STOP

Before using this product, please read the Limited Use License statement below:

Important Limited Use License information for pCpGfree-siRNA Kit

The purchase of the pCpGfree-siRNA Kit conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes.

The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes.

Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic, or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research.

If the purchaser is unwilling to accept the limitations of this limited use statement, InvivoGen is willing to accept return of the product with a full refund. The product must be returned in resaleable condition. For information on purchasing a license to this product for purposes other than research, contact InvivoGen, 3950 Sorrento Valley Blvd. Suite 100, San Diego, CA 92121. Tel: 858-457-5873 Fax: 858-457-5843.

pCpGfree-siRNA

A CpG-free plasmid specifically designed for the production of siRNAs *in vivo*

Catalog # kcpgf-sirna

For research use only

Version # 14L08-MM

PRODUCT INFORMATION

Content:

• 50 µg **pCpGfree-siRNA** plasmid provided as lyophilized DNA
• 20 µg **pCpGfree-siRNA-Scramble,** a control plasmid expressing a scramble sequence.
si-Scr sequence: GCATATGTGCGTACCTAGCAT
• 1 disk of lyophilized **GT115**
GT115 genotype: *F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZM15 ΔlacX74 recA1 endA1 Δdcm uidA::pir-116 ΔsbcC-sbcD*
• 10 µg of lyophilized **OL559 sequencing primer** (forward)
OL559 sequence: 5' GATAAGTAACTTGACCTAAGTG 3' (22 mer)
MW: 7072 g/mol. Tm: 57.1˚C
• 10 µg of lyophilized **OLSI3' sequencing primer** (reverse)
OLSI3' sequence: 5' GCATTCTAGTTGTGGTTTGTCC 3' (22 mer)
MW: 6738 g/mol. Tm: 58.4˚C
• 4 pouches of **Fast-Media® Zeo XGal**

Storage and Stability:

- Products are shipped at room temperature.
- Upon receipt, resuspend lyophilized DNA and store at -20°C. Avoid repeated freeze-thaw cycles.
- Store the disk of lyophilized GT115 *E. coli* at -20 °C.
- Store sequencing primers at -20˚C.
- Store Fast-Media® Zeo XGal at room temperature. Fast-Media® pouches are stable 18 months when stored properly.

Quality control:

- Plasmid construct has been confirmed by restriction analysis and sequencing.
- Plasmid DNA was purified by ion exchange chromatography and lyophilized.

GENERAL PRODUCT USE

InvivoGen has developed a new family of plasmids that are completely devoid of CpG dinucleotides. These plasmids yield high levels of transgene or shRNA expression both *in vitro* and *in vivo*, and in contrast to CMV-based plasmids allow sustained expression *in vivo*. pCpGfree plasmids contain elements that naturally lack CpG dinucleotides, were modified to remove all CpGs, or entirely synthesized such as genes encoding selectable markers or reporters.

pCpGfree-siRNA is a plasmid that combines the backbone of the pCpGfree plasmids with the siRNA expression cassette of the psiRNA plasmids. It is designed for long lasting expression of siRNAs *in vivo* as the plasmid does not induce inflammatory responses and gene silencing by methylation in vertebrate hosts.

PLASMID FEATURES

All the elements required for replication and selection of the plasmid in *E. coli* and gene expression in mammalian cells are completely devoid of CpG dinucleotides.

Elements for expression in *E. coli*

• Origin of replication: The *E. coli* R6K gamma ori has been modified to remove all CpGs. This origin is activated by the R6K specific initiator protein π, encoded by the *pir* gene[1].
• Bacterial promoter: E2MK is a CpG-free version of the bacterial EM7 promoter that drives the expression of the resistance gene.
• Selectable marker: The Zeocin™ resistance gene is a small gene (<400 bp) that contains numerous CpG dinucleotides. A synthetic new allele was created that contains no CpGs.

Elements for expression in mammalian cells

• 7SK prom: 7SK is an abundant and evolutionarily conserved small nuclear RNA discovered in the mid-70s. It is transcribed by RNA polymerase III from one or more genes belonging to a family of interspersed repeats in the mammalian genome. The high conservation and abundance of 7SK suggest an important physiological function of this RNA. Recent studies suggest that 7SK acts as a negative regulator of the RNA polymerase II elongation factor P-TEFb, and that this activity is influenced by stress response pathways. The human 7SK promoter presents a strong permissivity for the nucleotide in the +1 position and recognizes a cluster of 4 or more T residues as a termination signal. This promoter is ideal for the production of shRNAs as it can generate high amounts of shRNAs.
The human 7SK promoter has been modified to remove all CpGs and coupled with the mouse CMV enhancer, also modified to remove all CpGs, to increase its activity[2].
• EM7-alpha-peptide: This bacterial expression cassette enables white/blue selection. Transformation of permissive bacterial strains, such as strains harboring the *lacZΔM15* allele, leads to alpha-complementation which results in the production of active β-galactosidase (LacZ). In such transformants, LacZ catalyses the hydrolysis of X-Gal to produce a blue precipitate.
• MAR: Matrix attached regions (MARs) are sequences typically AT-rich that are able to form barriers between independently regulated domains[3]. pCpG plasmids contains two MARs, from the 5' region of the human IFN-ß gene or ß-globin gene that were chosen because they are naturally CpG-free. The MARs are placed between the bacterial and mammalian transcription units.

References:

1. Wu F. *et al.,* 1995. A DNA segment conferring stable maintenance on R6K gamma-origin core replicons. J Bacteriol. 177(22):6338-45.
2. Xia XG. *et al.* 2003., An enhanced U6 promoter for synthesis of short hairpin RNA. Nucleic Acids Res. 31(17):e100.
3. Bode J. *et al.*, 1996. Scaffold/matrix-attached regions: topological switches with multiple regulatory functions. Crit Rev Eukaryot Gene Expr. 6(2-3):115-38.

TECHNICAL SUPPORT
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METHODS

Plasmid resuspension

Quickly spin the tube containing the lyophilized plasmid to pellet the DNA. Resuspend DNA in the appropriate amount of sterile H_2O to obtain a plasmid solution at 1µg/µl. Store resuspended plasmid at -20 °C.

Plasmid amplification

pCpGfree-siRNA is a low yield plasmid DNA. In order to enhance the yield, we suggest to increase the quantity of DNA of your culture before using a column. To do so, prepare a larger culture than recommended in the protocol provided by the manufacturer (for example instead of preparing 250 ml, prepare 2 liters). Lyse the culture and precipitate the DNA with isopropanol. Resuspend the pellet in the volume indicated by the protocol and treat with RNAse before using the column. Then follow the manufacturer's protocol.

Plasmid preparation

• Cloning with Bbs I

Digest pCpGfree-siRNA plasmid with Bbs I (NEB, 2 units enzyme/µg plasmid DNA). Elute the large fragment (2926 bp) using a 0.7% low-melting agarose gel, and if necessary dilute the purified DNA fragment to obtain a solution at 0.1 µg/µl.

siRNA sequence design

- With the help of the **siRNA Wizard** (www.sirnawizard.com), choose a sequence of 19-21 nucleotides within the coding region of the gene of interest. The **siRNA Wizard** offers two search options: the "Standard Search" uses default criteria to analyze your gene of interest whereas the "Advanced Search" lets you manually set the selection criteria.

The first nucleotide of the siRNA sequence can either be an A or a G. Sequences that contain 4 or 5 consecutive As or Ts should be avoided, as they might act as termination signals for polymerase III.

- Using the **siRNA Wizard**, design two complementary oligonucleotides compatible Bbs I /Bbs I.

Cloning into pCpGfree-siRNA

1- Annealing of siRNA insert

- Dissolve the oligonucleotides (forward and reverse) at a concentration of 100 µM.
- Prepare the annealing solution by mixing the following components:

Forward oligonucleotide (100 µM)	2 µl
Reverse oligonucleotide (100 µM)	2 µl
0.5 M NaCl	6 µl
H_2O to a final volume of	30 µl

- Incubate 2 minutes at 80 °C then stop the heating and maintain in water bath until the temperature reaches 35 °C.
- Use annealed siRNA insert immediatly or store at -20 °C for further use.

Note: Purification of the annealed oligonucleotide solution is not necessary for efficient ligation of the siRNA insert.

2- Ligation of siRNA insert into pCpGfree-siRNA

- Prepare the ligation solution by mixing the following components:

Digested pCpGfree-siRNA	1 µl (100 ng)
Annealed siRNA insert	1 µl
T4 DNA Ligase	1 µl (1 unit)
10X ligation buffer	2 µl
H_2O to a final volume of	20 µl

- Incubate the mixture at 16 ºC overnight (or at 27 °C for 2 hours).

Reconstitution of *E. coli* GT115 strain

Use sterile conditions to do the following:

- Reconstitute *E. coli* GT115 by adding 1 ml of LB medium in the tube containing the paper disk. Let sit for 5 minutes. Mix gently by vortexing for 1-2 minutes.
- Streak bacteria taken from this suspension on a LB agar plate.
- Place the plate in an incubator at 37 °C overnight.
- Isolate a single colony and grow the bacteria in LB or TB medium.
- Prepare competent cells utilizing protocol of choice.

Note: Alternatively, we recommend the use of ChemiComp GT115, frozen chemically competent E. coli cells.

3- Transformation of GT115

We recommend using *E. coli* GT115 strain, a *sbcCD* deletion mutant, which is more compatible with hairpin harboring plasmids than standard laboratory strains which are $sbcCD^+$.

- Place the appropriate number of competent cells on ice (100 µl per ligation or transformation reaction).

Note: If competent cells are frozen, allow the cells to thaw on ice for 2-5 minutes.

- Introduce 10 µl of ligation product (or 1 µg supercoiled plasmid DNA) in pre-chilled 1.5 ml tubes and return tubes to ice.
- Gently flick the cells twice to homogenize and add 100 µl of cells to each DNA-containing tubes.
- Mix by tapping gently and place in ice immediately.
- Incubate the tubes in ice for 30 minutes.
- Incubate the tubes in a 42 °C water bath for exactly 30 seconds, then place the tubes back in ice for 1-2 minutes.
- Add 900 µl of room temperature SOC (or LB) medium to each reaction. (Practice sterile techniques to avoid contamination.)
- Incubate tubes at 37˚C for 1h30 with shaking at 250 rpm.
- Spread each transformation reaction (150 µl if using a ligation product or 100 µl of 10^{-1} and 10^{-2} dilutions if using a supercoiled plasmid) onto agar plate prepared with Fast-Media® Zeo XGal, to take advantage of the white/blue selection.

Notes: The white/blue selection will allow you to discriminate between blue parental clones and white recombinant clones.

To increase the number of transformants, spread the remaining transformed cells first concentrated by low speed centrifugation.

- Incubate plates at 37 ˚C overnight.

Note: The blue/white selection is more visible after 24h incubation.

4- DNA extraction and sequencing of the siRNA insert

- Extract plasmid DNA from 5 white clones.
- Confirm the presence of the siRNA insert on a 3% agarose gel (NuSieve GTG FMC type) after digestion with Spe I and Nhe I (2h at 37°C).

pCpGfree-siRNA containing a siRNA insert will generate two bands: 2619 bp and 363 bp (for an siRNA insert consisting of a 21 bp stem and 7 bp loop).

- Verify the sequence of the siRNA insert of 3 positive clones using the sequencing primers provided: OL559 (forward primer) and OLSI3' (reverse primer).

Transfection of mammalian cells

- Transfect your cell line with a recombinant plasmid containing the expected siRNA insert using standard transfection procedures.

Note: We recommend using LyoVec™, a transfection reagent optimized for pCpGfree-siRNA.

Analysis of siRNA-induced silencing

siRNA-induced silencing of your gene of interest can be determined by using different techniques, such as Northern analysis or quantitative RT-PCR at the RNA level, or Western Blot at the protein level.

TECHNICAL SUPPORT
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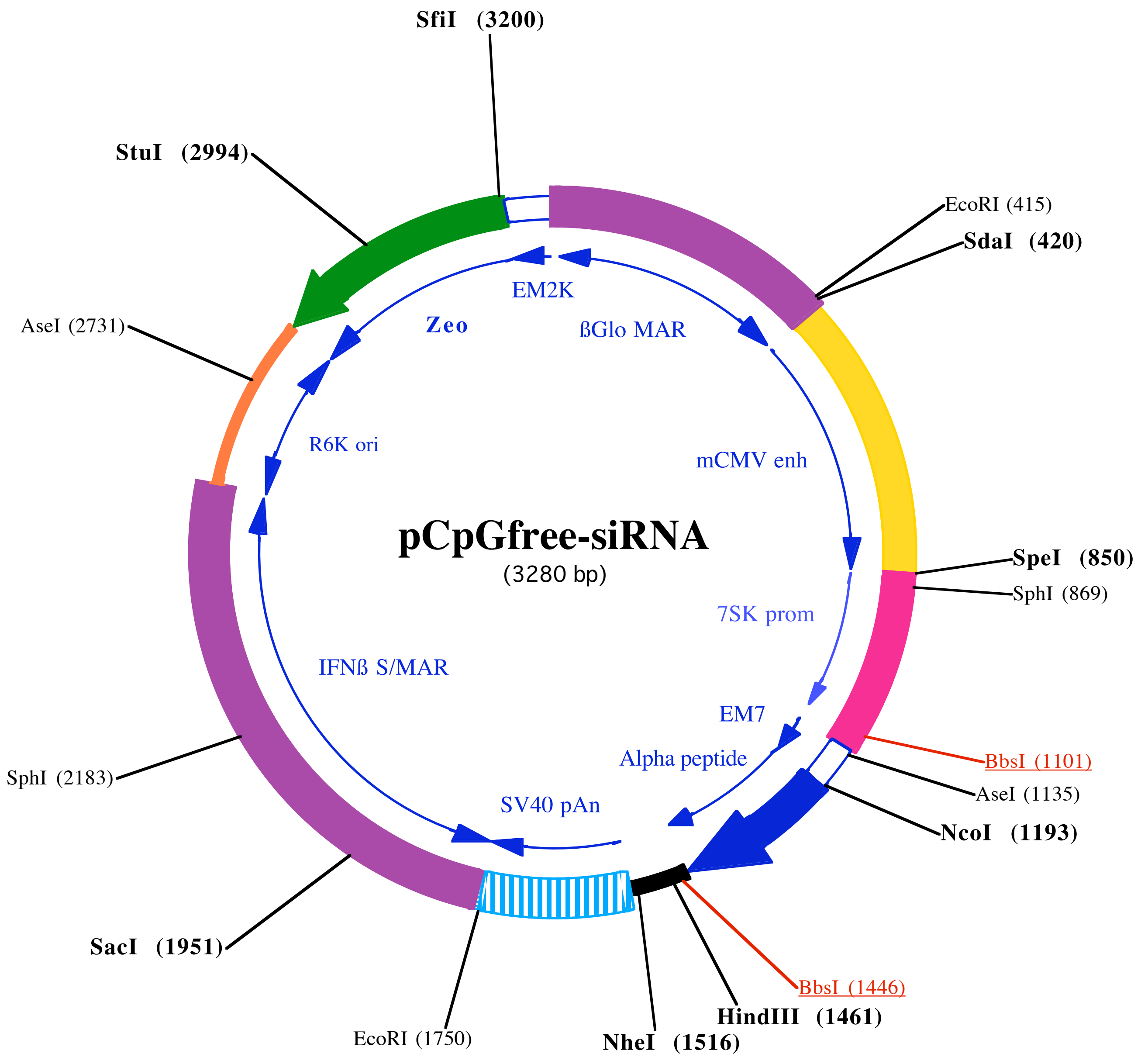

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 336 TTTTATGAATAAAAAATTATTAGCAATCAATATTGAAAACCACTGATTTTTGTTTATGTGAGCAAAC
                                 SdaI (420)
                        EcoRI (415)
 403 AGCAGATTAAAAGGAATTCCTGCAGGAGTCAATGGGAAAAACCCATTGGAGCCAAGTACACTGACTC
 470 AATAGGGACTTTCCATTGGGTTTTGCCCAGTACATAAGGTCAATAGGGGGTGAGTCAACAGGAAAGT
 537 CCCATTGGAGCCAAGTACATTGAGTCAATAGGGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTC
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                                                       SpeI (850)       SphI (869)
 805 ATGGGTTTTTCCCATTATTGGCACATACATAAGGTCAATAGGGGTGACTAGTGCTGCAGTATTTAGC
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 939 CAAACTTTAGCATTTTGGGAATAAATGATATTTGCTATGCTGGTTAAATTAGATTTTAGTTAAATTT
1006 CCTGCTGAAGCTCTAGTATGATAAGTAACTTGACCTAAGTGTAAAGTTGAGATTTCCTTCAGGTTTA
                                   BbsI (1101)                  AseI (1135)
1073 TATAGTCCCTATCAGTGATAGAGACCTCGGTCTTCACCTGAGGTTTTCAAAAGTAGTTGACAATTA
                                                              NcoI (1193)
1140 ATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTATAGGAGGGCCACCATGGACCCTGT
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1207 TGTGCTGCAAAGGAGAGACTGGGAGAACCCTGGAGTGACCCAGCTCAACAGACTGGCTGCCCACCCT
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                                                BbsI (1446)   HindIII (1461)
1408 TGACCTCCCAGAGGCTGACACTGTGTAACCCTGAGCTAGGAAGACTTTTTGGAAAAGCTTAATTAAG
  71 sAspLeuProGluAlaAspThrVal •••
                                                NheI (1516)
1475 CTGTACCACTATCAGCTGGTGTGGTGCCAGAAGTAAACCTGAGCTAGCTGGCCAGACATGATAAGAT
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               EcoRI (1750)
1743 GTGGTATGGAATTCAGTCAATATGTTCACCCCAAAAAGCTGTTTGTTAACTTGCCAACCTCATTCT
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1810 AAAATGTATATAGAAGCCCAAAAGACAATAACAAAAATATTCTTGTAGAACAAAATGGGAAAGAATG

1877 TTCCACTAAATATCAAGATTTAGAGCAAAGCATGAGATGTGTGGGGATAGACAGTGAGGCTGATAAA

SacI (1951)

1944 ATAGAGTAGAGCTCAGAAACAGACCCATTGATATATGTAAGTGACCTATGAAAAAAATATGGCATTT

2011 TACAATGGGAAAATGATGGTCTTTTTCTTTTTTAGAAAAACAGGGAAATATATTTATATGTAAAAAA

2078 TAAAAGGGAACCCATATGTCATACCATACACACAAAAAAATTCCAGTGAATTATAAGTCTAAATGGA

SphI (2183)

2145 GAAGGCAAAACTTTAAATCTTTTAGAAAATAATATAGAAGCATGCCATCAAGACTTCAGTGTAGAGA

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2279 AGACTTATTTTTAAAATTAAAAAACCATTAAGAAAAGTCAGGCCATAGAATGACAGAAAATATTTGC

2346 AACACCCCAGTAAAGAGAATTGTAATATGCAGATTATAAAAAGAAGTCTTACAAATCAGTAAAAAAT

2413 AAAACTAGACAAAAATTTGAACAGATGAAAGAGAAACTCTAAATAATCATTACACATGAGAAACTCA

2480 ATCTCAGAAATCAGAGAACTATCATTGCATATACACTAAATTAGAGAAATATTAAAAGGCTAAGTAA

2547 CATCTGTGGCTTAATTAAAATCAGCAGTTCAACCTGTTGATAGTATGTACTAAGCTCTCATGTTTAA

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AseI (2731)

2681 GTACTAAGCTCTCATGTTTCATGTACTAAGCTCTCATGTTTGAACAATAAAATTAATATAAATCAGC

2748 AACTTAAATAGCCTCTAAGGTTTTAAGTTTTATAAGAAAAAAAAGAATATATAAGGCTTTTAAAGGT

2815 TTTAAGGTTTCCTAGCTTTAGTCCTGTTCCTCAGCTACAAAATGGACACAATTTCCAGCAGGGTCTC

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StuI (2994)

2949 GAAATTTGTGCTGACTACTTCTGACCATTCTGCATAAAGCTCATCTAGGCCTCTGACCCAGACCCAA

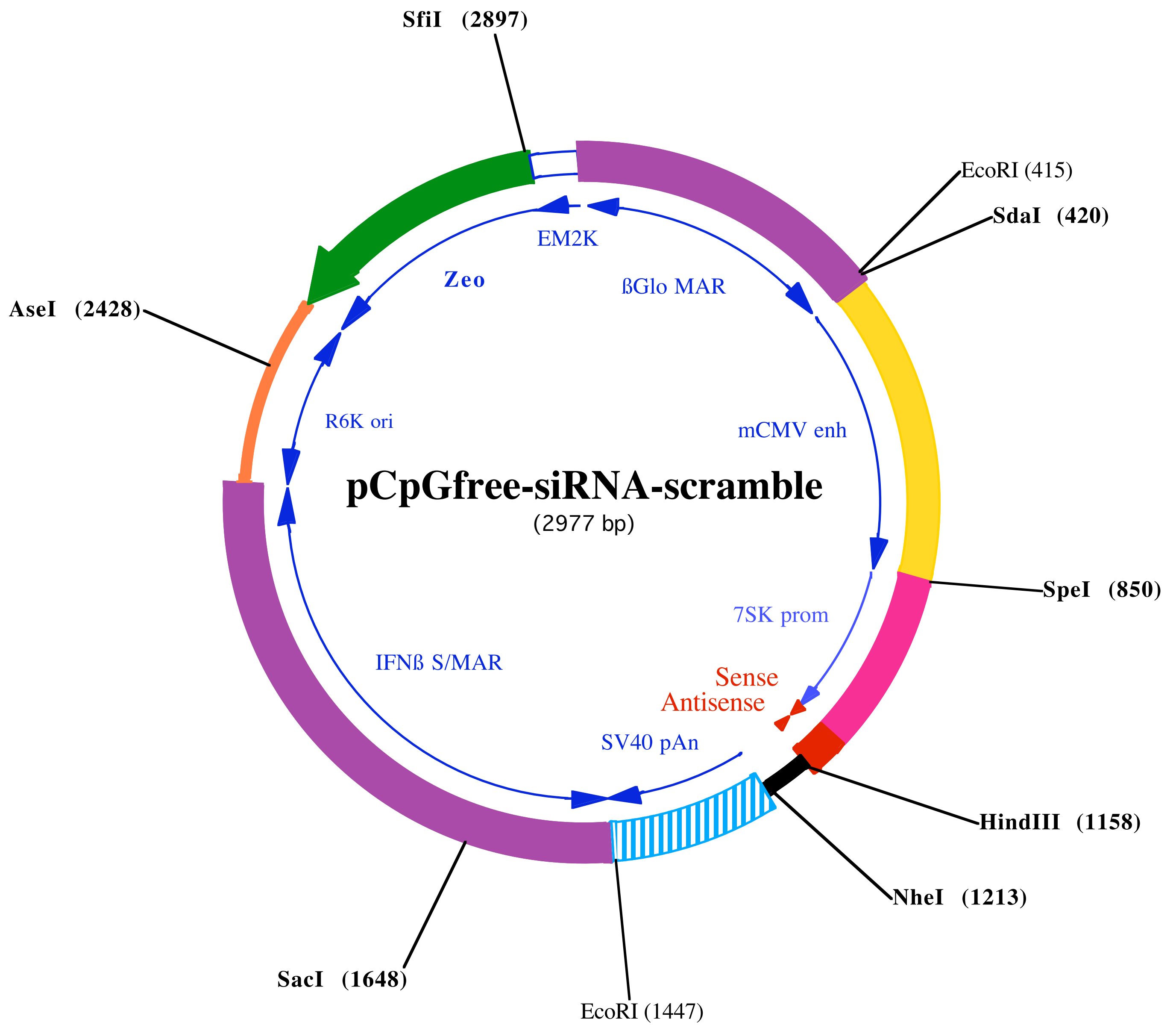
3016 GCAAGGGTGTTGTCAGGGACAACTTGGTCCTGAACTGCTGAGATGAAGAGGGTGACATCATCTCTGA

3083 CAACACCAGCAAAATCATCTTCAACAAAGTCTCTGGAGAATCCTAATCTGTCAGTCCAGAACTCTAC

SfiI (3200)

3150 AGCCCCTGCAACATCCCTTGCTGTGAGGACTGGGACTGCAGAAGTGAGTTTGGCCATGATGGCCCTC

3217 CTATAGTGAGTTGTATTATACTATGCAGATATACTATGCCAATGTTTAATTGTCAACTACTGTT



InvivoGen

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 202 ATAAATAATAAATCTCTTTGTTCAGCTCTCTGTTTCTATAAATATGTACCAGTTTTATTGTTTTTAG
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 336 TTTTATGAATAAAAAATTATTAGCAATCAATATTGAAAACCACTGATTTTTGTTTATGTGAGCAAAC

                                 SdaI (420)
                         EcoRI (415)
 403 AGCAGATTAAAAGGAATTCCTGCAGGAGTCAATGGGAAAAACCCATTGGAGCCAAGTACACTGACTC
 470 AATAGGGACTTTCCATTGGGTTTTGCCCAGTACATAAGGTCAATAGGGGGTGAGTCAACAGGAAAGT
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 738 ACACTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCCAGTACAAAAGGTCAATAGGGGGTGAGTCA

                                                         SpeI (850)
 805 ATGGGTTTTTCCCATTATTGGCACATACATAAGGTCAATAGGGGTGACTAGTGCTGCAGTATTTAGC
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1006 CCTGCTGAAGCTCTAGTATGATAAGTAACTTGACCTAAGTGTAAAGTTGAGATTTCCTTCAGGTTTA
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                                   HindIII (1158)
1140 GCACATATGCTTTTTGGAAAAGCTTAATTAAGCTGTACCACTATCAGCTGGTGTGGTGCCAGAAGTA

                 NheI (1213)
1207 AACCTGAGCTAGCTGGCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATG
1274 CAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCT
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                                                    EcoRI (1447)
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                                                    SacI (1648)
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2078 ATAAAAAGAAGTCTTACAAATCAGTAAAAAATAAAACTAGACAAAAATTTGAACAGATGAAAGAGAA

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2279 GTTGATAGTATGTACTAAGCTCTCATGTTTAATGTACTAAGCTCTCATGTTTAATGAACTAAACCCT

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2547 TACAAAATGGACACAATTTCCAGCAGGGTCTCTGAGGGCAAATTCCCTTCCCCAAGGTTGTTCACCA

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2681 AAAGCTCATCTAGGCCTCTGACCCAGACCCAAGCAAGGGTGTTGTCAGGGACAACTTGGTCCTGAAC

2748 TGCTGAGATGAAGAGGGTGACATCATCTCTGACAACACCAGCAAAATCATCTTCAACAAAGTCTCTG

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2949 ATGCCAATGTTTAATTGTCAACTACTGTT
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Fast-Media® Zeo Agar X-Gal

Microwaveable media for selection and propagation of Zeocin™ resistant *E. coli*

Catalog # fas-zn-x

For research use only

Version # 13F26-MM

PRODUCT INFORMATION

Contents:

- 20 individual sealed pouches of Fast-Media® Zeo Agar X-Gal. Each pouch contains the necessary amount of powder for the preparation of **200 ml** of **Lysogency Broth (LB) based solid medium** supplemented with **Zeocin™, IPTG and X-Gal**. Lysogeny Broth is also known as Luria Broth.

Fast-Media® Zeo Agar X-Gal are used for the detection of blue/white resistant colonies after transformation by a vector carrying the **LacZ** gene.

Effective concentration: Zeocin™ 25 µg/ml, IPTG 100 µg/ml, X-Gal 100 µg/ml

Storage and stability:

- Fast-Media® Zeo Agar X-Gal are shipped at room temperature, and must be stored in a dry and cool place. They are stable for 2 years at room temperature.
- When properly prepared, Fast-Media® Zeo Agar X-Gal broths are stable for 4 weeks at 4˚C, retaining sterility and selective properties.

Quality control:

The high quality and performance of each formulation are tested with *E. coli* K12 derived strains. *E. coli* transformed with a plasmid carrying the Zeocin™ resistance gene are used as positive controls for Fast-Media® Zeo Agar X-Gal.

METHOD

For customer convenience, the following procedure is directly printed on each pouch.

1. Pour the pouch contents into a clean borosilicate glass bottle or flask.
2. Add 200 ml of distilled or deionized water.
3. Mix thoroughly by swirling the glass bottle or flask.
4. Heat in a microwave oven on MEDIUM power setting (about 450W) until bubbles start to appear (about 3 minutes).

Do not heat in a closed container.

5. Swirl gently to mix the preparation and re-heat for 30 seconds. Swirl gently again.
6. Repeat step 4 if necessary until the medium is completely dissolved. Do not overboil.
7. Allow the medium to cool to 50-55 °C before use.

Caution: *Any solution heated in a microwave oven may become superheated and suddenly boil when moved or touched. Handle with extreme care. Wear heat-proof gloves.*

***Note**: Do not repeat this above procedure once the medium is prepared because the antibiotic will be adversely affected.*

SPECIAL HANDLING

Caution should be exercised during handling of Fast-Media® due to potential allergenic properties of antibiotics. Wear protective gloves, do not breathe the dust.

FAST-MEDIA® FEATURES

Fast-Media® offer researchers a quick and convenient way to prepare 200 ml of sterile *E.coli* growth medium in about five minutes using a **microwave** instead of an autoclave.

Fast-Media® is supplied with a choice of antibiotics for selection (see table below), and chromogenic substrates, for the growth or selection of *E. coli* transformant colonies, as well as detection of blue/white colonies. Fast-Media® Base is supplied without selective antibiotics.

Several Fast-Media® are available:

• **Fast-Media® TB,** Terrific Broth based liquid medium
• **Fast-Media® LB,** Lysogency Broth (LB) based liquid medium
• **Fast-Media® Agar,** LB based solid medium
• **Fast-Media® Agar X-Gal,** LB based solid medium containing IPTG and X-Gal
• **Fast-Media® Agar X-Gluc** LB based solid medium containing X-Gluc.

Fast-Media®	Agar	Agar X-Gal	Agar X-Gluc	LB	TB
Base	X			X	
Ampicillin	X	X		X	X
Blasticidin	X	X			X
Hygromycin	X	X			X
Kanamycin	X	X		X	X
Puromycin	X				X
Zeocin™	X	X	X		X

RELATED PRODUCTS

Product	Catalog Code
Fast-Media® Zeo TB	fas-zn-l
Fast-Media® Zeo Agar	fas-zn-s
Fast-Media® Zeo Agar X-Gluc	fas-zn-g