

### 2.6.1 PCR directly from bacterial colonies (Colony PCR protocol)

Colony PCR is a suitable technique for screening colonies before isolation of plasmid DNA. The main advantage is that many colonies should be screened in parallel before plasmid purification. Primers located flanking the Multiple Cloning Site such as pUC/M13 forward and reverse primers should be used. All pSpark® DNA cloning vectors have sites for annealing such pUC/M13 primers. Also, you should use the same primers used for amplification of cloned DNA, that is, insert specific primers. 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. Some protocols recommend to use also colony PCR to check orientation of the cloned PCR product by using for example a pUC/M13 forward primer located in the vector with a specific reverse primer of the insert but such PCR by definition has no positive control and we always recommend to set up a positive control in the PCR mainly to check if master mix was properly prepared.

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

#### Protocol for colony PCR

See material required but not supplied with kit on **Section 1.6**

1. For each bacterial colony to be screened prepare a 1.5 mL microcentrifuge tube with 30-40 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.



**ADVICE:** *Please remember*

Note

- ✓ *To mark both the PCR tube and the inoculated tube or plate that will be used to grow positive colonies after colony PCR.*
- ✓ *To include a positive control of amplification if possible. A negative colony PCR does not always mean that the colony has not the desired insert.*

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 master mix containing Taq DNA polymerase (or equivalent), dNTPs at 200 µM final concentration, buffer for Taq reaction, MgCl<sub>2</sub> at 2 mM, forward and reverse primers each at 1 µM final concentration and water to 30 µL per 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.



**ADVICE:** *Please follow the guidelines of your Taq polymerase supplier for optimal conditions for PCR and use the above conditions only as a suggestion*

Note

6. Spin boiled tubes from **Step 4** at 14500 rpm for 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. Begin PCR program (use an extension time of about 1min per kb; 72°C for extension if Taq polymerase is used and 52 °C of annealing temperature if pUC/M13 forward and reverse primers are used). Use 25-30 cycles.



**ADVICE:** *Please follow the guidelines of your Taq polymerase supplier for optimal conditions for PCR and use the above conditions only as a suggestion*

Note

9. Check the amplifications by agarose gel electrophoresis.

### 2.6.2 Isolation of plasmid DNA

1. Take 4-6 positive colonies (from colony PCR, see **Section 2.5.1**) or 8-12 colonies (if inoculated directly from the plate without colony PCR), inoculate them in LB with antibiotic (about 6-8 mL each culture) and culture them overnight at 37 °C.

2. Isolate plasmid DNA using any kit for plasmid DNA miniprep. In particular we recommend isolating plasmid by silica based minispin columns because DNA of high quality is obtained.



Note

**ADVICE:** *Alternative methods such as CTAB method can also be used but plasmid will be contaminated with both salts and genomic DNA from bacteria and thus a higher amount of restriction endonucleases will be needed for digestions and a lower quality of sequencing reads will be obtained.*

3. Analyse the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. We recommend to use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert or if primers with restriction sites were used for amplification to check the size of the insert by digestion with those enzymes. We recommend to save clones in both orientations in such cases where multiple vector variants will be made in the future with such insert (see **Section 2.5.4** below for storage of sequenced clones)

### 2.6.3 Sequencing

After plasmids with the correct insert size, orientation and restriction pattern are identified at least two clones should be sequenced to confirm that the sequence is correct. With the use of second generation high fidelity DNA polymerases mainly Phusion® and iProof™ but also KAPAHiFi™ DNA polymerases we have found no polymerase mistakes in over 800.000 bases sequenced to date. Also, in certain cases you may wish to sequence the junction points of vector and insert.



Note

**ADVICE:** *The use of either pUC/M13 forward or reverse is highly recommended. Refer to the map of pSpark® DNA cloning vector used for cloning on **page 4** for sequence surrounding the Multiple Cloning Site. If specific primers are used for sequencing then the first 30-60 bp of sequence are sometimes not seen. Please check with your supplier of sequencing services.*

### 2.6.4 Long term storage of sequence-verified clones

Once you have identified correct clones, be sure to prepare a glycerol stock for long-term storage.

#### Protocol for storage of sequenced clones

1. Streak the original colony out on LB plates containing antibiotic or re-transform again into *E. coli* the sequenced-verified clones.
2. Isolate a single colony and inoculate into 3-4 mL of LB containing antibiotic.

3. Grow overnight at 37 °C with shaking at 150-200 rpm. Centrifuge 3 mL of culture, discard supernatant and resuspend pellet in 700 µL of fresh LB (without antibiotic).
4. Mix 700 µL of resuspended culture from **Step 3** with 300 µL of sterile pure glycerol and transfer to a sterile 1.5 mL microcentrifuge vial.
5. Store at -80 °C.

Clones stored at -80 °C using the above protocol can be recovered up to 5 years later. For recovery, streak into LB plates with antibiotic, isolate a colony, inoculate into liquid LB media with antibiotic and purify plasmid by miniprep.



Note

**ADVICE:** *Copy number of plasmid is reduced during long term storage at -80 °C. If after miniprep a low yield of plasmid is obtained then re-transform into E. coli and purify again plasmid from such recently transformed colonies. This is especially relevant for low copy number plasmids.*

### 3. TROUBLESHOOTING

For questions not addressed here, please contact us at [www.canvaxbiotech.com](http://www.canvaxbiotech.com) or alternatively contact your local Distributor.

PROBLEM	CAUSE	SOLUTION
<b>No colonies (even in the control insert reaction)</b>	1.- Any component is missing in the ligation reaction.	Repeat ligation and transformation and include controls.
	2.- Any of the reagents of the kit is not working properly.	Prepare individual test reactions for the T4 DNA ligase, the ligation buffer and the pSpark® DNA cloning vector. If any is damaged, use a new aliquot or a new kit.
	3.- Competent cells are damaged or with very low efficiency.	Check the transformation efficiency of <i>E. coli</i> competent cells. A transformation efficiency lower than $1 \times 10^7$ cfu/ $\mu$ g is not recommended.
<b>Colonies only in the control insert reaction</b>	1.- Any component is missing in the specific insert reaction only.	Repeat ligation and transformation and include controls.
	2.- A non proofreading DNA polymerase has been used for amplification by PCR.	Amplify your PCR insert with a proofreading polymerase (see <b>Appendix III</b> ).
	3.- PCR insert is degraded or damaged.	Check quality of insert by gel electrophoresis and verify it has not been exposed for more than 1 minute to short wave UV light to avoid formation of pyrimidine dimers.
	4.- A very low amount or no PCR insert have been used for ligation. 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 (see <b>Section 2.3.1</b> ) in a new ligation reaction.
	5.- Salts and/or ethanol present in the purified PCR insert.	Repeat PCR and purification from agarose for a new ligation and transformation.
	6.- Ligation is not optimal.	Optimise the ligation by trying other insert to vector ratios. (see <b>Section 2.3.1</b> )
	7.- Insert is unstable or toxic to <i>E. coli</i> .	Try to use a transcription-free pSpark® DNA cloning system specifically developed for cloning of toxic inserts or a low copy and transcription-free pSpark® DNA cloning system specifically developed for cloning of unstable inserts. (See <b>Section 2.3.3</b> )
<b>White colonies without insert of interest</b>	1.- A plasmid containing the same antibiotic resistance than pSpark® DNA cloning vector was used and unpurified PCR product was used for ligation .	Gel purify your PCR insert or use a pSpark® DNA cloning system with dual antibiotic resistance developed specifically for these situations (pSpark®III).
	2.- The PCR insert product has multiple bands and is used unpurified directly for cloning.	Gel purify your PCR insert or screen more colonies by colony PCR.

	3.- The PCR insert has a single band but also primer dimers and unpurified PCR product was used for ligation.	Clean up you PCR insert by a minispin column or screen more colonies by colony PCR.
	4.- There is a contamination with nucleases (eg. endonucleases) in any of the reagents used.	Always wear gloves, use sterile pipet tips and top quality reagents. Repeat the ligation and transformation with new reagents.
<b>High ratio of blue-to-white colonies for both the PCR insert and the control insert reaction</b>	1.-The T4 DNA ligase used has exonuclease activity.	Use only the supplied T4 DNA ligase or a top quality T4 DNA ligase.
	2.- There is a contamination with exonucleases in any of the reagents used.	Use only high quality reagents and kits for cloning into pSpark® DNA cloning systems.
<b>High ratio of blue-to-white colonies for only the PCR insert</b>	1.- Insert has an open reading frame (ORF).	Screen blue colonies
	2.- Insert behave as a promoter in <i>E. coli</i> .	Screen blue colonies
	3.- Insert has few or only a single leaky stop codon (very frequently observed with short inserts).	Screen blue colonies
	4.- Insert has a high frequency of frameshifting in <i>E. coli</i> .	Screen blue colonies
	5.-PCR insert is contaminated with nuclease activity.	Change reagents used for PCR and or PCR product purification and repeat the ligation and transformation.
	6.- Primer dimers were cloned.	Gel purify PCR product or screen white colonies by colony PCR.

## 4. FREQUENTLY ASKED QUESTIONS (FAQs)

### About pSpark® DNA cloning systems (Family blunt ends: pSpark I, II, III, IV and V)

As a top quality service to our customers, FAQs are continuously updated in our web site ([www.canvaxbiotech.com](http://www.canvaxbiotech.com)) so please check the online manual version to see if FAQs have been updated.

#### Q1.- What is the pSpark® DNA cloning system?

**A1:** The pSpark® DNA cloning system is a family of cloning vectors for blunt ends DNA cloning. They are based on a novel patent pending technology for background reduction. Vectors are digested with EcoRV and both ends are treated to reduce background to less than 1%. Also, they are not based on transcription of toxic genes to reduce background and for this reason, ORF and promoters can also be cloned into pSpark® DNA cloning systems without cloning bias or artifacts.

#### Q2.- Which polymerases can be used for amplifications to be cloned into pSpark® DNA cloning systems?

**A2:** Any proofreading (high fidelity) DNA polymerase could be used for amplification. This includes polymerase blends, first generation proofreading DNA polymerases and second generation proofreading DNA polymerases (see Section 2.2.2.1 and Appendix III).

#### Q3.- I have used an enzyme blend containing Taq DNA Pol and a proofreading DNA Pol (e.g. Expand™ High Fidelity PCR System, a Taq/Tgo blend) for amplification. Should I clone this PCR product into pSpark® DNA cloning systems? Do I need any additional step (e.g. a blunting step)? Which are the expected results?

**A3:** Yes. pSpark® DNA cloning systems could be used for cloning PCR products amplified with enzyme blends without any additional step such as a blunting step. In fact, when such inserts were cloned in parallel into a T/A based vector and into pSpark® DNA cloning systems a slightly higher number of colonies were obtained with pSpark® DNA cloning systems using 10 times less DNA. **No additional blunting step is needed** because such amplified DNA is a mix of both 3'-Adenine overhanging and blunt ended DNA molecules.

#### Q4.- I have used Taq (or Tth) DNA polymerase for amplification. Should I clone this PCR product into pSpark® DNA cloning systems? Do I need any additional step?

**A4:** Taq amplified PCR products can not be cloned directly into pSpark® DNA cloning systems. However, if **an additional blunting step is done** with for example a proofreading thermostable DNA polymerase or T4 DNA polymerase, then Taq amplified products can be cloned into pSpark® DNA cloning systems. Blunting kits that include a phosphorylation enzyme (e.g. NEB E1201L, Quick Blunting™ Kit) are not needed albeit they can be used as ligation is not inhibited by phosphorylated PCR products or primers.

#### Q5.- Do I need to purchase phosphorylated primers or to phosphorylate PCR product for cloning into pSpark® DNA cloning systems? Do primers need any tail at their 5'-end for cloning into pSpark® DNA cloning systems?

**A5:** No, there is no need to purchase phosphorylated primers not to phosphorylate PCR product. Any primer you already have in your lab can be used for cloning into pSpark® DNA cloning systems. Phosphorylated primers do not inhibit ligation and thus can also be used. There is no need to add any specific sequence at 5'-ends of primers for cloning into pSpark® DNA cloning systems.

#### Q6.- How are transformants selected when using pSpark® DNA cloning systems?

**A6:** All pSpark® DNA cloning vectors have ampicillin resistance for selection of transformants and one vector has dual ampicillin/kanamycin antibiotic resistance. All inserts can be amplified by PCR with pUC/M13 forward and reverse primers and in vitro transcribed with either T7 or SP6 RNA polymerases. Blue white screening is possible in all vectors except pSpark® DNA cloning systems IV and V that are transcription free.

#### Q7.- Do I need to change any protocol, E. coli strain or media I am using now to change to pSpark® DNA cloning systems?

**A7:** No. Just set up a ligation according to Section 2.3.1 (or Quick Protocol for Advanced Users), incubate ligation up to 60 minutes at 22 °C (or overnight if needed) and follow all the regular protocols you are familiar with. Use a 3:1 to

5:1 (**recommended**) insert-to-vector molar ratio as starting point. That corresponds exactly to 30 ng of insert per kb for the recommended 5:1 insert-to-vector ratio. As a rule of thumb you can use 30 ng per kb of insert to be cloned, that is, 15 ng, 30 ng or 60 ng if cloning an insert of 0.5 kb, 1 kb or 2 kb. If cloning an unpurified PCR product use 1  $\mu$ L of PCR directly for ligation.

**Q8.- If no change is made in my current protocols, which are the expected results?**

**A8:** pSpark® DNA cloning based systems are about **50 times more efficient** than any T/A based cloning kit. As a result you will need about **10 times less DNA** to obtain **4-5 times more colonies** with a very low **background (about 1%)** and a very low percentage of cloning reaction failures and artefacts (see **Section 2.4.5**).

**Q9.- Many suppliers and users do not recommend the fast transformation protocol (Section 2.4.3) to be used to transform ligations. Why do you recommend this protocol for cloning into pSpark® DNA cloning systems?**

**A9:** pSpark® DNA cloning systems are by far the most efficient cloning kits developed to date and in fact this supplied fast transformation protocol has been developed and tested to be used with pSpark® DNA cloning systems. Using this fast protocol of only 5 minutes transformation, cells with a transformation efficiency of  $4 \times 10^7$  cfu/ $\mu$ g and the supplied control insert, about 1000 positive white colonies are obtained. If you feel uncomfortable with this protocol, use the standard protocol, but as a suggestion to save your precious time we recommend to test this protocol. We do not know, however, if for example, the method to prepare competent cells, the E. coli strain used, the temperature inside air flow cabinet, the transformation protocol used or the plating method, have influence in outcome. To date we have no customer who have tested this fast transformation protocol for cloning into pSpark® with unsatisfactory results. If you test this protocol in parallel with the standard transformation protocol with unsatisfactory results please contact us to know more about your experience and to make it available to the rest of the scientific community.

**Q10.- Should I use my own T4 DNA ligase for cloning into pSpark® DNA cloning systems?**

**A10:** Yes, provided it is a very high quality T4 DNA ligase. We have developed one of the best T4 DNA ligases available today on the market, that is, a highly active ligase with the lowest nuclease activity of any T4 DNA ligase you can find anywhere. If you use another T4 DNA ligase please check both the total number of white colonies and the ratio of white positive colonies to blue negative colonies using the supplied Control Insert. This ratio is about 100 for the supplied T4 DNA ligase. If you obtain less than 1000 white colonies (using cells with  $4 \times 10^7$  cfu/ $\mu$ g) and a ratio of less than 20 then maybe our T4 DNA ligase can help you in your laboratory (see in our web site [www.canvaxbiotech.com](http://www.canvaxbiotech.com) other T4 DNA ligases tested at Canvax)

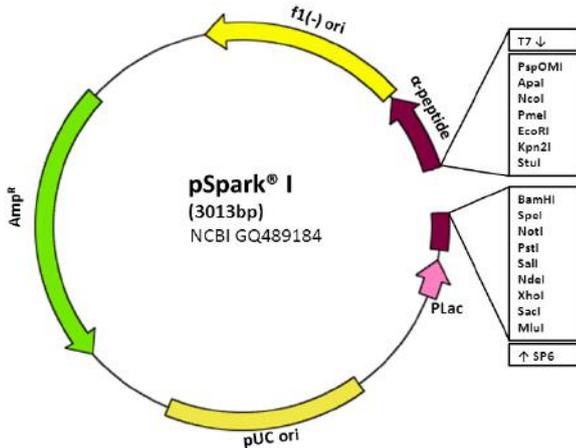
**Q11.- Are pSpark® DNA cloning vectors mixed or bound to any protein?**

**A11:** No. The vector vial has only a highly stable DNA vector and this contributes to the very high stability of pSpark® DNA cloning vectors that could be even shipped at room temperature (only samples and kits without ligase). We have stored freeze-dried pSpark® DNA cloning vectors up to 30 days at 20-24 °C with no loss in cloning efficiency. However, we recommend the storage of vector at -20 °C.

## 5. APPENDIX

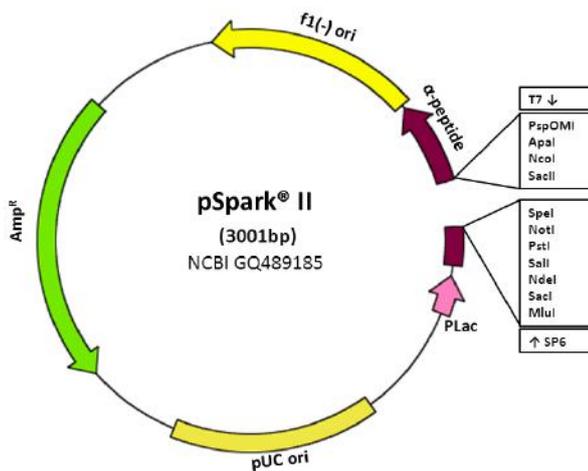
### 5.1 Appendix I: Maps and features for pSpark® vectors

#### 1.- pSpark® I (Cat. N°. C0001)



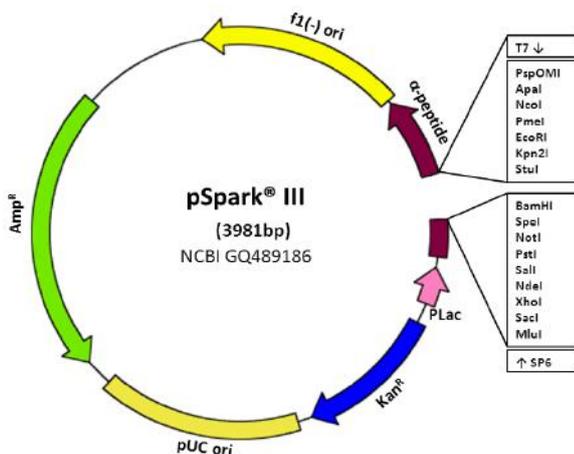
T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10-117
SP6 RNA polymerase transcription initiation site	137
SP6 RNA polymerase promoter	138-154
pUC/M13 reverse sequencing primer binding site	170-189
lacZ start codon	178
lac operator	179-300
Kanamycin resistance gene	-
$\beta$ -lactamase coding region	1335-2195
Phage f1 region	2376-2821
pUC/M13 forward sequencing primer binding site	2962-2978
T7 RNA polymerase promoter	2997-3013

#### 2.- pSpark® II (Cat. N°. C0002)



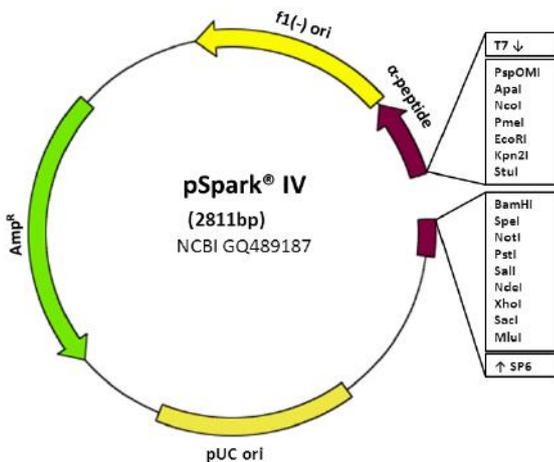
T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10-105
SP6 RNA polymerase transcription initiation site	125
SP6 RNA polymerase promoter	126-142
pUC/M13 reverse sequencing primer binding site	158-177
lacZ start codon	164
lac operator	165-288
Kanamycin resistance gene	-
$\beta$ -lactamase coding region	1323-2183
Phage f1 region	2364-2809
pUC/M13 forward sequencing primer binding site	2962-2978
T7 RNA polymerase promoter	2986-3001

#### 3.- pSpark® III (Cat. N°. C0003)



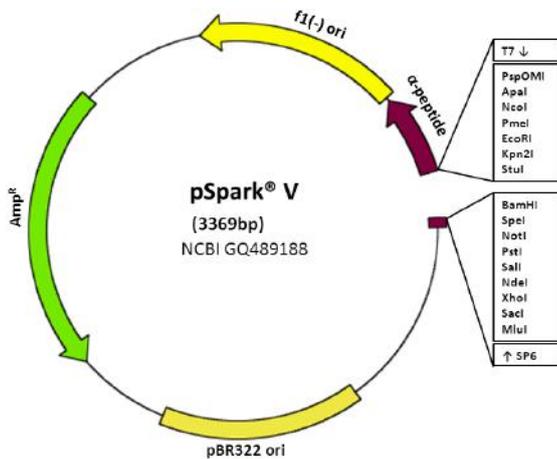
T7 RNA Polymerase transcription initiation site	1
Multiple cloning region	10-117
SP6 RNA polymerase transcription initiation site	137
SP6 RNA polymerase promoter	138-154
pUC/M13 reverse sequencing primer binding site	170-189
lacZ start codon	178
lac operator	179-300
Kanamycin resistance gene	4000-1368
$\beta$ -lactamase coding region	2303-3163
Phage f1 region	3344-3789
pUC/M13 forward sequencing primer binding site	3942-3958
T7 RNA polymerase promoter	3966-3981

#### 4.- pSpark® IV (Cat. N°. C0004)



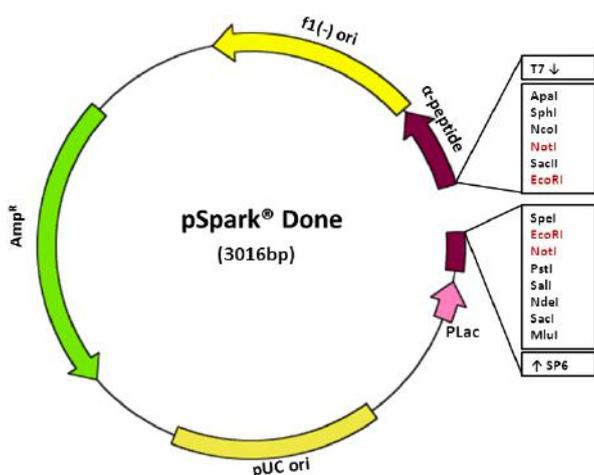
T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10-117
SP6 RNA polymerase transcription initiation site	137
SP6 RNA polymerase promoter	138-154
pUC/M13 reverse sequencing primer binding site	170-189
lacZ start codon	-
lac operator	-
Kanamycin resistance gene	-
β-lactamase coding region	1133-1993
Phage f1 region	2174-2619
pUC/M13 forward sequencing primer binding site	2772-2788
T7 RNA polymerase promoter	2796-2811

#### 5.- pSpark® V (Cat. N°. C0005)



T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10-105
SP6 RNA polymerase transcription initiation site	125
SP6 RNA polymerase promoter	126-142
pUC/M13 reverse sequencing primer binding site	158-177
lacZ start codon	-
lac operator	-
Kanamycin resistance gene	-
β-lactamase coding region	1692-2551
Phage f1 region	2731-3177
pUC/M13 forward sequencing primer binding site	3330-3346
T7 RNA polymerase promoter	3354-3369

#### 6.- pSpark® Done (Cat. N°. C0006)

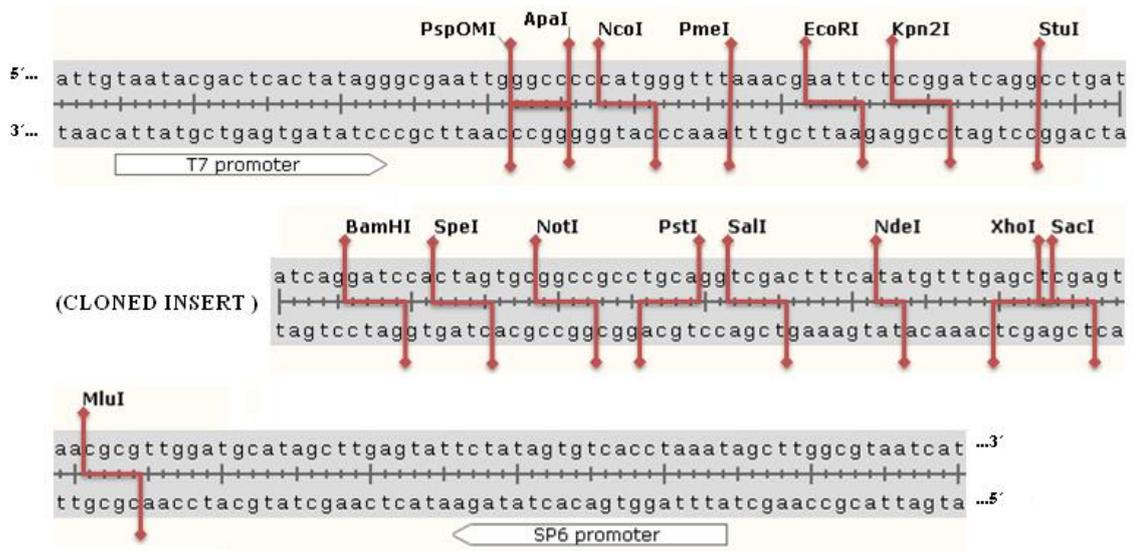


T7 RNA polymerase transcription initiation site	3
Multiple cloning region	10-120
SP6 RNA polymerase transcription initiation site	140
SP6 RNA polymerase promoter	141-159
pUC/M13 reverse sequencing primer binding site	177-193
lacZ start codon	200
lac operator	201-217
Kanamycin resistance gene	-
β-lactamase coding region	1338-2198
Phage f1 region	2381-2836
pUC/M13 forward sequencing primer binding site	2977-2993
T7 RNA polymerase promoter	3000-2

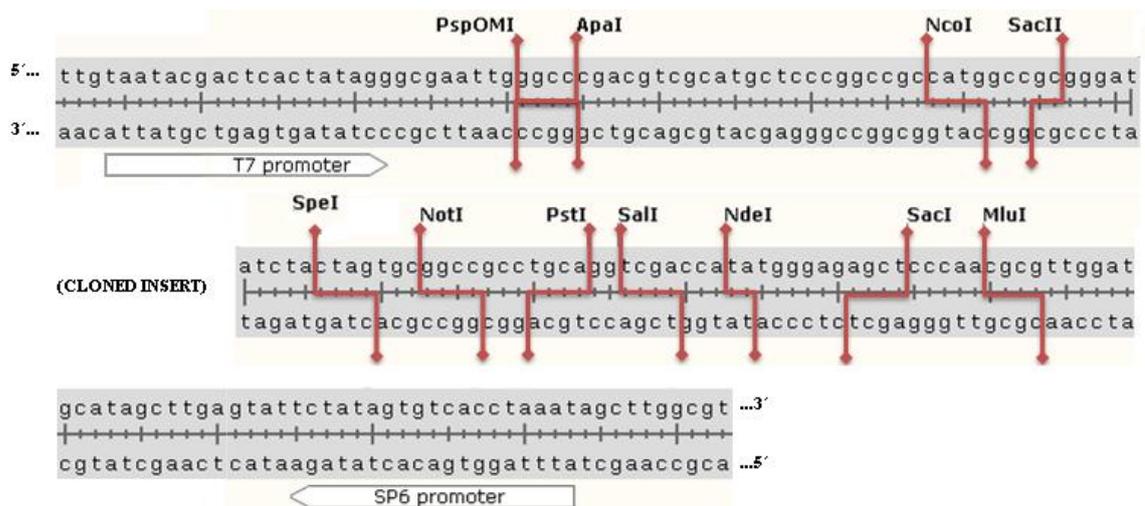
A single digest with **EcoRI** or **NotI** will release inserts cloned into the pSpark®-Done Vector. Double digests can also be used to release inserts.

## 5.2 Appendix II: Sequence of the Multiple Cloning Site (MCS)

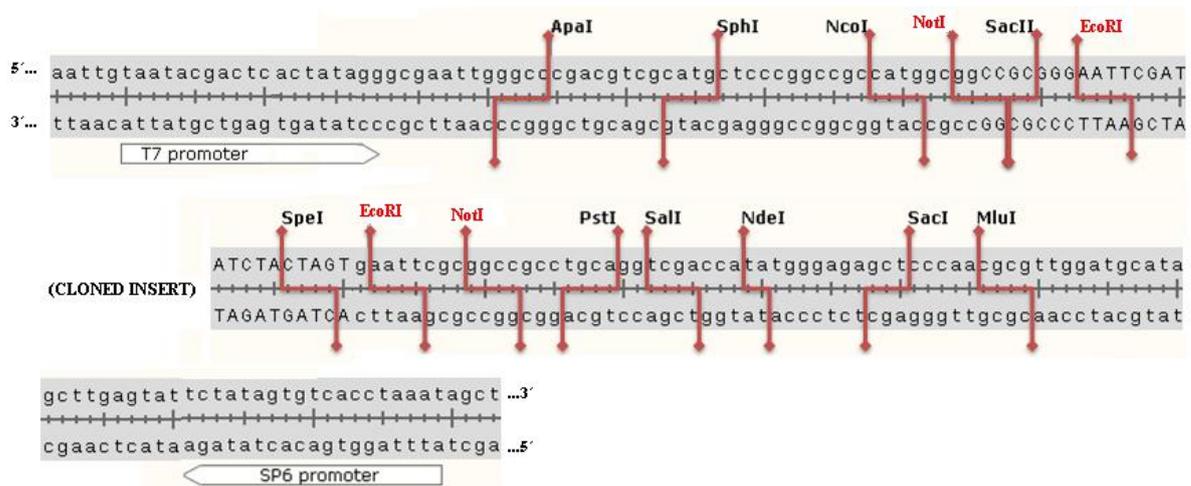
### Sequence of MCS (pSpark® I, III, IV and V)



### Sequence of MCS (pSpark® II)



### Sequence of MCS (pSpark® Done)



### 5.3 Appendix III: Preparation of media and reagents

#### Ampicillin: 50 mg/mL stock solution (1000x stock)

1. Dissolve 2.5 g of ampicillin in about 40 mL of distilled water and bring to 50 mL with distilled water.
2. Filter-sterilize using 0,22 µm filters and store in sterile aliquots at -20 °C

#### Kanamycin: 30 mg/mL stock solution (1000x stock)

1. Dissolve 1.5 g of kanamycin in about 40 mL of distilled water and bring to 50 mL with distilled water.
2. Filter-sterilize using 0.22 µm filters and store in sterile aliquots at -20 °C

#### IPTG: 100 mM stock solution

1. Dissolve 1.2 g of IPTG (M.W. 238.3 g/mol) in about 40 mL of distilled water and add more water to 50 mL final volume.
2. Filter-sterilize using 0.22 µm filters and store in sterile aliquots at -20 °C.

#### X-Gal stock solution 400x (2% (w/v))

1. Dissolve 400 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in 20 mL of N,N'-dimethylformamide (Sigma, D4551).
2. Cover with aluminum foil and store at -20 °C.

### **LB medium (per liter)**

1. Mix
  - 10 g Tryptone (1% (w/v) final concentration)
  - 5 g Yeast extract (0.5% (w/v) final concentration)
  - 5 g NaCl (0.5% (w/v) final concentration for a low salt LB medium)
2. Dissolve in distilled water with a magnetic stirrer, adjust pH to 7.0 with NaOH 1 M and add distilled water to 1 L. If LB-agar is to be prepared add 15 g. of agar per liter of liquid LB.
3. Autoclave at 121 °C for 20 minutes. Allow the medium to cool to 45-50 °C before adding ampicillin to a final concentration of 50 µg/mL or kanamycin to a final concentration of 30 µg/mL.
4. For LB-agar plates preparation pour 25 mL of medium into standard petri dishes (85 mm diameter) and let plates to cool before storage at 4 °C. Plates should be stored for up to 1 month at 4 °C.

### **LB plates with antibiotic/IPTG/X-Gal**

Make the LB plates with ampicillin or kanamycin as above; then supplement with 134 µM final concentration of IPTG (134 µL of IPTG 100mM per 100 mL of LB/antibiotic) and with 0.005% final concentration of X-Gal (250 µL of X-Gal 400x per 100 mL of LB/antibiotic). Plates should be stored for up to 1 month at 4 °C.

### **SOC Medium (per liter)**

1. Mix
  - 20 g of Tryptone (2% (w/v) final concentration)
  - 5 g of Yeast Extract (0.5% (w/v) final concentration)
  - 0,5 g of NaCl (0,05% (w/v) final concentration)
2. Dissolve with a magnetic stirrer in 900 ml of distilled water and add 10 mL of KCl 250 mM (2.5 mM final concentration).
3. Autoclave at 120 °C for 20 minutes and cool to room temperature.
4. Add 10 mL of filter-sterilized 1 M MgCl<sub>2</sub> per liter (10 mM final concentration) and 20 mL of filter-sterilized 1 M glucose per liter (20 mM final concentration).
5. Bring with distilled sterile water to 1 liter. Store at 4 °C.

**5.4 Appendix III: Classification of thermostable DNA polymerases according to suitability for blunt-end cloning and list of some proofreading or high fidelity DNA polymerases that produce blunt ends and could be used for pSpark® based DNA cloning.**

**Table 3. Classification of thermostable DNA polymerases according to their suitability for blunt-end cloning.**

DNA Pol	Prototypes	Ends	Relative fidelity to Taq	Percentage of clones with mutations		Characteristics
				1 kb	5 kb	
<b>Non proofreading</b>	Taq Tth	Single base 3'-overhang	1x	>15%	>80%	<ol style="list-style-type: none"> <li>1.- Most widely used</li> <li>2.- Robust for up to 5 kb</li> <li>3.- High percentage of mutations</li> <li>4.- Only useful for T/A based cloning</li> </ol>
<b>Blend of polymerases</b>	Expand™ High Fidelity	Both blunt and single base 3'-overhang	1.5-3x	5-10%	25-50%	<ol style="list-style-type: none"> <li>1.- Highly robust for up to 15-30 kb</li> <li>2.- Significant percentage of mutated molecules</li> <li>3.- Useful for both T/A and pSpark® based cloning</li> </ol>
<b>High fidelity (1<sup>st</sup> generation)</b>	Pfu/Pwo Tgo KOD HiFi Pfx50™ Platinum® Pfx	Blunt ends	4-8x	2-4%	10-20%	<ol style="list-style-type: none"> <li>1.- Difficult to be optimized for yields, variable results and long amplification times.</li> <li>2.- Amplification of less than 5kb.</li> <li>3.- Useful for pSpark® based cloning.</li> </ol>
<b>High fidelity engineered polymerases (2<sup>nd</sup> generation)</b>	Phusion® PfuUltra™ iProof™ KAPAHiFi™	Blunt ends	>20x	<1%	<5%	<ol style="list-style-type: none"> <li>1.- Highly robust, minimal optimization and high yield</li> <li>2.- Amplification of up to 10-15kb.</li> <li>3.- High speed: 15-60 seconds per kilobase.</li> <li>4.- Recommended for pSpark® based cloning.</li> </ol>

**Table 4. List of some proofreading or high fidelity DNA polymerases that produce blunt ends and could be used for pSpark® based DNA cloning.**

Polymerase	Type of high fidelity polymerase	Supplier	Web site
Pfu	1st generation	Stratagene	<a href="http://www.stratagene.com">www.stratagene.com</a>
Pwo	1st generation	Roche	<a href="http://www.roche-applied-science.com">www.roche-applied-science.com</a>
Tgo	1st generation	Roche	<a href="http://www.roche-applied-science.com">www.roche-applied-science.com</a>
Vent®	1st generation	NEB	<a href="http://www.neb.com">www.neb.com</a>
Deep Vent®	1st generation	NEB	<a href="http://www.neb.com">www.neb.com</a>
KOD HiFi	1st generation	Toyobo/Novagen	<a href="http://www.novagen.com">www.novagen.com</a>
Pfx50™	1st generation	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Herculase®II	1st generation	Stratagene	<a href="http://www.stratagene.com">www.stratagene.com</a>
Platinum Pfx™	1st generation	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
ProofStart	1st generation	Qiagen	<a href="http://www.qiagen.com">www.qiagen.com</a>
EXL® DNA pol	Blend	Stratagene	<a href="http://www.stratagene.com">www.stratagene.com</a>
TaqPlus®	Blend	Stratagene	<a href="http://www.stratagene.com">www.stratagene.com</a>
Expand™ High Fidelity PCR	Blend	Roche	<a href="http://www.roche-applied-science.com">www.roche-applied-science.com</a>
AccuTaq™ DNA pol	Blend	Sigma-Aldrich	<a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a>
Platinum® Taq High Fidelity	Blend	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Advantage HF	Blend	Clontech/Takara	<a href="http://www.clontech.com">www.clontech.com</a>
PrimeSTAR™ HS DNA pol	Blend	Takara	<a href="http://www.takara-bio.com">www.takara-bio.com</a>
AccuPrime™ Taq DNA pol	Blend	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
AccuPrime™ Pfx DNA pol	Blend	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
ThermalAce™ DNA pol	Blend	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
PfuUltra™	2nd generation	Stratagene	<a href="http://www.stratagene.com">www.stratagene.com</a>
PfuUltra™II	2nd generation	Stratagene	<a href="http://www.stratagene.com">www.stratagene.com</a>
Phusion® DNA pol	2nd generation	Finnzymes/NEB	<a href="http://www.finnzymes.fi">www.finnzymes.fi</a> <a href="http://www.neb.com">www.neb.com</a>
Velocity DNA pol	2nd generation	Bioline	<a href="http://www.bioline.com">www.bioline.com</a>
iProof™ DNA pol	2nd generation	Biorad	<a href="http://www.bio-rad.com">www.bio-rad.com</a>
KAPAHiFi™	2nd generation	Kapa Biosystems	<a href="http://www.kapabiosystems.com">www.kapabiosystems.com</a>

**Warranty:** Canvax Biotech SL warrants that the product will meet the specifications stated on this **Product Manual** and agrees to replace the product free of charge if the product does not conform to the specifications but notice for replacement must be given within 30 days. In consideration of the above promises by Canvax Biotech SL, the purchaser agrees to and accepts the following conditions: (1) That this warranty is in lieu of all other warranties, expressed or implied; (2) That ALL WARRANTIES OF MERCHANTABILITY OR OF FITNESS FOR A PARTICULAR PURPOSE ARE HEREBY EXCLUDED OR WAIVED; (3) That the purchaser's sole remedy shall be to obtain replacement of the product free of charge from Canvax Biotech SL; and (4) That this remedy is in lieu of all other remedies or claims for damages, consequential or otherwise, which the buyer may have against Canvax Biotech SL.

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## NOTES