canvax

# pSpark® TA DNA cloning system

## Manual for cat. nº :

## C0020 (pSpark® TA)

## C0021 (pSpark® TA Done)

**Upon Receipt**
**Store Kits at -20ºC**

**PRODUCT MANUAL**
**Version 3.0**
**Last updated: July 2012**

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# MATERIALS PROVIDED, KIT STORAGE AND EXPIRATION DATE

| | pSpark® TA DNA Cloning Vector (50 ng/µL) 20 rxn (20 µL) | |
|---|---|---|
| Item | TA | TA Done |
| T4 DNA Ligase (5 Weiss Units/µL) | 100 U (20 µL) | 100 U (20 µL) |
| 5x T4 DNA Ligase Buffer | 200 µL | 200 µL |
| 10x PEG 6000 | 50 µL | 50 µL |
| Control Insert (600 bp) | 5 µL | 5 µL |

See on the kit label and vector vial label for information.

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

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

**IMPORTANT:** *pSpark® TA DNA cloning kit* ***MUST be stored at -20ºC in a non-frost free freezer since temperature rises above 0ºC daily in frost-free freezers. If properly stored, kits are guaranteed for 9 months from the date of purchase.***

Note

**ADVICE:** *Storage temperatures above -20ºC are not recommended. But in cases of an accident such as power failure, stored vector should be tested with supplied control insert before discarding it.*
*Please note that T4 DNA ligase is extremely temperature sensitive and storage temperatures above -20ºC are not tolerated.*

# 1. INTRODUCTION

## 1. Description kit

**pSpark® TA and pSpark® TA Done Vector** are based on a classical TA technology for cloning single 3´-adenine overhanging DNA. The vectors are prepared by digestion of **pSpark® TA** cloning vector at EcoRV site and the subsequent addition of a single thymidine at each 3´-end to allow cloning Taq polymerase amplified DNA fragments. It offers greater efficiency than the most TA vectors on the market although with less background of blue colonies.

**Table 1. Optimal DNA Cloning vector vs. your DNA amplification**

| DNA Polymerase | PCR Insert | Choose DNA Cloning vector |
|---|---|---|
| **Blend** | Blend (both 3´-A and blunt) | pSpark® TA (-TA Done) DNA Cloning Vector |
| **Non-proofreading** | 3´-A | pSpark® TA (-TA Done) DNA Cloning Vector |

## 2. Principles and advantages.

**pSpark® TA and pSpark® TA Done Vector** are based on a **classical TA technology** for **cloning of single 3´-adenine overhanging DNA**, for example DNA amplified by PCR with Taq DNA polymerases. The vectors are prepared by digestion of pSpark® TA DNA cloning vector at EcoRV site (see maps in section 11) and the subsequent addition of a single thymidine at each 3´-end to allow cloning of Taq amplified DNA fragments. **pSpark® TA Done** vector offer all of the advantages of the **pSpark® TA** Vector with the added convenience of recognition sites for **EcoRI** and **NotI** flanking the insertion site. Thus, several options exist to remove the desired insert DNA with a single restriction digestion

**pSpark® TA DNA cloning vector and pSpark® TA Done DNA cloning vector** are as easy to use as the other common TA DNA cloning vectors avaiable on the market.

Any non-proofreading DNA polymerase like Taq DNA polymerase or Tth DNA polymerase may be used for amplification cloning into pSpark® TA (-TA Done) DNA cloning vector to obtain hundreds of positive colonies.

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 (ORFs).

**Table 2. Main advantages of pSpark® TA (-TA Done) DNA cloning vectors.**

| Property | pSpark® TA (-TA Done) DNA cloning vectors | More information (Section) |
|---|---|---|
| **Efficient** | 1. Two times more efficient than common TA cloning vector on the market.<br>2. Over **700 positive colonies** expected under optimal conditions. | **9.4**<br>**9.4** |
| **Fast&Easy** | 1. **No** steps after PCR<br>2. Use of **PCR product directly** for cloning is possible. | **6.**<br>**6.** |
| **Flexible** | 1. **No** special competent cells needed<br>2. **No** special primer design needed<br>3. Use of **competent cells** with **subcloning efficiency** possible albeit or lower number of colonies is obtained.<br>4. **No** primer phosphorylation required<br>5. A ligation time from **60 minutes to overnight.** | **9.1**<br>**7.1**<br>**9.1**<br>**7.1**<br>**8.3** |
| **Robust** | 1. **No cloning bias** due to **transcription** of toxic genes.<br>2. Low **background:** around 4%: 3-5 times less background than others TA DNA cloning vectors available on the market. | **2.**<br>**9.4** |

## 3. Specialized Applications of the pSpark® TA (-TA Done) DNA cloning kits:

- Cloning of non-proofreading PCR fragments into pSpark® TA (-TA Done) DNA Cloning vectors.
- Production of ssDNA.
- Blue/white screening for recombinants.
- In vitro transcription from T7/SP6 dual-opposed promoters.

## 4. The family of pSpark® DNA cloning vectors.

All pSpark® DNA cloning vectors 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 vector have binding sites for pUC/M13 Forward and Reverse primers and thus cloned insert can be amplified or sequenced with those primers.

Two versions of the MCS have been developed for pSpark® DNA cloning systems: 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.

**Table 3** shows the available variants of pSpark® DNA cloning kits and features of each one. Two variants for cloning TA are **pSpark® TA** and **pSpark® TA Done** vectors developed on classical **TA technology** for **cloning of single 3´-adenine overhanging DNA.**

**Table 3. Family of pSpark® DNA cloning vectors, main characteristics and applications.**

| Vector | OriC (copy number) | MCS | Blue/white feature | Antibiotic | Size (bp) | Application |
|---|---|---|---|---|---|---|
| **pSpark®-TA** | **pUC (High)** | **Classical** | **Yes** | **Amp** | **3001** | **General cloning** |
| **pSpark®-TA Done** | **pUC (High)** | **Classical** | **Yes** | **Amp** | **3015** | **General cloning** |
| **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 |

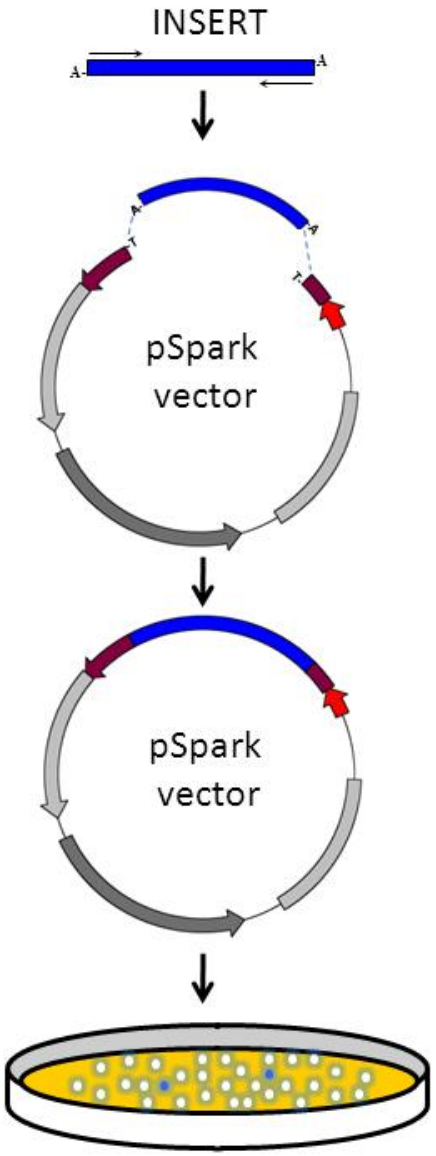
***Note:*** *All pSpark® DNA cloning vectors have f1 ori, pUC/M13 Forward and Reverse primers binding sites and T7/SP6 RNA polymerases binding sites.*

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

1. The appropiate DNA polymerase
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 I** for preparation of Media and Reagents).
    - Incubator at 37ºC.
    - LB Ampicillin.
    - Orbital shaker at 37ºC and 230-260 rpm for plasmid purification
    - Water bath with regulated temperature exactly at 42ºC
    - Ice bath
    - SOC medium (Optional)
    - Timer
    - Taq DNA polymerase and dNTPs (if colonies are screened by colony PCR)
    - Sterile tubes (both 0,2mL tubes for PCR and 1,5mL microcentrifuge tubes for ligation, transformation and colony PCR)
    - Sterile pipet tips (and optional sterile toothpick to use in protocol for colony PCR)

# DETAILED PROTOCOL

## 6. Experimental Outline.




**1. Amplify your gene of interest** **(See section 7).**

**2. Ligate (1 hour)** **(See section 8)**

**3. Transform (1hours 45 minutes)** **(See section 9)**

**4. Grow and plate Incubate 37 ºC overnight (See section 9 and 10)**

# 7. PCR.

## 7.1 PCR Primers design.

One of the main advantages of pSpark® TA (-TA Done) DNA cloning vectors 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 successful cloning into pSpark® TA (- TA Done) DNA cloning vector. However, the use of PIG sequences at 5´ends of primers used in the PCR allows the cloning of large fragments with high efficiency. The addition of the sequence PIG, GTTTCTT increase by 9 times the efficiency of cloning a PCR fragment of 3,5 kb.

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 bindings.

2. Primer hairpins with a melting temperature higher than 46-48ºC 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 4-6ºC 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 Tm 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.

## 7.2. PCR Amplification

**DNA polymerase selection:** It is necessary to use a non-proofreading DNA polymerase to obtain a PCR insert suitable for cloning into pSpark® TA (-TA Done) DNA cloning vector. Any thermostable DNA polymerase that produces at least a fraction of molecules with both 3´-A ends could be used to amplify by PCR the DNA to be cloned into pSpark® TA (-TA Done) DNA cloning vector (also see section 6.2**). It is also advisable to use PIG sequences to clone**

**fragments larger than 2 kb. Typically, these sequences are added in the 5 'ends of primers to facilitate cloning of inserts.**

**EXTREMELY IMPORTANT**: *Please do not use proofreading DNA polymerases for amplification. High fidelity amplified DNA has blunt ends that* ***CAN NOT*** *be cloned into pSpark® TA or pSpark® TA Done DNA cloning vector, unless an additional 3´-adenine addition step is done.*

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 homogenous band with only primers-dimers can be purified by any high quality PCR clean-up kit for cloning into pSpark® TA (or pSpark® TA Done) DNA cloning vector. In several cases crude unpurified PCR products can be used directly for ligation into pSpark® TA (-TA Done) DNA cloning vector 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® TA (or pSpark® TA Done) Vector and a plasmid template DNA has been used for PCR, please check that plasmid template has a different antibiotic resistance than pSpark® TA (-TA Done) 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.*

On the other hand, PCR products often contain numerous spurious bands that must be purified away from correct product. **Agarose gels** electrophoresis **is highly recommended** to size fractionate desired fragments, for 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® TA (-TA Done) Vector 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 ultraviolet light. Exposure to short wavelength ultraviolet 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. 360nm) 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.*

## 8. Ligation

### 8.1 Amount of insert needed for ligation into pSpark® TA and pSpark® TA Done Vector.

The concentration of PCR product should be estimated either by comparison to quantitative DNA mass standards on an agarose gel, by absorbance at 260nm/280nm quantification using for example a Nanodrop™ Spectrofotometer or by using a fluorescent assay. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation:

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

If initial experiments with your PCR products are suboptimal, ratio optimisation may be necessary. Ratios of insert to vector from 5:1 to 1:1 provide good initial parameters.
pSpark® TA (TA Done) DNA Cloning vectors have been optimized using an insert to vector ratio of 3:1. Optimal ratio of insert to vector is from 3:1 to 5:1.

**Example of amount of insert calculation:**

How much 1 kb PCR product should be added to a ligation in which 50 ng of pSpark® TA (-TA Done) DNA vector (3.0 kb) will be used if a 3:1 insert to vector ratio is used?

$$\frac{50 \text{ ng vector} \times 1000 \text{ pb insert} \times 3}{3000 \text{ pb vector} \times 1} \sim \textbf{50 ng of insert}$$

Note

***RULE OF THUMB:***

1. *Use **50 ng** of insert per kilobase for a ligation of **purified PCR** product.*
2. *Use **2µL** of **unpurified PCR** product for a ligation.*

See in our web site (www.canvaxbiotech.com) for additional information.

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

The pSpark® TA DNA cloning kits are designed and tested for routine cloning of PCR products up to 4 kb in length with high efficiency.

Note

**ADVICE**: *pSpark® TA DNA cloning kits 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 analyse 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 nonspecific 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 **$5x10^7$ cfu/µg** is strongly recommended **for cloning of long PCR products**.

## 8.3 Protocol for ligation using the pSpark® TA (-TA Done) DNA cloning vector.

1. Briefly centrifuge the pSpark® TA 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**

Set up the ligation reaction by mix in the following reagents:

| Reagent | Cloning Reaction | Control Reaction | Background Reaction |
|---|---|---|---|
| pSpark®-TA ( or TA Done)DNA cloning vector (50 ng/µL) | 1µL | 1µL | 1µL |
| 5x T4 DNA Ligase Buffer | 2µL | 2µL | 2µL |
| PCR product | X µL* | -- | -- |
| Control Insert DNA (600 bp) | -- | 1µL | -- |
| T4 DNA ligase (5 Weiss units/µL) | 1µL | 1µL | 1µL |
| Molecular Biology grade Water to a final volume of | 10µL | 10µL | 10µL |
| **See **Section 8.1** for insert to vector ratio* | | | |

4. Mix the reactions by pipetting slowly. Incubate the ligation reactions one hour at 22ºC in either a thermoblock, a thermocycler, a water bath or at bench if room temperature is between 20-24ºC.

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

**IMPORTANT:** *The supplied 5X T4 DNA Ligase Buffer contains ATP, which degrades during temperature fluctuations. Usually a white precipitate is formed on 5X T4 DNA Ligase Buffer. This buffer with a precipitate at the bottom could 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.*


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 any 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) for details.*

c) *If your template DNA for PCR is a plasmid that shares the same antibiotic resistance than pSpark® DNA cloning vector we advise to set up a control ligation without pSpark® DNA cloning vector but containing insert, T4 DNA Ligase, 5x T4 DNA Ligase Buffer, and water to check if insert produces colonies.*

# 9. Transfomation

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

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 42ºC for a critical time period and then returned to ice before plating.

## 9.2 Standard protocol for transformation.

Please see **Section 5.** for Additional Material Required.

**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. 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 µL of each ligation reaction to a sterile 1,5 mL microcentrifuge tube on ice. Set up another tube on ice with 25 pg uncut plasmid for determination of the transformation efficiency of the competent cells.

**3.** Remove a tube of frozen Competent Cells 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. 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 selection antibiotic is ampicillin then plate 60 µL of each transformation culture onto pre-warmed LB/antibiotic/IPTG/X-Gal plates. If 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 37ºC and incubate for 1-1.5 hours at 37ºC with shaking (~150 rpm) before plating into LB/antibiotic/IPTG/X-Gal plates. After incubation, centrifuge cells at 2500g for 10 minutes and resuspend in 50-100 µL of liquid media for plating.

Note

**ADVICE**: (a) *If cells transformed with ligation reactions from **Step 7** are incubated before plating for 1 hour at 37ºC with shaking (~150 rpm) in 950 µL of SOC medium the number of transformants is about two fold higher. This step is not needed in routine cloning into ampicillin pSpark® TA (-TA Done) DNA cloning vector because of their very high cloning efficiency (see **Section 9.5.**).*

*(b) For the transformation control if expected transformation efficiency of cells is higher than $4x10^7$ cfu/µg then at least a 1:10 dilution with SOC medium is recommended for plating. For example, competent cells with $2x10^8$ cfu/µg 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.

## 9.3 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 µL 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. Precipitation based methods have in general low recovery and thus we recommend a spin column purification method.

Protocols for *E. coli* electrotransformation have been developed for both 1mm gap and 2mm 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), BTX (www.btxonline.com) or Eppendorf AG (www.eppendorf.com).

### 9.4. Expected results

White colonies will generally contain insert but if crude (unpurified) PCR is used directly for ligation into pSpark® TA (-TA Done) DNA cloning vector 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 2000bp 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 colour 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.

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® TA and pSpark® TA Done Vector do not use transcription to reduce background.

But in most situations blue colonies are negative that is they are the results of self-vector recircularization.

**EXTREMELY IMPORTANT**: *Use only the supplied T4 DNA Ligase to perform ligations into pSpark® TA*(-TA Done) *DNA cloning vector 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® TA DNA cloning vector, albeit the number of positive colonies is about 2 to 4 fold lower and the number of blue 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® TA DNA cloning vector.*

**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 $1x10^7$ 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?

25 pg = 0,025 ng DNA----------250 colonies (cfu)
1000 ng = 1 µg DNA---------- X (transformation efficiency)
Transformation efficiency = $\frac{250 \text{ cfu} \times 1000 \text{ng}/\mu\text{g}}{0{,}025 \text{ng}} = 1x10^7$ cfu/µg DNA

**Expected results for cloning of amplified DNA**: pSpark®TA(-TA Done) DNA cloning vector produces about 2 times more colonies than any other TA cloning kit available on the market. At Canvax our scientist have found that 1 µL of a very high yield PCR could be transformed directly without purification and most of the times generates 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 primer dimers.

Background of pSpark® TA (-TA Done) DNA vector self-ligation in a ligation reaction without insert produces less than 4% 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 a 600 bp insert and transformation into cells with a transformation efficiency of $4x10^7$ cfu/µg produces over 600-700 white colonies and less than 20 blue colonies. But if ligation is carried out under non-optimal conditions background could be higher.

**Expected Result for the Control Insert Transformation:** After plating 60 µL of the Control Insert transformation reaction, about 600 white colonies are expected if using cells with a transformation efficiency of about $4x10^7$ 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 800 bp while empty colonies should give an amplification of about 200bp.

## 10. 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 37ºC that favour blue colour 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.

## 10.1 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 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. 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 5**

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.


Note

**ADVICE**: *Please remember*

- ✓ *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 means that the colony has no the desired insert.*

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 master mix containing Taq DNA polymerase (or equivalent), dNTPs at 200 µM final concentration, buffer for Taq reaction, $MgCl_2$ at 2mM, 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.


Note

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

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. Begin PCR program (use an extension time of about 1 min per kb; 72ºC for extension if Taq polymerase is used and 52ºC of annealing temperature if pUC/M13 forward and reverse primers are used). Use 25-30 cycles.


Note

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

9. Check the amplifications by agarose gel electrophoresis.

### 10.2 Isolation of plasmid DNA.

1. Take 4-6 positive colonies (from colony PCR, see **Section 9.1**) 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 37ºC.

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 were multiple vector variants will be made in the future with such insert (see **Section 10.4** below for storage of sequenced clones)

### 10.3 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.


Note

**ADVICE**: *The use of either pUC/M13 forward or reverse is highly recommended. Refer to the map of any pSpark® DNA cloning vectors used for cloning on* ***section 11*** *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.*

### 10.4 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 37ºC 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 -80ºC 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.

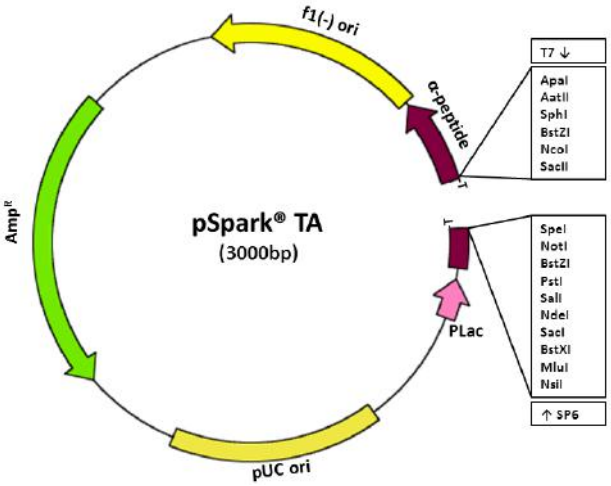

Note

**ADVICE:** *Copy number of plasmid is reduced during long term storage at -80ºC. 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.*

## 11. pSpark®-TA (-TA Done): Cloning Vector Map and sequence reference points

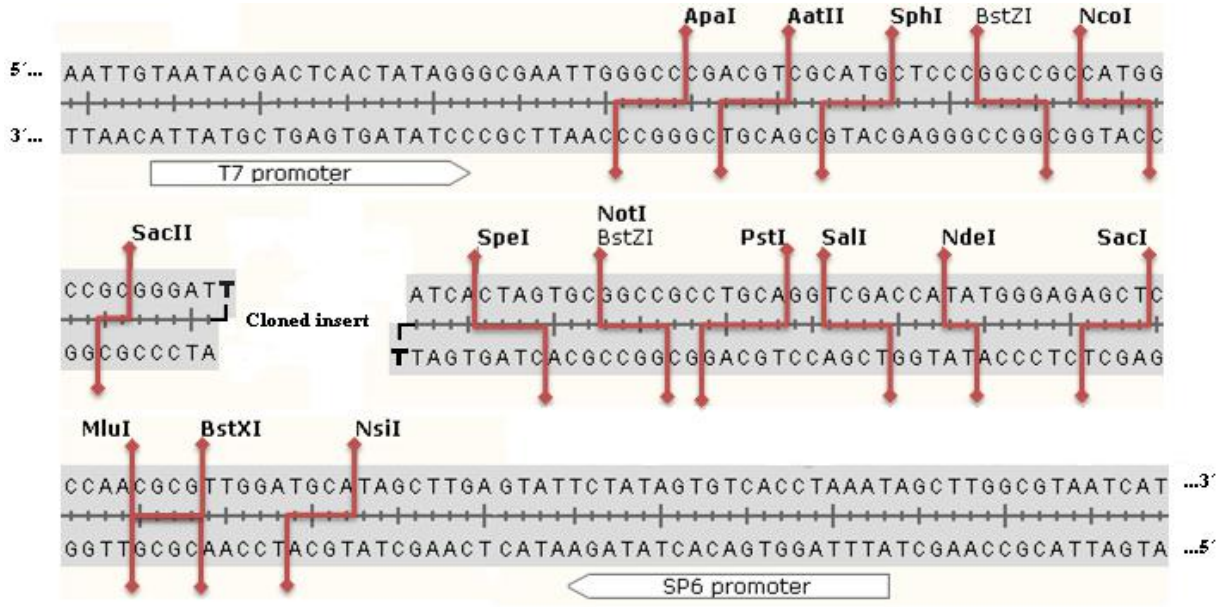
The map below shows the features of pSpark®-TA DNA cloning vector, pSpark®-TA Done DNA cloning vector and the sequence surrounding the advanced Multiple Cloning Site (cMCS). The arrows indicate the start of transcription for the T7 and SP6 polymerases.

### 11.1.- pSpark® TA (Cat. Nº. C0020)



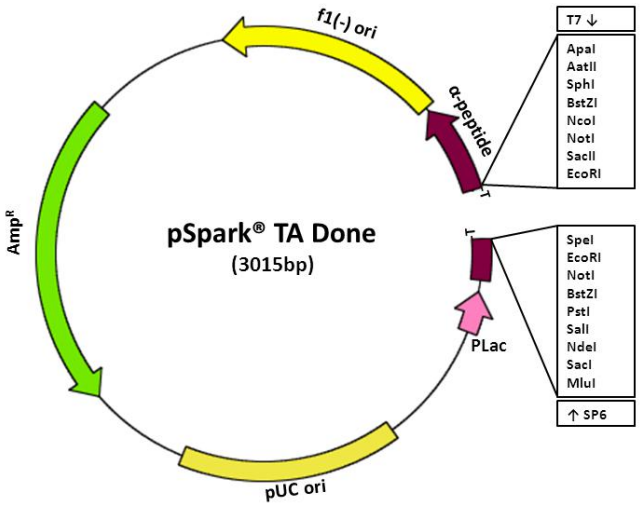

| | |
|---|---|
| T7 RNA Polimerase Transcrption initiation site | 1 |
| Multiple cloning region | 10-113 |
| SP6 RNA Polymerase Transcription initiation site | 125 |
| SP6 RNA Polymerase promotor | 127-145 |
| pUC/M13 Reverse Sequencing Primer Binding site | 163-179 |
| lacZ start condon | 164 |
| lac opetartor | 187-203 |
| Kanamicine Resistence gen | - |
| β-lactamase coding region | 1323-2183 |
| Phage f1 region | 2367-2822 |
| pUC/M13 Forward Sequencing Primer Binding site | 2962-2978 |
| T7 RNA polymerase promotor | 2986-3001 |

### Sequence of Multiple Cloning Site (cMCS)



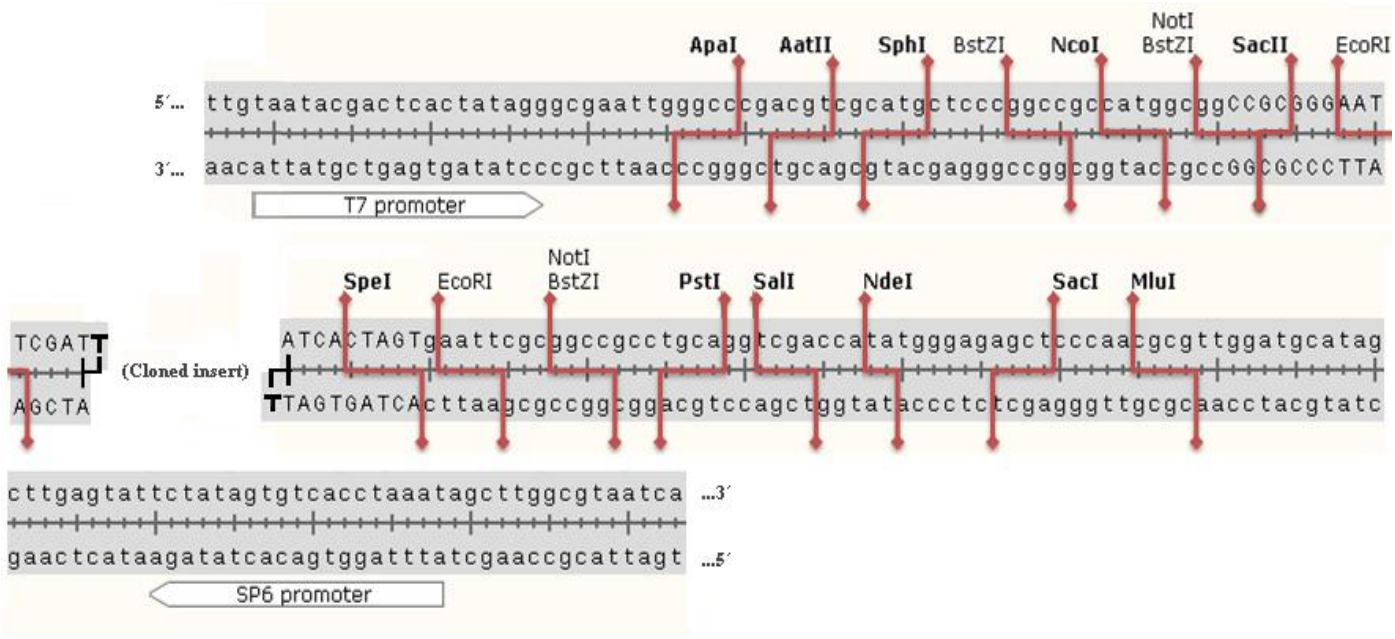

*Inserts can be sequenced using the SP6 Promoter Primer , T7 Promoter Primer, pUC/M13 Forward Primer, or pUC/M13 Reverse Primer.*

## 11.2.- pSpark® TA Done (Cat. Nº. C0021)




| | |
|---|---|
| T7 RNA Polimerase Transcrption initiation site | 1 |
| Multiple cloning region | 10-117 |
| SP6 RNA Polymerase Transcription initiation site | 137 |
| SP6 RNA Polymerase promotor | 138-156 |
| pUC/M13 Reverse Sequencing Primer Binding site | 174-190 |
| lacZ start condon | 197 |
| lac opetartor | 198-214 |
| Kanamicine Resistence gen | - |
| β-lactamase coding region | 1335-2195 |
| Phage f1 region | 2378-2833 |
| pUC/M13 Forward Sequencing Primer Binding site | 2974-2992 |
| T7 RNA polymerase promotor | 2954-3015 |

## Sequence of Multiple Cloning Site (cMCS)




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

| *Sequences and melting temperatures of pUC/M13 primers are the following:* | | |
|---|---|---|
| **Primer** | **Sequence** | **Melting Temperature** |
| pUC/M13 Forward | 5′-GTAAAACGACGGCCAGT-3′ | 52ºC |
| pUC/M13 Reverse | 5′-AGGAAACAGCTATGACCATG-3′ | 58ºC |

Complete sequence of pSpark®-TA and pSpark® -Ta Done can be found on the web site: www.canvaxbiotech.com

# 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)** | 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 *E. coli* competent cells. A transformation efficiency lower than $1x10^7$ cfu/µg is not recommended |
| **Colonies only in the Control Insert Reaction** | 1-. Any component is missing in the specific insert reaction only | Repeat ligation and transformation and include controls. |
| | 2-. A wrong DNA polymerase has been used for amplification. | Amplify your PCR insert with the appropriate DNA polymerase. (**see section 7.2.1**) |
| | 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 **Section 8.1**) 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 **Section 8.1**) |
| | | |
| **White colonies without insert of interest** | 1-. A plasmid containing the same antibiotic resistance than pSpark® TA DNA cloning vector was used and unpurified PCR product was used for ligation. | Gel purify your PCR insert. |
| | 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. endonu-cleases) 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. |
| **High ratio of blue-to-white colonies for both the PCR insert and the Control Insert Reaction** | 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 vectors. |
| **High ratio of blue-to-white colonies for only the PCR insert** | 1-. Insert has an open reading frame (ORF) | Screen blue colonies |
| | 2-. Insert behave as a promoter in *E. coli* | 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 *E. coli* | 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 |

# Frequently Asked Questions (FAQs) about pSpark® TA (-TA Done) DNA cloning vector.

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

**Q1-. Do I need to purchase phosphorylated primers or to phosphorylate PCR product for cloning into pSpark® TA DNA cloning vector? Do primers need any tail at their 5´-end for cloning into pSpark® TA DNA cloning vector?**

***A1:* 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® TA (-TA Done) DNA cloning vector. 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® TA DNA cloning vector.*

**Q2-. How transformants are selected when using pSpark® TA DNA cloning vector?**

***A2:*** *All pSpark® DNA cloning vectors have ampicillin resistance for selection of transformants. 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.*

**Q3-. Should I use my own T4 DNA ligase for cloning into pSpark® TA (or TA Done) DNA cloning vector?**

***A3:* 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. If you obtain less than 700 white colonies (using cells with $2x10^7$ cfu/µg) and more than 4% of blue colonies then maybe our T4 DNA ligase can help you in your laboratory (see in our web site www.canvaxbiotech.com other T4 DNA ligases tested at Canvax)*

**Q4-. Do pSpark® TA DNA (TA Done) cloning vector is mixed or bound to any protein?**

***A4:* No***. Vector vial has only a highly stable DNA vector and this contributes to the very high stability of pSpark® TA DNA cloning vector that could be even shipped at room temperature (only samples and kits without ligase). However, we recommend storage of vector at -20ºC.*

**Q5-. Which polymerases can be used for amplifications to be cloned into pSpark® TA (-TA Done) DNA cloning vectors?**

***A5****: For cloning into pSpark® TA DNA cloning vector any non-proofreading DNA polymerase could be used.*

**Q6-. 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® TA (or TA Done) DNA cloning vector? Do I need any additional step?**

***A6:* Yes***. pSpark® TA and pSpark® TA Done Vector could be used for cloning PCR products amplified with enzyme blends without any additional step, because such amplified DNA is a mix of both 3´-Adenine overhanging and blunt ended DNA molecules.*

# Appendix

## 12. Appendix I: 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 -20ºC

**Kanamycin: 30mg/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 um filters and store in sterile aliquots at -20ºC

**IPTG: 100mM 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 400mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in 20 mL of N,N´-dimethylformamide (Sigma, D4551).
2. Cover with aluminium foil and store at –20°C.

**LB medium (per liter)**

1. Mix

   10g Tryptone (1% (w/v) final concentration)

   5g Yeast extract (0,5% (w/v) final concentration)

   5g 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 1M 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 121ºC for 20 minutes. Allow the medium to cool to 45-50ºC 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 (85mm diameter) and let plates to cool before storage at 4ºC. Plates should be stored for up to 1 month at 4ºC.

**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 4ºC.

**SOC Medium (per litre)**

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 120ºC for 20 minutes and cool to room temperature.
4. Add 10 mL of filter-sterilized 1M $MgCl_2$ per litre (10 mM final concentration) and 20 mL of filter-sterilized 1M Glucose per litre (20 mM final concentration).
5. Bring with distilled sterile water to 1 litre. Store at 4ºC.

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