



Table of Contents

Description	1
System Components	2
DNase Shotgun [®] Cleavage	4
Single dA [™] Tailing	5
Ligation to pSCREEN [™] T-Vector	6
Transformation of NovaBlue(DE3) Competent Cells	7
Screening	7
Plasmid Preparation and Sequencing	11
References	12
pSCREEN-1b(+) Vector Map and Sequence	13

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Description	NovaTope Library Construction and Screening System	69279-3
	NovaTope Library Construction System	69280-3

The NovaTope System* is a powerful new technique for protein analysis that allows rapid functional screening of peptide domains. The method is based on the creation of a library of bacterial clones, each of which expresses a small peptide derived from the protein under study. The library is screened by standard colony lift methods using an antibody or other ligand of interest as the probe. Positive clones can be analyzed directly by DNA sequencing to determine the precise amino acid sequence of the target epitope. Using this system monoclonal antibody epitopes or other contiguous functional domains can be localized to 10-20 amino acids within 1 week. If desired, the peptide can be produced in large quantities from the bacterial recombinant.

In practice, the library is constructed using DNase I to randomly cleave the starting gene into fragments averaging 50 to 150 bp in size. The enzyme is used in the presence of Mn²⁺, which causes double strand cleavage of the DNA molecule (1). The DNA fragments are fractionated by electrophoresis on an agarose gel. Following elution from the gel, the DNA is treated successively with T4 DNA polymerase and *Tth* DNA polymerase, which repairs and then adds a single dA residue to each 3' end. The prepared DNA fragments are ligated into a plasmid vector containing single dT overhangs, which are complementary to the inserted fragments. This novel cloning strategy has several advantages, including the prevention of tandem inserts, low non-recombinant background, and elimination of the need for special linkers and additional fractionation steps. The vector is designed for the expression of small peptides since they are produced as part of a larger fusion protein, which prevents breakdown by cellular proteases. Expression is controlled by a T7 promoter. For screening, ligation reactions are transformed into a bacterial host carrying the gene for T7 RNA polymerase, which results in the accumulation of the fusion protein in the cell. Colonies are transferred to nitrocellulose filters, lysed and screened with the relevant antibody or ligand as the probe. Plasmid DNA from positive clones is prepared and epitope DNA sequence determined by standard double stranded sequencing methods. The pSCREEN-1 vector also contains an f1 origin of replication for the production of single stranded plasmid DNA for mutagenesis.

* The NovaTope method is covered by U.S. Patents owned by Associated Universities and Novagen, Inc. A non-distribution agreement accompanies the products. Commercial customers must obtain a license from Associated Universities before purchase.



The NovaTope Library Construction and Screening System contains all components needed to construct and screen 10 epitope libraries with mouse antibodies. The System includes the following kit modules:

- DNase Shotgun Cleavage Kit
- Single dA Tailing Kit
- DNA Ligation Kit
- pSCREEN T-Vector Kit
- ColonyFinder[®] Immunoscreening Kit
- pSCREEN Vector Primers

The NovaTope Library Construction System includes all of the above kits except the ColonyFinder Immunoscreening Kit, which is available separately.

The methods and procedures developed for the NovaTope System can be used in a variety of applications, including the generation of genomic DNA libraries, shotgun cloning, PCR[†] product cloning, and colony immunoscreening. Therefore, each of the kit modules is also available separately.

[†]PCR is covered by U.S. Patent Numbers 4,683,195 and 4,683,202 owned by Hoffmann-La Roche.

System Components

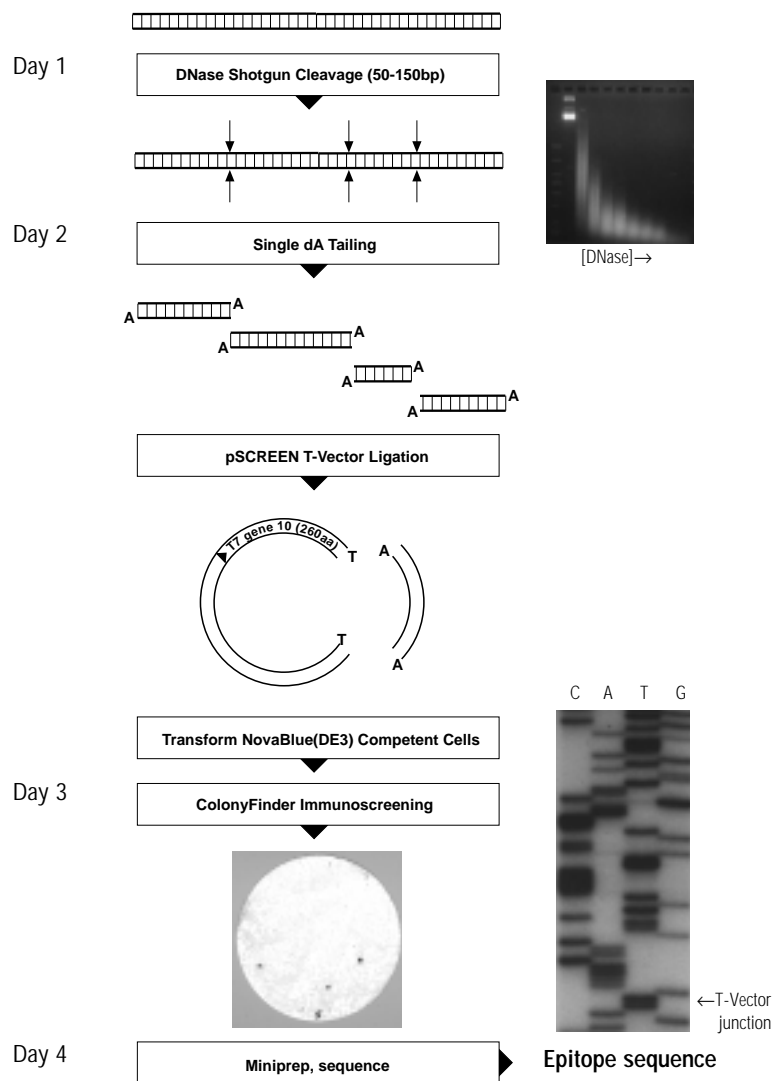
DNase Shotgun Cleavage Kit	69281-3
• 50 U DNase I, ds qualified	
• 350 µl 10X DNase I Buffer	
• 350 µl 10X MnCl ₂	
• 400 µl 6X Stop Buffer	
• 50 lanes PCR Markers	
Single dA Tailing Kit	69282-3
• 25 U T4 DNA Polymerase	
• 50 µl 10X Flush Buffer	
• 50 µl 10X dNTP Mix	
• 25 µl 100 mM DTT	
• 25 U <i>Tth</i> DNA Polymerase	
• 200 µl 10X dA Tailing Buffer	
• 500 ng Positive Control HSV•Tag [®] 36mer	
DNA Ligation Kit	69838-3
• 100 U T4 DNA Ligase	
• 500 µl 10X Ligation Buffer	
• 250 µl 100 mM DTT	
• 250 µl 10 mM ATP	
• 1.5 ml Nuclease-free water	
Regular pSCREEN T-Vector Kit	69917-3
• 2 µg pSCREEN T-Vector	
• pSCREEN T-Vector Positive Control Insert	
• 5 × 200 µl NovaBlue (DE3) Competent Cells	
• 4 × 2 ml SOC medium	
• Test Plasmid	
ColonyFinder Immunoscreening Kit	69283-3
• 125 ml Colony Denaturing Solution	
• 40 ml 10% Gelatin	
• 35 ml 20% Tween 20	



- 40 µl Goat Anti-Mouse IgG Alkaline Phosphatase Conjugate
- 1.5 ml each BCIP/NBT Color Development Substrates
- 20 ml 20X Alkaline Phosphatase Buffer
- 4 µg Positive Control Antibody (HSV•Tag monoclonal antibody)
- Positive Control Clone (glycerol stock)

pSCREEN Vector Primers

- 500 pmol T7 gene 10 primer
- 500 pmol T7 terminator primer





DNase Shotgun Cleavage

DNase I Digestion

The method is based on the observation that bovine pancreatic DNase I causes double strand scission of DNA in the presence of Mn^{2+} (1,2). Because cleavage is random and can be controlled by varying the enzyme concentration, temperature and/or incubation time, this method is very useful as the initial fragmentation step in the generation of representative libraries having virtually any insert size range. The following protocol uses a fixed amount of DNA with increasing dilutions of DNase I to find conditions that produce the desired fragment size range. For simplicity we recommend using 10 μ g of target DNA per reaction, which will produce more than enough fragments for several libraries (on a molar basis, assuming relatively small (<500 bp) insert sizes are desired). Because of the amount of target DNA required, it is usually most convenient to use plasmid DNA rather than purified insert DNA. Note that the target DNA must be fairly concentrated (~1.5–5 μ g/ μ l) for these reactions to keep the volume small for gel loading purposes. The target DNA must be free of all traces of Mg^{2+} , because this would cause single strand cleavage by the enzyme. Under these conditions, 1 μ l of DNase I diluted 1:200 or 1:300 usually gives the highest quantity of 50–150 bp fragments in preparation for cloning into the pSCREEN T-Vector.

An alternative approach may be more appropriate in cases where these quantities of target DNA are not readily available, with large target DNA that cannot be easily concentrated, or when large fragments are needed (which reduces the number of molecules produced on a molar basis). In these cases the test reactions can be scaled proportionately to find the appropriate enzyme:DNA ratio, and then the appropriate reaction performed again on a larger scale to produce enough material for cloning. When scaling up it is important to maintain the same DNA and enzyme concentrations, as well as ratios, as were used on the trial scale reactions. If larger volumes are used for scaled up reactions, we recommend extracting the reaction twice with phenol:CIAA (1:1) after adding the Stop Buffer. The DNA can then be precipitated with ethanol (as described below) to concentrate it prior to gel loading.

1. Immediately before use, dilute the DNase I with 1X Buffer + $MnCl_2$ as follows. First make 200 μ l dilution buffer by adding 20 μ l 10X DNase I Buffer (0.5 M Tris-HCl pH 7.5, 0.5 mg/ml BSA) and 20 μ l 10X $MnCl_2$ (100 mM) to 160 μ l sterile deionized H_2O . Make a 1:133 dilution by adding 1 μ l of DNase I to 132 μ l of this solution. Mix thoroughly by gently flicking the tube (avoid vigorous mixing, because DNase I is susceptible to surface denaturation). Add 30 μ l of this dilution to a tube containing 15 μ l buffer to obtain the 1:200 dilution. Repeat the same procedure twice more to obtain 1:300 and 1:450 dilutions.
2. Set up the following reactions (add all components except the enzyme in 0.5 ml microcentrifuge tubes at room temperature).

Sample	10X DNase I Buffer	10X $MnCl_2$	DNA	DNase I	H_2O
1.	0.9 μ l	0.9 μ l	10 μ g	1 μ l of 1:133	to 10 μ l
2.	0.9 μ l	0.9 μ l	10 μ g	1 μ l of 1:200	to 10 μ l
3.	0.9 μ l	0.9 μ l	10 μ g	1 μ l of 1:300	to 10 μ l
4.	0.9 μ l	0.9 μ l	10 μ g	1 μ l of 1:450	to 10 μ l

3. Start the reactions by adding the enzyme and mixing gently. Incubate at room temperature for exactly 10 minutes. Add 2 μ l (0.2 vol) 6X Stop Buffer to stop the reactions. The Stop Buffer contains 100 mM EDTA, 30% glycerol, and tracking dyes.
4. Samples may be analyzed by agarose gel electrophoresis. If small fragments (<1000 bp) are desired, 1 μ l samples should be analyzed on a 2% gel beside the PCR Markers (50, 150, 300, 500, 750, 1000, 1500 and 2000 bp). Use 5 μ l Markers per lane as supplied in loading buffer.

Fractionation of DNA Fragments

The method of fractionation and purification of the reaction products will depend on the fragment size range and personal preference. Any procedure giving good recovery



and purity can be used; however, it should be noted that small fragments (<200 bp) bind poorly to most commercial resins used for DNA purification. Therefore, we recommend recovering small fragments from agarose gels by electroelution, using low melting point agarose, or using a high-quality agarase that is available from several suppliers. The following protocol has been successfully used in conjunction with electroelution of 50–150 bp fragments with the Elutrap (Schleicher and Schuell).

1. Pool cleavage reactions containing fragments in the 50-150 bp size range and run on a 2% molecular biology grade agarose gel containing 0.5 µg/ml ethidium bromide. Up to 30 µg of DNA in this size range can be loaded into a 10mm wide comb in a gel that is 0.75–1 cm thick. Run the PCR Markers in an adjacent lane.
2. Excise the band corresponding to 50–150 bp fragments and electroelute the DNA following the device manufacturer's instructions.
3. Following elution, extract the sample sequentially with 1 volume TE-buffered phenol, 1 volume of phenol:CIAA (1:1; CIAA = chloroform:isoamyl alcohol 24:1), and 1 volume CIAA. Transfer the final aqueous phase to a fresh tube.
3. Optional: At this point the DNA can be further purified using an Elutip (Schleicher and Schuell) according to the manufacturer's instructions. We observe slightly better results (more efficient cloning) if this step is included.
4. Precipitate the DNA by adding 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. Leave on ice for 30 min. Centrifuge at 12,000 × g for 15 min. Drain carefully, rinse the pellet with 70% ethanol, dry, and resuspend DNA in 30 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Determine the DNA concentration by reading the A₂₆₀ of a 3 µl sample in 300 µl water (1A₂₆₀ unit = 50 µg/ml). Expect an overall recovery of about 20%, or 6 µg DNA if starting with 30 µg. If the average fragment size is 100 bp, 6 µg corresponds to about 100 pmol of DNA, or 200 pmol ends. The DNA is now ready for further treatment, such as Single dA Tailing, in preparation for cloning.

Single dA Tailing

The Single dA Tailing Kit module is for the preparation of DNA having any type of end for T-cloning. The kit supplies the reagents needed to blunt the target DNA ends and add a single 3' dA residue in consecutive reactions, without the need for precipitation. The products of the reaction are suitable for direct ligation into the pSCREEN T-Vector. The Positive Control 36mer in the kit is the blunt-ended HSV•Tag 36mer that encodes the 11 aa peptide recognized by the HSV•Tag monoclonal antibody, thus allowing the entire cloning and screening procedure to be verified.

DNase I digested fragments are treated in consecutive reactions to prepare for T-cloning. Following treatment with T4 DNA polymerase in the presence of all four dNTPs to ensure flush ends, a single 3' dA residue is added using *Tth* DNA polymerase. The *Tth* enzyme preferentially adds a dA residue even though all four dNTPs are present.

The starting DNA should be at a minimum concentration of 0.1 µg/µl in water or TE buffer. Other buffers have not been extensively tested; however, it is likely that most restriction enzyme buffers are compatible with T4 and *Tth* DNA polymerase activities for this purpose.

1. Assemble the following components in a microcentrifuge tube:
 - 1 µg DNA (up to 30 pmol of ends; this is equivalent to 1 µg of DNA with an average size of 100 bp)
 - 2.5 µl 10X Flush Buffer (0.5 M Tris-HCl pH 8.0, 50 mM MgCl₂, 1 mg/ml BSA)
 - 2.5 µl 10X dNTP Mix (1 mM each dCTP, dGTP, dTTP, 10 mM dATP)
 - 1.25 µl 100 mM DTT
 - 0.5 µl T4 DNA Polymerase (1–2 units)
 - Sterile deionized H₂O to 25 µl
2. Start the reaction by adding the enzyme. Mix gently by stirring with the pipet tip and incubate at 11 °C for 20 minutes. Stop the reaction by heating for 10 minutes at 75 °C.



3. Following the heat inactivation step, the entire flushing reaction is used for Single dA Tailing. In a microcentrifuge tube, combine:
 - 1 µg DNA (e.g., entire flushing reaction, 25 µl)
 - 8.5 µl 10X dA Tailing Buffer (100 mM Tris-HCl pH 9.0, 0.5 M KCl, 0.1% gelatin, 1% Triton X-100)
 - 0.5 µl *Tth* DNA Polymerase (1.25 units)
 - Sterile deionized H₂O to 85 µlStart the reaction by adding the enzyme and mixing gently with the pipet tip.
4. Incubate at 70 °C for 15 minutes.
5. Add 1 volume of CIAA, vortex vigorously for 60 seconds, and centrifuge at 12,000 × g for 1 minute. Transfer the aqueous phase to a fresh tube. If 1 µg DNA was used, the final concentration of DNA is 11.8 ng/µl. Store at -20 °C.

Positive Control 36mer Reaction

To monitor performance of test DNA, the Positive Control 36mer can be used in a parallel reaction. Following the reaction the fragment can be ligated into the pSCREEN T-Vector and transformed into NovaBlue(DE3) competent cells. The 36mer (shown below) encodes a sequence that will produce an in-frame peptide fusion capable of reacting with the HSV•Tag monoclonal antibody only if a single 3' dA is added.

The reaction is performed on a small scale to conserve reagents. Combine:

- 2 µl Positive Control 36mer (100 ng)
- 1.25 µl 10X dNTP Mix
- 4.25 µl 10X dA Tailing Buffer
- 1.25 µl 10X Flush Buffer
- 33.5 µl H₂O
- 0.3 µl *Tth* DNA Polymerase

Incubate and extract as above. The final DNA concentration is 2.35 ng/µl.

```
5' -CAGCCTGAACTCGCTCCAGAGGATCCGGAAGATTAA-3'  
3' -GTCGGACTTGAGCGAGGTCTCCTAGGCCCTTCTAATT-5'
```

Positive Control 36mer Sequence

Ligation to pSCREEN T-Vector

The pSCREEN T-Vector is designed for expression of inserts as stable fusion proteins driven by T7 RNA polymerase. It is in the pET family but contains a high copy number origin of replication for superior plasmid yields. The vector has been prepared by digestion with *EcoR* V followed by the addition of single 3' dT residues at each end. Inserts having single 3' dA overhangs can be ligated directly into the vector.

The T-cloning site in pSCREEN-1b(+) is placed such that target sequences are expressed as a fusion containing the first 260 aa of the T7 gene 10 protein (see map and cloning region sequence on pg 13). The fusion partner ensures high level expression and helps protect the target sequence from proteolytic degradation. Because the DNase Shotgun procedure creates randomly cleaved fragments, statistically one in six inserts will encode a protein corresponding to an open reading frame in the starting DNA.

The pSCREEN T-Vector Kit module contains the pSCREEN Positive Control T-Vector insert to monitor performance of cloning and immunoscreening procedures. The pSCREEN Positive Control T-Vector Insert produces an in-frame peptide capable of reacting with the HSV•Tag monoclonal antibody.

Inserts Processed with the Single dA Tailing Kit

DNAs having single 3' dA overhangs as produced by the Single dA Tailing reaction are suitable for direct ligation with the pSCREEN T-Vector.



1. For a standard reaction, 50 ng (0.025 pmol) of pSCREEN T-Vector is ligated with 0.2 pmol (50 ng of a 500 bp fragment) amplified product in a volume of 10 μ l. Assemble the following components in a 1.5 ml tube.
 - 1 μ l 10X Ligase Buffer (10X = 200 mM Tris-HCl pH 7.6, 100 mM MgCl₂, 250 μ g/ml acetylated BSA)
 - 0.5 μ l 100 mM DTT
 - 0.5 μ l 10 mM ATP
 - 1 μ l 50 ng/ μ l pSCREEN T-Vector
 - 0.5 μ l T4 DNA Ligase (2-3 Weiss units)
 - X μ l Target DNA (0.2 pmol; corresponds to ~6 ng of 100 bp DNA)
 - Y μ l water
 - 10 μ l total volume
2. Add the ligase last and gently mix by stirring with a pipet tip. Incubate at 16 °C 2 h to overnight.
3. To test the efficiency of ligation, use 2 μ l (5 ng) of the pSCREEN Positive Control T-Vector Insert provided with the kit in place of the target DNA in the above reaction. The molar ratio of insert to vector is about 10:1 under these conditions.

Transformation

NovaBlue(DE3) competent cells are provided in 0.2 ml aliquots. The standard transformation reaction calls for 20 μ l cells, so each tube contains enough cells for 10 transformations. Cells can be refrozen at -70 °C and reused; however, transformation efficiencies will decline several-fold with each freeze-thaw cycle. For optimal performance, dispense the cells into desired aliquots after the initial thaw to avoid multiple cycles.

1. Thaw the required number of tubes of cells on ice and mix gently to assure that the cells are evenly suspended. Place the required number of 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill.
2. Pipet 20 μ l aliquots of cells into the pre-chilled tubes.
3. (Optional) To determine transformation efficiency, add 1 μ l (0.2 ng) Test Plasmid to one of the tubes containing cells. Gently flick the tube to mix. Plate only 5 μ l of the final transformation mix (see below).
4. Add 1 μ l of ligation reaction directly to the cells. Stir gently to mix.
5. Place the tubes on ice for 30 min.
6. Heat the tubes for exactly 40 seconds in a 42 °C water bath; do not shake.
7. Place on ice for 2 min.
8. Add 80 μ l of room temperature SOC medium to each tube.
9. Shake at 250 rpm at 37 °C for 1 hour.
10. Spread 50 μ l* of each transformation on LB agar plates containing 50 μ g/ml carbenicillin (Cat. No. 69101-3)(or ampicillin) plus 15 μ g/ml tetracycline.
11. Let the plates sit on the bench for several minutes to allow excess liquid to be absorbed, and then invert and incubate overnight at 37 °C.

* The appropriate amount of transformation mixture to plate will vary with the efficiency of both the ligation and the competent cells. As little as 2 μ l will yield several hundred transformants under highly efficient conditions (e.g., with cells giving >4 x 10⁸ cfu/ μ g).

Screening

Because of the features required for T7 expression and gene 10 sequences, blue/white screening of recombinants is not possible with the pSCREEN vector. However, if the ligation conditions above are followed, more than 60% of the resulting colonies should contain inserts. The presence of inserts can be rapidly determined by direct colony PCR as described below. If antibodies or other detectable ligands that bind to the target gene product are available, it is possible to screen directly for the desired clone on colony lift filters. Reagents for colony immunoscreening with mouse antibodies are



included in the NovaTope Library Construction and Screening System and are available separately as the ColonyFinder Immunoscreening Kit (Cat. No. 69283-3).

Rapid Screening for Inserts by Colony PCR

Vector-specific primers that will amplify just the insert and immediate flanking sequences are the T7 gene 10 primer (Cat. No. 69341-3) and the T7 terminator primer (Cat. No. 69337-3). In the absence of an insert, this primer combination amplifies a 341 bp fragment. Using a vector-specific primer in combination with an insert-specific primer will also reveal the orientation of the insert.

1. Pick a pSCREEN colony from an agar plate using a 200 μ l pipet tip or sterile toothpick. Choose colonies that are at least 1mm in diameter and try to get as many cells as possible. If a "copy" of the colony is desired, touch the pipet tip to a plate before transferring the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a 1.5 ml tube containing 50 μ l of sterile water. Vortex to disperse the pellet.
3. Place the tubes in boiling water or a heat block at 99 °C for 5 minutes to lyse the cells and denature DNases.
4. Centrifuge at 12,000 \times g for 1 min to remove cell debris.
5. Transfer 10 μ l of the supernatant to a fresh 0.5 ml tube for PCR. Leave on ice until use.
6. Make a master reaction mix as follows:

Per reaction:

- 31.8 μ l sterile water
- 1 μ l dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
- 1 μ l 5' primer, 5 pmol/ μ l
- 1 μ l 3' primer, 5 pmol/ μ l
- 5 μ l 10X buffer (10X = 100 mM Tris-HCl pH 8.8 at 25 °C, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100)
- 0.25 μ l (1.25 U) AmpliTaq DNA polymerase (Perkin-Elmer)

Mix together the above components in a single tube using amounts corresponding to the number of reactions desired. (It is convenient to multiply the amounts by X.5, where X is the number of reactions, in order to account for pipetting losses.)

7. Add 40 μ l of the master mix to each sample, mix gently, add 2 drops of mineral oil, cap the tubes and put the samples in a thermal cycler (Perkin-Elmer). Process for 35 cycles for 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a final extension at 72 °C for 5 min.
8. To analyze the reaction products, first remove the oil overlay by adding 100 μ l of chloroform. Add 5 μ l of 10X loading dye to the top aqueous phase and load 10–25 μ l per lane on a 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide.

Notes:

- a. Novagen's primers are designed and sold for use in the Polymerase Chain Reaction (PCR) process covered by patents owned by Hoffmann-La Roche. Use of the PCR process requires a license. A license for research may be obtained by purchase and use of authorized reagents and DNA thermal cyclers from The Perkin-Elmer Corporation or by otherwise negotiating a license with Perkin-Elmer.
- b. As an optional step, a "hot start" procedure can be used in which the cell lysate samples are prewarmed to 80 °C before the addition of the master mix.



Colony Screening by Protein Expression

General Considerations

The number of clones required to achieve a given probability that a given sequence will be present in a gene library is $N = \ln(1-P)/\ln(1-1/n)$, where N = the number of clones required, P = the probability desired (e.g., 0.99), and $1/n$ = the fractional proportion of the total sequence represented by target sequence (3). In a pSCREEN library that will be screened by expression of target DNA, this number must be multiplied by a factor of 6 to account for 3 possible reading frames and 2 possible orientations.

For example, if a pSCREEN library having an average insert size of 100 bp is prepared from a 2000 bp cDNA clone, $[\ln(1-.99)/\ln(1-100/2000)] \times 6 = 540$ recombinants would need to be screened for a single epitope. In practice several plates having 1–2000 colonies each provide more than enough recombinants for screening with a given antibody.

In contrast, to scan a bacterial genome (3×10^7 bp) for epitopes with a library having an average insert size of 100 bp would require screening 8.4×10^6 clones. In this case it would be more practical to construct and screen an initial library having larger inserts, followed by the creation of secondary libraries with smaller inserts from positives isolated from the first library. For example, if the initial library had an average insert size of 1500 bp, 5.5×10^5 clones would need to be screened. This number is more feasible for the construction and screening procedures.

Immunoscreening

When using this system to detect epitopes it is important to note that in most cases only contiguous epitopes will be expressed. Conformation-dependent epitopes consisting of different protein domains will probably not be formed unless they are encoded on a single DNA fragment and fold properly in the bacterial host. Therefore, not all antibodies are suitable for mapping with this technique (or any other peptide fragmentation or synthetic method). Signal to noise ratios obtained with this procedure depend on several factors. First, the amount of antigen that is deposited on the filter varies with each fusion protein based on its expression level and stability in the host cells. (This system tends to minimize variability by producing a strongly expressed, stable gene 10 fusion protein.) Second, the quality (titer and affinity) of the screening antibody is of obvious importance. Generally, antibodies that produce good signals on Western blots are likely to produce good results in immunoscreening. Like conformation-dependent antibodies, those directed against carbohydrate determinants are not suitable for immunoscreening. Third, crude antisera and ascites occasionally contain IgG components that bind to *E. coli* proteins that can produce high backgrounds on colony lifts. The effect of these factors can be minimized by using the least amount of antibody that still gives a strong signal with a given amount of antigen and gives little reactivity with *E. coli* proteins. Various antibody dilutions can be easily tested by a dot blot assay using known amounts of antigen in parallel with similar amounts of *E. coli* extract (prepared by sonicating *E. coli* to break the cells and centrifuging to remove cell debris). In general, dilutions from 1:200 to 1:10,000 are appropriate for antisera, ascites fluids, or purified antibodies, whereas hybridoma tissue culture supernatants usually require dilutions from 1:10 to 1:100.

If non-specific background due to cross-reactivity with filter-bound *E. coli* proteins continues to be a problem, the antibody preparation can be preabsorbed with an *E. coli* lysate. As a starting point, incubate 1 mg/ml extract with antisera diluted 1:1000 for 30 minutes prior to incubating the antisera with filters. The amount of extract and the antibody dilution can be varied to optimize signal to noise ratios.

The recommended colony immunoscreening procedure is described below. Reagents for this method are contained in the ColonyFinder Immunoscreening Kit (Cat.# 69283-3). This procedure can be modified for screening with other ligands, and the conditions may need to be optimized for particular applications. For this purpose it is useful (when feasible) to clone the entire target sequence into pSCREEN such that the domain of interest is expressed, and use this clone as a positive control in the optimization experiments.



Reagents for 50 filters (82 mm diameter):

125 ml	Colony Denaturing Solution (20 mM Tris-HCl pH 7.9, 6 M urea, 0.5 M NaCl)
40 ml	10% Gelatin
35 ml	20% Tween 20
40 µl	Goat anti-Mouse IgG Alkaline Phosphatase conjugate
20 µl	Positive Control Antibody (HSV•Tag monoclonal antibody)
	Positive Control Clone (expresses HSV•Tag epitope), glycerol stock
1.5 ml	BCIP, 42 mg/ml
1.5 ml	NBT, 83 mg/ml
20 ml	20X Alkaline Phosphatase Buffer

The T7 RNA polymerase carried by host strain NovaBlue(DE3) drives the expression of fusion proteins from pSCREEN plasmids. Under most conditions these proteins accumulate as insoluble inclusion bodies. Although the T7 RNA polymerase is under *lacUV5* control, substantial amounts of protein are produced even in the absence of IPTG induction. Inclusion bodies appear to provide the advantage of sequestering expressed proteins so that they are less susceptible to proteolytic breakdown, and so that potentially harmful products do not affect cell viability.

1. Plate transformants in NovaBlue(DE3) at the desired density (up to 10,000 on an 82 mm plate; usually 1–2000 is recommended) on LB plates containing 50 µg/ml carbenicillin and 15 µg/ml tetracycline. The approximate number of colonies obtained per microliter of transformation should be known from initial plating experiments. We recommend screening fresh transformants to minimize the probability of biasing the library due to disproportionate growth rates of individual clones during amplification. Colonies are ready for screening even when small (0.5–1 mm in diameter), this size is usually reached within 17–18 h at 37 °C. Chill plates at 4 °C for one-half hour prior to making colony lifts so that the agar will not stick to the nitrocellulose.
2. Carefully overlay the plates with nitrocellulose filters (e.g., Schleicher and Schuell BA85). Wear gloves and handle the filters by the edges. Bend slightly and allow the center to touch the plate first. Filters may be marked by poking an 18 gauge needle in 3 asymmetric places into the filter and plate. If desired, needles may be dipped into waterproof ink before stabbing into the filter and plate. Number both the filter and the plate. After one minute of contact, carefully peel the filter off of the plate. Colonies should stick to the filter, however, we have observed that sufficient protein is deposited so that strong signals are still produced even if colonies do not remain on the filter. Plates may be returned to 37 °C for several hours to regenerate colonies.
3. Lyse the colonies by putting them into a chloroform vapor chamber. In a fume hood, place paper towels in a large glass or Pyrex dish. Lightly dampen the paper towels with water. Put the filters (colony side up) on the paper towels. In a small beaker, place a few Kimwipes or other absorbent paper material and soak with chloroform. Cover dish tightly with Saran wrap and leave at room temperature for 15 min.
4. Remove the filters from the chamber and place colony side up on a piece of Whatman 3MM paper saturated with Colony Denaturing Solution. Use just enough of the solution to fully saturate the paper, because too much liquid will result in smearing of the signal. If done in a Petri dish this will require ~2.5 ml of solution per filter. Leave in contact with the solution for 15 min at room temperature.
5. Perform the following incubations and washes at room temperature with gentle agitation. Filters may be placed in petri dishes (up to 5 per dish, given adequate agitation). Liquefy the 10% gelatin (supplied) by brief heating in a water bath or microwave oven. Block non-specific protein binding sites by immersing filters in TBST + 1% gelatin for 30 minutes (use about 5 ml/filter). Most colonies will fall off the filters as soon as they are put in the blocking solution. TBST is 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20. It can be prepared as a 10X stock containing 0.02% sodium azide and stored at room tem-



- perature.
6. Wash with TBST (without gelatin) twice for 15 minutes each. Remove any colony debris by rubbing filter with a Kimwipe. Failure to remove colony debris results in a combination of high background and low signal and thus positives cannot be distinguished from background.
 7. Incubate filters with primary antibody diluted in TBST for 30 minutes (5 ml per filter; less can be used if filters are incubated together). If using the Positive Control Clone, dilute the Positive Control HSV•Tag Antibody 1:2000 to 0.1 µg/ml). Many diluted antibodies can be used at least 4 times within a 2 week period, if they are preserved with 0.05% sodium azide and stored at 4 °C.
 8. Remove antibody solution and wash filters three times with 15–20 ml TBST per filter for 10 minutes each.
 9. Incubate filters for 30 minutes with Goat anti-Mouse IgG Alkaline Phosphatase conjugate diluted 1:10,000 in TBST. This diluted antibody conjugate can also be reused up to 4 times. Add sodium azide to 0.05% and store at 4 °C.
 10. Remove the antibody solution and wash filters three times with 15–20 ml TBST for 10 minutes each.
 11. After the last wash, place the filters on paper towels to absorb excess liquid, but do not allow them to dry out. Dilute the 20X stock of Alkaline Phosphatase Buffer to 1X with water and add 4 µl/ml of each of the NBT and BCIP Color Development Substrates. Transfer the filters to Petri dishes and add enough color development solution to cover each filter completely (about 6 ml). Strong signals generally develop within 1–10 minutes. Incubate until the background of the filters shows all of the colonies. This will make it easier to identify the original positive colony to rescreen. To stop color development, rinse the filters several times in water and allow to air dry. If colony density of the plate is high, it will be necessary to rescreen the colonies to obtain an isolated, pure colony. Restreak from the positive colony area to fresh plates. Rescreen the following day using the above protocol, and reuse the antibody solutions.

Plasmid Preparation and Sequencing

The pSCREEN-1b(+) vector can be used for double-stranded dideoxy sequencing of supercoiled plasmid DNA obtained using standard miniprep procedures, which is the simplest method and produces satisfactory results using T7 DNA polymerase, *Taq* DNA polymerase, Klenow fragment or reverse transcriptase as the enzyme. The plasmid has a very high copy number (pUC-based origin of replication) and sufficient quality and quantities of DNA are produced from minipreps in the NovaBlue(DE3) host. The following mini-prep protocol is a slight modification of one presented in reference 3, and works very well for pSCREEN plasmids in NovaBlue(DE3).

1. Using a sterile loop, toothpick or pipet tip, transfer a well-isolated colony into 3 ml of LB broth supplemented with 50 µg/ml carbenicillin in a Falcon 2059 culture tube. Cap loosely and incubate with shaking at 37 °C for 6 hours to overnight.
2. Transfer 1.5 ml of culture into a 1.5 ml microcentrifuge tube and centrifuge at 12,000 × g for 1 min.
3. Remove the medium by aspiration, leaving the pellet as dry as possible.
4. Resuspend the cells in 100 µl of ice-cold 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA. Pipet up and down to make sure that the pellet is completely suspended.
5. Add 200 µl of freshly prepared 0.2 N NaOH, 1% SDS. Mix by inversion and let sit on ice for 3 min.
6. Add 150 µl of ice-cold 3 M NaOAc, pH 5.2. Mix by inversion and leave on ice for 5 min.
7. Centrifuge at 12,000 × g for 5 min. Transfer the clear supernatant to a fresh



- tube, avoiding the pellet, which tends to break up easily. Spin again if too much particulate matter remains in the supernatant.
8. Add 400 μ l phenol:CIAA (1:1), vortex for 30 seconds, and centrifuge at 12,000 \times g for 1 min at room temperature.
 9. Transfer the top aqueous phase to a fresh tube and add 800 μ l ethanol. Vortex, leave at room temperature for 2 min, and centrifuge at 4 $^{\circ}$ C, 12,000 \times g for 5 min.
 10. Decant the supernatant and add 400 μ l ethanol to the pellet. Spin briefly, pour off the ethanol and allow the pellet to air dry in an inverted position for about 10 minutes.
 11. Resuspend the pellet in 30 μ l of TE buffer containing 20 μ g/ml RNase and incubate at 37 $^{\circ}$ C for 15 min. Expect a yield of about 5–8 μ g plasmid DNA.
 12. At this point the DNA can be analyzed by restriction digestion, etc., but it should be further processed to remove RNA breakdown products before sequencing the double stranded plasmid. This can be simply accomplished by precipitation with polyethylene glycol. Add 10 μ l of 30% PEG-8000, 1.5 M NaCl (autoclave this solution before use to remove possible DNase contamination), vortex thoroughly, and incubate on ice for 60 min.
 13. Centrifuge at 12,000 \times g at 4 $^{\circ}$ C for 10 min. Carefully remove the supernatant, leaving the small transparent DNA pellet behind. Rinse the pellet successively with 70% ethanol and then 100% ethanol as above, and let air dry.
 14. Resuspend the DNA in 20 μ l TE. The plasmid is now suitable for alkali denaturation and double stranded sequencing. Detailed protocols for sequencing with double stranded and single stranded templates are available from many manufacturers of sequencing kits. References 4–6 are also useful. In most cases, inserts are small enough to allow complete sequencing of both strands using the T7 gene 10 primer and T7 terminator primer. Analysis of a collection of positive clones should reveal a common segment that encodes the target epitope.

References

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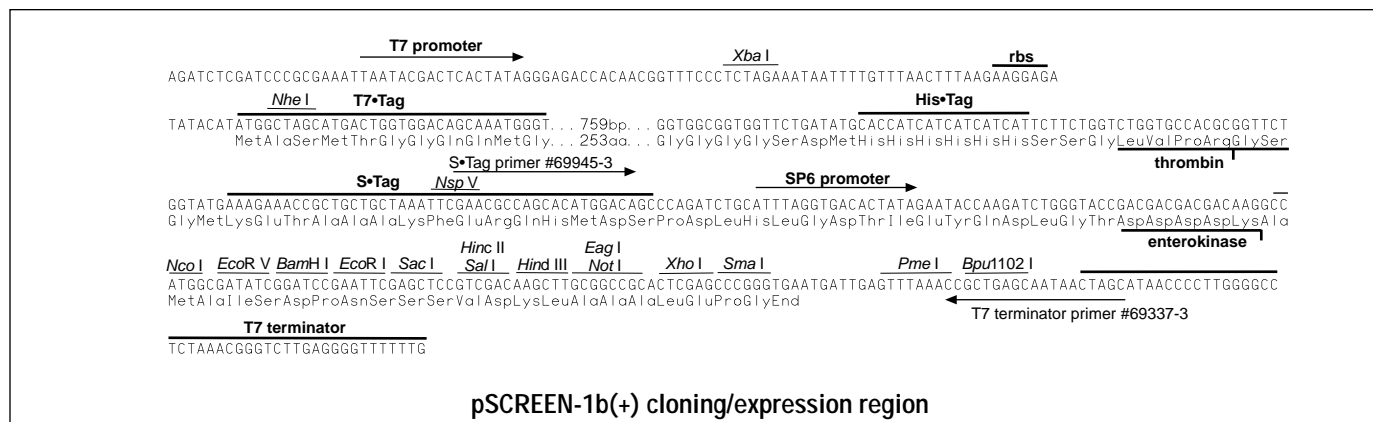
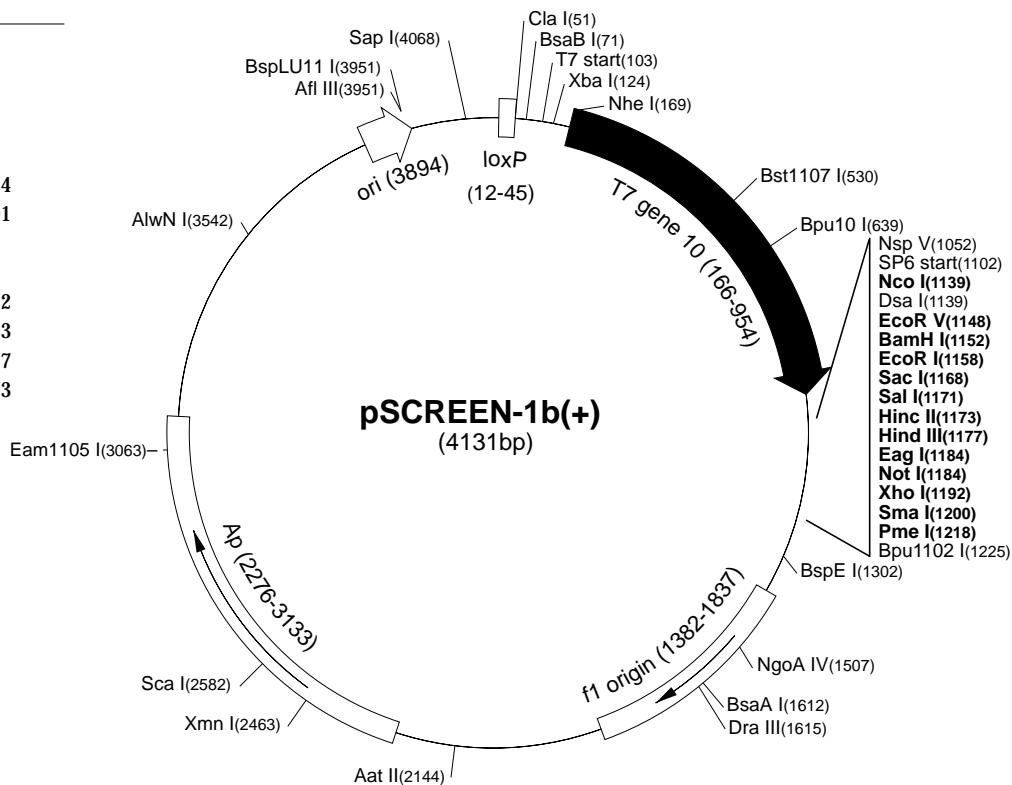


pSCREEN-1b(+) Vector

pSCREEN[™]-1b(+) is the plasmid product of *loxP*-cre mediated autosubcloning from the λSCREEN-1 vector (pSCREEN-1b(+) is also available separately). Like pEX/*lox*[®](+) and pTOPE[®]-1b(+), it contains the T7 gene 10 260aa coding sequence, T7 transcription and translation signals, f1 origin of replication and high copy number pUC replication origin. However, pSCREEN-1b(+) also contains His•Tag[®] and S•Tag[™] coding sequences and SP6 promoter immediately upstream from an expanded multiple cloning region. Unique sites are shown on the circle map below. The f1 origin is oriented so that infection with helper phage will produce virions containing single stranded DNA that corresponds to the T7 RNA polymerase coding strand. Therefore, single stranded sequencing should be performed using the T7 terminator primer.

pSCREEN-1b(+) sequence landmarks

<i>loxP</i> site	12-45
T7 promoter	86-102
T7 transcription start	103
T7 gene 10 coding seq.	166-954
His•Tag coding sequence	979-996
S•Tag coding sequence	1030-1074
SP6 promoter	1085-1101
SP6 transcription start	1102
Multiple cloning region (<i>Nco</i> I- <i>Pme</i> I)	1139-1222
T7 terminator	1236-1283
f1 origin	1382-1837
<i>bla</i> coding sequence	2276-3133
pUC origin	3894





pSCREEN-1b(+) Restriction Sites

Enzyme	# Sites	Locations	Enzyme	# Sites	Locations	Enzyme	# Sites	Locations			
AatII	1	2144	DraI	4	1218 2485 3177 3196	Sau96I	8	1252 1330 1618 2083 2699			
AccI	2	529 1172	DraIII	1	1615	2921	2938	3017			
AccIII	4	1768 2017 2759 3999	DrdI	3	1659 1980 3849	Sau3AI	22				
AcII	55		DrdII	3	506 968 1607	Scal	1	2582			
AflIII	1	3951	Dsal	1	1139	ScrFI	14				
AluI	16		EaeI	3	1184 2670 4112	SfaNI	9	818 1833 1873 1909 2003			
AlwI	14		EagI	1	1184	2362	2611	2802 3854			
Alw21I	5	1168 1898 2395 2480 3641	Eam1105I	1	3063	Sfcl	6	98 1097 1389 2817 3495			
Alw44I	3	1894 2391 3637	EarI	3	1320 2264 4068	3686					
AlwNI	1	3542	Ecil	3	2907 3735 3881	Smal	1	1200			
ApaBI	1	1894	Eco57I	2	2397 3409	Sspl	3	1796 1820 2258			
ApoI	3	1048 1158 1806	EcoO109I	2	1252 2083	StyI	3	204 1139 1247			
AvaI	2	1192 1198	EcoRI	1	1158	TaqI	13				
Avall	2	2699 2921	EcoRII	4	1310 3790 3803 3924	TaqII	8	532 1588 1710 2361 2378			
BamHI	1	1152	EcoRV	1	1148	2531	2716	4055			
BanI	6	5 940 1008 1117 1571	FauI	7	84 1340 1404 1473 1956	TfiI	4	547 813 3977 4117			
3110			1966	4111		Thal	17				
BanII	3	1168 1199 1541	FokI	5	580 1981 2624 2911 3092	Tsel	21				
BbvI	21		FspI	2	1361 2840	Tsp45I	7	297 790 1090 1434 2014			
BccI	9	327 560 570 982 1605	GdiII	3	1184 2670 4112	2590	2801				
1622			HaeI	4	1138 3477 3929 3940	Tsp509I	11				
Bce83I	5	1289 2459 3327 3568 3866	HaeII	4	1457 1465 3711 4081	Tth1111I	4	442 3328 3360 3367			
BceffI	3	482 1585 3452	HaeIII	16		UbaJI	13				
Bcgl	6	679 713 1162 1196 2525	Hgal	6	505 1390 1973 2531 3261	VspI	3	85 2888 4123			
2559			3839			XbaI	1	124			
Bfal	8	59 125 170 1236 1459	HgiEI	4	453 930 1889 3369	XhoI	1	1192			
2870			HhaI	22		XmnI	1	2463			
BglI	2	1371 2945	Hin4I	10	53 304 518 530 592						
BglIII	3	66 1077 1110	733	803 1150 2988 3062							
Bpml	3	248 315 2994	HincII	1	1173	Enzymes that do not cut pSCREEN-1b(+):					
Bpu10I	1	639	HindIII	1	1177	AflIII	Agel	Apal	AscI	AvrII	BaeI
Bpu1102I	1	1225	Hinfl	10	92 547 813 1660 1682	BbsI	BclI	Bmgl	BseRI	BsgI	BsmI
BsaI	4	99 463 655 2997	3064	3581 3977 4052 4117	BsmFI	BspGI	BspMI	BsrGI	BssHII		
BsaAI	1	1612	HphI	14		BstEII	BstXI	Bsu36I	Eco47III	EcoNI	FseI
BsaBI	1	71	KpnI	3	9 944 1121	HpaI	MluI	MscI	MunI	NarI	NruI
BsaHI	2	2141 2523	Maell	15		Nsil	Pacl	PmlI	PshAI	Psp5II	PstI
BsaJI	6	1 204 1139 1198 1247	MaellI	15		PvuII	RleAI	RsrII	SacII	SexAI	SfiI
3791			MbolI	9	989 1337 1473 2281 2390	SgfI	SgrAI	SnaBI	SpeI	SphI	SrfI
BsaWI	5	943 1302 2767 3598 3745	2468	3223 3294 4085	Sse8387I	StuI	SunI	Swal	Tth1111I	XcmI	
BsaXI	2	1664 4097	MmeI	3	1637 3558 3742						
Bsbl	2	1705 1955	MnlI	25							
BscGI	8	1264 1518 1977 2515 3037	MseI	22							
3061			MslI	4	1088 2292 2651 2810						
Bsil	4	737 2087 2394 3778	MspI	20							
BsiEI	7	1187 1342 1581 2545 2694	MspA1I	8	335 928 1039 1224 1961						
3617			2427	3368 3613							
BsII	15		MwoI	22							
BsmAI	7	99 463 655 2026 2068	NciI	10	3 392 1199 1200 1991						
2221			2026	2527 2549 2878 3574							
BsmBI	2	2026 2068	NcoI	1	1139						
BsoFI	34		NdeI	2	164 1889						
Bsp24I	10	176 208 441 473 2131	NgoAIV	1	1507						
2163			NheI	1	169						
Bsp1286I	7	1168 1199 1541 1898 2395	NlaIII	13							
2480			NlaIV	17							
BspEI	1	1302	NotI	1	1184						
BspLU11I	1	3951	NspI	2	2038 3955						
BsrI	11		NspV	1	1052						
BsrBI	4	346 1468 2221 4022	Pfi1108I	2	512 3044						
BsrDI	2	2829 3003	PfiMI	2	1065 1113						
BsrFI	3	592 1507 2978	PleI	6	86 1668 1676 3072 3575						
Bst1107I	1	530	4060								
BstYI	10	66 1077 1110 1152 2416	PmeI	1	1218						
2433			Psp1406I	4	887 1825 2461 2834						
Cac8I	13		PvuI	2	1342 2694						
CjeI	22		RcaI	3	2118 2223 3231						
CjePI	22		RsaI	9	7 198 291 320 645						
Clal	1	51	942	1119 1906 2582							
CviJI	64		SacI	1	1168						
CviRI	12		Sall	1	1171						
Ddel	16		SapI	1	4068						
DpnI	22										



pSCREEN-1b(+) Sequence

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151 GAAGGAGATA  TACATATGGC  TAGCATGACT  GGTGGACAGC  AAATGGGTAC
201 TAACCAAGGT  AAAGGTGTAG  TTGCTGCTGG  AGATAAACTG  GCGTTGTCTT
251 TGAAGGTATT  TGCGCGTGAA  GTCCTGACTG  CGTTCGCTCG  TACCTCCGTG
301 ACCACTTCTC  GCCACATGGT  ACGTTCCATC  TCCAGCGGTA  AATCCGCTCA
351 GTTCCCTGTT  CTGGGTCGCA  CTCAGGCAGC  GTATCTGGCT  CCGGGCGAGA
401 ACCTCGACGA  TAAACGTAAG  GACATCAAAC  ACACCGAGAA  GGTAATCACC
451 ATTGACGGTC  TCCTGACGGC  TGACGTTCTG  ATTTATGATA  TTGAGGACGC
501 GATGAACCAC  TACGACGTTT  GCTCTGAGTA  TACCTCTCAG  TTGGGTGAAT
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1301 ATCCGGATAA  CCTGGCGTAA  TAGCGAAGAG  GCCCGCACCG  ATCGCCCTTC
1351 CCAACAGTTG  CGCAGCCTGA  ATGGCGAATG  GACGCGCCCT  GTAGCGGCGC
1401 ATTAAGCGCG  GCGGGTGTGG  TGGTTACGCG  CAGCGTGACC  GCTACACTTG
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1551 GTTCCGATTT  AGAGCTTTAC  GGCACCTCGA  CCGCAAAAAA  CTTGATTTGG
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1951 GCCAACACCC  GCTGACGCGC  CCTGACGGGC  TTGTCTGCTC  CCGGCATCCG
2001 CTTACAGACA  AGCTGTGACC  GTCTCCGGGA  GCTGCATGTG  TCAGAGGTTT
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pSCREEN-1b(+) Sequence, cont'd

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2201 ATACATTCAA ATATGTATCC GCTCATGAGA CAATAACCCT GATAAATGCT
2251 TCAATAATAT TGAAAAAGGA AGAGTATGAG TATTCAACAT TTCCGTGTCTG
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2351 GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT
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