psiRNA-h7SKhygro G1 Kit

A simple and innovative tool to create siRNAs

Catalog # ksirna3-h21

For research use only

Version # 06G06-SV

PRODUCT INFORMATION

Content:

• 20 µg of lyophilized **psiRNA-h7SKhygro G1** plasmid
• 20 µg of lyophilized **psiRNA-h7SKh-EGFP** plasmid, a control plasmid expressing a siRNA targeting the EGFP gene
siEGFP sequence: GCAAGCTGACCCTGAAGTTCA
• 20 µg of lyophilized **psiRNA-h7SKh-Luc**,a control plasmid expressing an siRNA targeting the luciferase GL3 gene
siLucGL3 sequence: GACTTACGCTGAGTACTTCGA
• 1 vial of **LyoComp GT116** and 1x 2 ml Reconstitutive Solution
GT116 genotype: *F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZM15 ΔlacX74 recA1 endA1 ΔsbcC-sbcD*
• 10 µg of lyophilized **OL559 primer** (forward)
OL599 sequence: 5' CGATAAGTAACTTGACCTAAGTG 3' (23 mer)
MW: 7072 g/mol. Tm: 57.1°C
• 10 µg of lyophilized **OL408 primer** (reverse)
OL408 sequence: 5' GCGTTACTATGGGAACATAC 3' (20 mer)
MW: 6141 g/mol. Tm: 55.3°C
• 4 pouches of **Fast-Media® Hygro XGal**

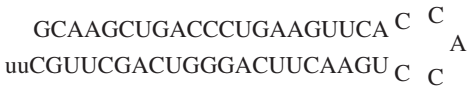
Storage and stability:

- Products are shipped at room temperature.
- Upon receipt, resuspend lyophilized DNA and store at -20°C. Avoid repeated freeze-thaw cycles.
- Store LyoComp GT116 and Reconstitutive Solution at 4°C for short term storage or at -20°C for long term storage. LyoComp cells are stable for 2 weeks when stored at 4°C and 6 months when stored at -20°C.
- Store sequencing primers at -20°C.
- Store Fast-Media® Hygro XGal at room temperature. Fast-Media® pouches are stable 18 months when stored properly.

Quality control:

- Plasmid DNA was prepared using affinity column and lyophilized.
- Plasmid construct has been confirmed by restriction analysis.
- The sequence between Cla I and Xba I restriction sites has been verified by sequencing.
- The transformation efficiency of LyoComp cells are evaluated periodically and are guaranteed to be stable for 6 months when properly stored (-20°C).

siEGFP Hairpin



OVERVIEW

RNA interference (RNAi) has recently emerged as a powerful technology for studying gene functions in eukaryotes. RNAi is a post-transcriptional process triggered by the introduction of small interfering RNA (siRNA) which leads to gene silencing in a sequence-specific manner. siRNAs consist of 19-21 mer double-stranded RNA with 2 nt 3' overhangs, or a 43-51 mer RNA molecule with a hairpin structure, called short hairpin RNA (shRNA) which are homologous to a region within the target gene[1-5]. Introduction of siRNAs in mammalian cells induces strong and specific suppression of the gene of interest. However, this effect is only transient due to the short lifespan of synthetic RNAs, which severely limits the applications of siRNAs. To overcome this limitation, InvivoGen has designed an efficient and simple-to-use vector, called psiRNA, that allows the production of siRNAs within the cells.

GENERAL PRODUCT USE

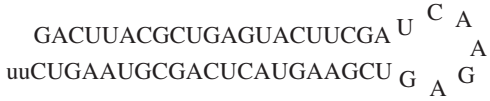
psiRNA is specifically designed for the cloning of small synthetic oligonucleotides (around 50-mer) that encode two complementary sequences of 21 nt, homologous to a segment of the gene of interest, separated by a short spacer region of 5-9 nt. The insert is cloned downstream of a RNA polymerase III promoter, the human 7SK or H1 promoter. It is transcribed into a short dsRNA with a hairpin structure (shRNA) consisting of a 21 bp double stranded region corresponding to the target sequence and a small loop formed by the spacer region.

psiRNA exploits the white-blue selection system. The cloning sites flank a bacterial lacZ α-peptide cassette allowing the discrimination between blue parental clones and white recombinant clones in *E. coli*. Although over 90% of the white clones have integrated a fragment, it is necessary to sequence the insert to verify the integrity of the sequence since a single base difference can lead to an inactive siRNA.

psiRNA-h7SK G1 plasmids offer two cloning options:

- Bbs I / Bbs I (although these sites are recognized by the same enzyme, they are different avoiding self-ligation of the plasmid)
- Acc 65I / Hind III

siLuc Hairpin



TECHNICAL SUPPORT
Toll free (US): 888-457-5873
Outside US: (+1) 858-457-5873
E-mail: info@invivogen.com
Website: www.invivogen.com

InvivoGen™
3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA

PLASMID FEATURES

• **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[6]. The high conservation and abundance of 7SK suggest an important physiological function of this RNA[7]. 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[8]. 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[9].

• **EM7-alpha-peptide:** The EM7-lacZ α-peptide cassette is a bacterial expression cassette enabling 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.

• **CMV enh / prom:** The human cytomegalovirus immediate-early gene 1 enhancer/promoter was originally isolated from the Towne strain and was found to be stronger than any other viral promoters.

• **EM7** is a bacterial promoter that enables the constitutive expression of the antibiotic resistance gene in *E. coli.*

• **Hygro:** Resistance to Hygromycin B is conferred by the *hph* gene from *E. coli*. The *hph* gene is driven by the CMV enhancer/promoter in tandem with the bacterial EM7 promoter allowing selection in both mammalian cells and *E. coli*.

• **βGlo pAn:** The human beta-globin 3'UTR and polyadenylation sequence allows efficient arrest of the transgene transcription[10].

• **pMB1 Ori** is a minimal *E. coli* origin of replication to limit vector size, but with the same activity as the longer Ori.

References

1. Elbashir SM. *et al.*, 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 15(2):188-200.
2. Elbashir SM. *et al.*, 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 411(6836):494-8.
3. Yu JY. *et al.*, 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci U S A. 99(9):6047-52.
4. Brummelkamp TR.*et al.*, 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science, 296:550-3.
5- Tiscornia G. *et al.*, 2003. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. PNAS 100(4):1844-1848
6. Murphy S. *et al.*, 1984. DNA sequences complementary to human 7 SK RNA show structural similarities to the short mobile elements of the mammalian genome. J Mol Biol. 177(4):575-90.
7. Wassarman DA, Steitz JA. 1991. Structural analyses of the 7SK ribonucleoprotein (RNP), the most abundant human small RNP of unknown function. Mol Cell Biol. 11(7):3432-45.
8. Blencowe BJ., 2002. Transcription: surprising role for an elusive small nuclear RNA. Curr Biol. 12(4):R147-9.
9. Czauderna F. *et al.*, 2003. Inducible shRNA expression for application in a prostate cancer mouse model. Nucleic Acids Res. 31(21):e127.
10. Yu J & Russell JE, 2001. Structural and functional analysis of an mRNP complex that mediates the high stability of human beta-globin mRNA. Mol Cell Biol, 21(17):5879-88.

METHODS

Plasmid resuspension

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

Plasmid amplification

psiRNA 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 psiRNA plasmid with Bbs I (NEB, 2 units enzyme/µg plasmid DNA). Elute the large fragment (3122 bp) using a 0.7% low-melting agarose gel and dilute the purified DNA fragment to obtain a solution at 0.1 µg/µl.

• Cloning with Acc 65I / Hind III

Digest psiRNA plasmid with Acc 65I and Hind III (with NEB enzymes, use NEBuffer 2 + BSA). Elute the large fragment (3092 bp) using a 0.7% low-melting agarose gel and 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 with either Bbs I/Bbs I or Acc 65I/Hind III (Figure 1).

Cloning into psiRNA (Figure 2)

1- Annealing of siRNA insert

- Dissolve the oligonucleotides (forward and reverse) at a concentration of 100 µM.
- Dilute to obtain each oligonucleotide solution at 25 µM.
- Prepare the annealing solution by mixing the following components:

Forward oligonucleotide (25 µM)	2 µl
Reverse oligonucleotide (25 µM)	2 µl
0.5 M NaCl	6 µl
H_2O to a final volume of	30 µl

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Toll free (US): 888-457-5873
Outside US: (+1) 858-457-5873
E-mail: info@invivogen.com
Website: www.invivogen.com

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3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA

- 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 psiRNA

- Prepare the ligation solution by mixing the following components:

Digested psiRNA	1 µl (100 ng)
Annealed siRNA insert	1 µl
T4 DNA Ligase	1 µl (1 unit)
10X ligation buffer	2 µl
H2O to a final volume of	20 µl

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

3- Transformation of LyoComp GT116

We recommend using *E. coli* GT116 strain, a *sbcCD* deletion mutant, which is more compatible with hairpin harboring plasmids than standard laboratory strains which are sbcCD$^+$.
E. coli GT116 is provided as lyophilized competent cells, called LyoComp GT116.

- Place the LyoComp GT116 vial in ice for 5 minutes.
- Add 1 ml cold reconstitutive solution and store in ice for 5 minutes.
- Gently homogenize and allow the cells to completely rehydrate in ice for 25-30 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® Hygro 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 (2 hours at 37°C).

psiRNA-h7SKhygro G1 containing a siRNA insert will generate two bands: 2417 bp and 741 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 OL408 (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 psiRNA.
- Two days after transfection, add Hygromycin B at the appropriate concentration (typically 50-100 µg/ml). Stable transfectants are usually individualized after 1-2 weeks.

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.

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Toll free (US): 888-457-5873
Outside US: (+1) 858-457-5873
E-mail: info@invivogen.com
Website: www.invivogen.com

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3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA

Figure 1

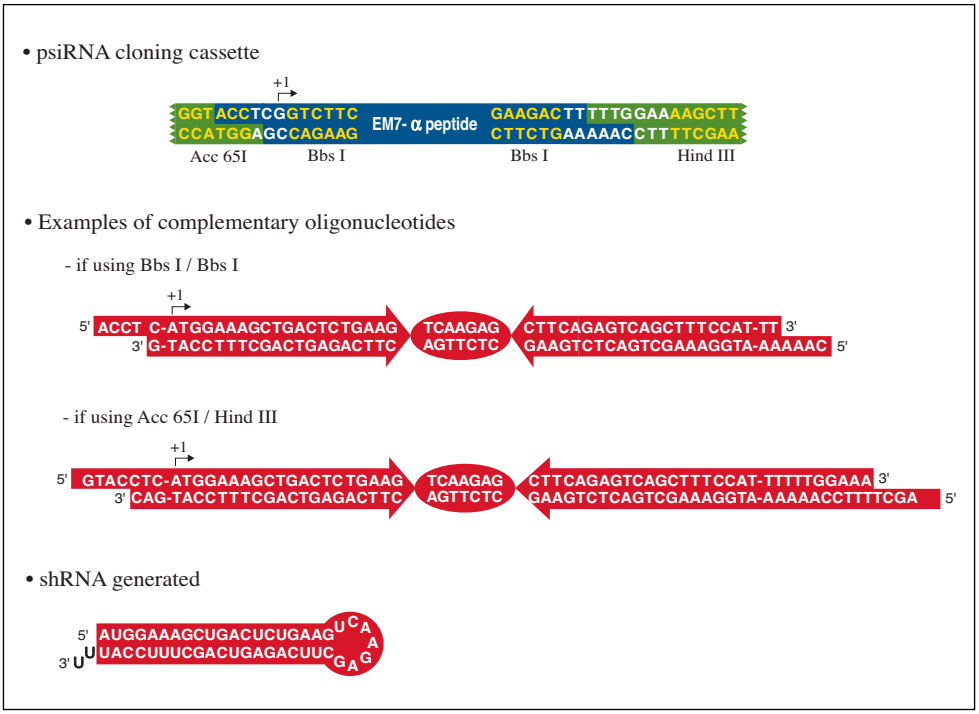
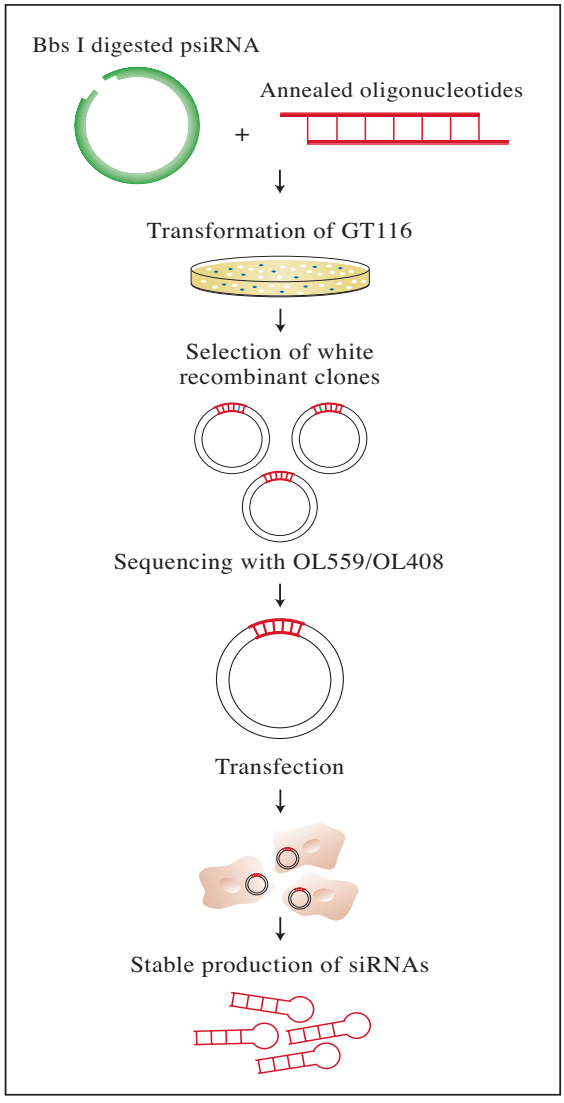
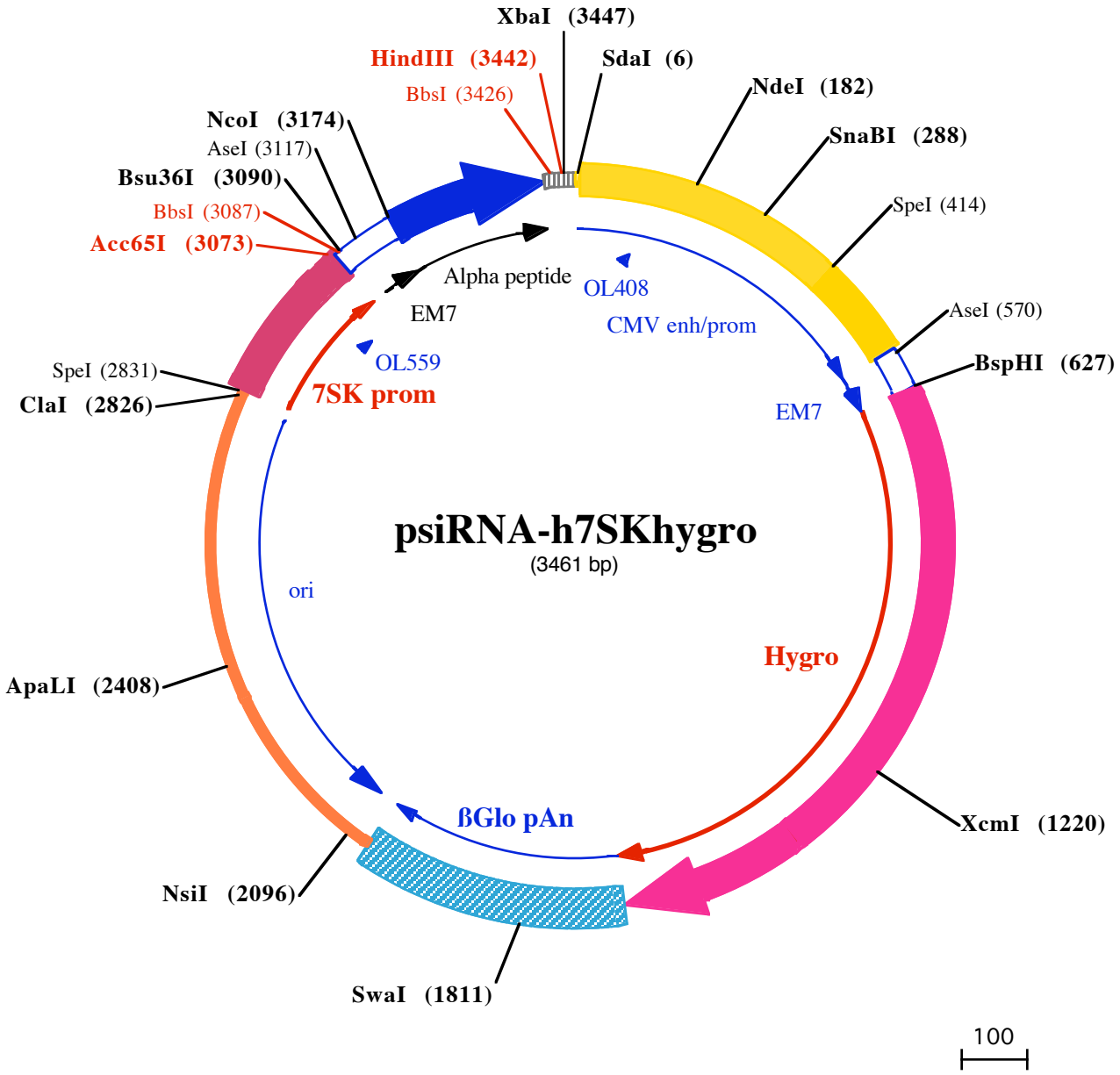


Figure 2




XbaI (3447)
HindIII (3442)
SdaI (6)
BbsI (3426)
NdeI (182)
NcoI (3174)
SnaBI (288)
AseI (3117)
Bsu36I (3090)
SpeI (414)
BbsI (3087)
Acc65I (3073)
Alpha peptide
OL408
EM7
CMV enh/prom
AseI (570)
BspHI (627)
SpeI (2831)
OL559
ClaI (2826)
7SK prom
EM7
psiRNA-h7SKhygro
(3461 bp)
ori
Hygro
ApaLI (2408)
ßGlo pAn
XcmI (1220)
NsiI (2096)
SwaI (1811)
100


SdaI (6)
NdeI (182)
SnaBI (288)
SpeI (414)
AseI (570)
BspHI (627)
XcmI (1220)
SwaI (1811)
NsiI (2096)
ApaLI (2408)
SpeI (2831)
ClaI (2826)
BbsI (3087)
Acc65I (3073)
Bsu36I (3090)
AseI (3117)
NcoI (3174)

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3201 GAGACTGGGAGAACCCTGGAGTGACCCAGCTCAACAGACTGGCTGCCCACCCTCCCTTTGCCTCTTGGAGGAACTCTGAGGAAGCCAGGACAGACAGGCC
   9▶rgAspTrpGluAsnProGlyVal ThrGlnLeuAsnArgLeuAlaAlaHisProProPheAlaSer TrpArgAsnSer GluGluAlaArgThrAspArgPr
3301 CAGCCAGCAGCTCAGGTCTCTCAATGGAGAGTGGAGGTTTGCCTGGTTCCCTGCCCCTGAAGCTGTGCCTGAGTCTTGGCTGGAGTGTGACCTCCCAGAG
  42▶oSer GlnGlnLeuArgSer LeuAsnGlyGluTrpArgPheAlaTrpPheProAlaProGluAlaVal ProGluSer TrpLeuGluCysAspLeuProGlu
                                                    XbaI (3447)
                                    BbsI (3426)          HindIII (3442)
3401 GCTGACACTGTGTAACCCTGAGCTAGGAAGACTTTTTGGAAAAGCTTCTAGACTTAATTAA
  76▶AlaAspThr Val •••
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LyoComp GT116

Lyophilized competent *E. coli* GT116 cells

Catalog # lyo-116

For research use only

Version # 05B03-SV

PRODUCT INFORMATION

Content:

- Lyophilized competent *E. coli* GT116 cells, provided in 1 vial containing enough material to perform 10 transformations after addition of 1ml reconstitutive solution.
- 2 ml of sterile reconstitutive solution.

GT116 Genotype: *F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZM15 ΔlacX74 recA1 endA1 ΔsbcC-sbcD*

Storage and stability:

- LyoComp cells are shipped at room temperature.
- Upon receipt, store LyoComp cells at -20°C.
- LyoComp cells are stable for 6 months when stored at -20°C.

Quality control:

The transformation efficiency of LyoComp cells are evaluated periodically and are guaranteed to be stable for 6 months when properly stored (-20°C).

DESCRIPTION / PROPERTIES

GT116 is a *sbcCD* deletion strain specifically engineered to support the growth of plasmid DNAs carrying hairpin structures, such as psiRNA a vector expressing small interfering RNA. Hairpin structures are known to be unstable in *E. coli* due to their elimination by a protein complex called SbcCD that recognizes and cleaves hairpins[1]. To increase their stability in *E. coli*, InvivoGen has developed GT116 by deleting the *sbcC* and *sbcD* genes. This modification significantly improves the number of recombinant clones harboring a plasmid with hairpin structures.

LyoComp GT116 was specifically designed for transformation with psiRNA ligation products or recombinant plasmids. LyoComp GT116 cells exihibit a lower competency than standard chemically competent cells but the competency is sufficient to allow successful cloning into psiRNA plasmids.

Reference

1. Connelly JC. et al., 1998. The SbcCD nuclease of escherichia coli is a structural maintenance of chromosomes (SMC) family protein that cleaves hairpin DNA. Proc. Natl. Acad. Sci. USA 95:7969-7974

TRANSFORMATION

The following protocol describes a method used to introduce DNA into bacterial host for efficient and convenient construction or maintenance of plasmid recombinants, and blue/white screening.

Additional required materials to be supplied by user:

- LB agar plates with appropriate antibiotic. For optimal results we recommend the use of InvivoGen's selective Fast-Media®.
- 37˚C shaking Incubator
- Ice bucket
- 42˚C water bath
- LB or SOC medium

Method:

Before starting:

- Prepare LB agar plates containing the appropriate antibiotic.
- Set water bath to 42˚C.
- Pre-chill appropriate number of 1.5 ml sterile tubes in ice.

1- Place the LyoComp cell vial (100 µl per ligation or transformation reaction) in ice for 5 minutes.
2- Add 1 ml cold reconstitutive solution per vial and store in ice for 5 minutes.
3- Gently homogenize and allow the cells to completely rehydrate in ice for 25-30 minutes.
4- Introduce 10 µl of ligation product (or 1 µg supercoiled plasmid DNA in pre chilled 1.5 ml tubes and return tubes to ice.
5- Gently flick the cells twice to homogenize and add 100 µl of cells to each DNA-containing tubes.
6- Mix by tapping gently and place in ice immediately.
7- Incubate the tubes in ice for 30 minutes.
8- Incubate the tubes in a 42˚C water bath for exactly 30 seconds, then place the tubes back in ice for 1-2 minutes.
9- Add 900 µl of room temperature SOC (or LB) medium to each reaction. (Practice sterile techniques to avoid contamination.)
10- Incubate tubes at 37˚C for 1h30 with shaking at 250 rpm.
11- Spread each transformation reaction (4 x 150 µl if using a ligation product or 100 µl of 10^{-1} and 10^{-2} dilutions if using a supercoiled plasmid) onto LB agar plates containing the appropriate antibiotic.
12- Incubate plates at 37˚C overnight.

TECHNICAL SUPPORT
Toll free (US): 888-457-5873
Outside US: (+1) 858-457-5873
E-mail: info@invivogen.com
Website: www.invivogen.com

InvivoGen
3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA

Fast-Media®

Microwaveable media for selection and propagation of *E. coli* transformants

Catalog # fas-xx-l, fas-xx-s, fas-xx-xgal

For research use only

Version # 05C28-SV

PRODUCT INFORMATION

Contents:

E. coli **Fast-Media®** are prepared as individual sealed pouches containing the necessary amount of powder for preparation of 200 ml of selective liquid or agar medium.

30 pouches are supplied for each order of TB or Agar and 20 pouches are supplied for each order of XGal Agar.

Storage and stability:

Fast-Media® are shipped at room temperature, and must be stored in a dry and cool place. They are stable for at least one year at room temperature.

When properly prepared, **Fast-Media®** plates or TB are stable several weeks at 4°C, and remain sterile and selective**.**

Quality control:

The high quality and performance of each formulation has been tested with some widely used and proprietary *E. coli* K12 derived strains*. These include DH5α, Top10, MC1061, XL1 blue, JM 109, TB1, GT100, GT110.

The adequate plasmids carrying the appropriate *E. coli* resistance genes are used as positive control.

**E. coli* recipient strains carrying the Tn5 transposon are resistant to Kanamycin and Zeocin™.

GENERAL PRODUCT USE

E. coli **Fast-Media®** are microwaveable ready-to-use solid or liquid media, supplied with a selective antibiotic, and chromogenic substrates (for five references), therefore designed for the growth or selection of *E. coli* transformant colonies, as well as detection of blue/white colonies.

- Fast-Media® Agar formulation is LB based agar medium supplemented with selective antibiotic, it's used for selection of resistant *E. coli* colonies after transformation by vectors carrying a selection resistance gene.

- Fast-Media® X-Gal formulation is a LB based agar medium supplemented with selective antibiotic, X-Gal and IPTG. It's used for detection of blue/white resistant colonies after transformation by a vector carrying *LacZ* gene.

- Fast-Media® TB formulation is a Terrific Broth based liquid medium supplemented with selective antibiotic. It's used for high cell density culture of transformed bacteria, and extraction of high quantity and quality of required plasmid.

FAST-MEDIA® FEATURES

E. coli **Fast-Media®** offer researchers a quick and convenient way to prepare 200 ml of liquid culture medium, or 8-10 agar plates in about five minutes USING A MICROWAVE INSTEAD OF AN AUTOCLAVE

E. coli **Fast-Media®** are available with a large variety of prokaryotic selective agents including Ampicillin, Blasticidin S, Hygromycin B, Kanamycin, Puromycin and Zeocin™ (see table below).**Fast-Media®** is also available with no selective agent (Base) that can be prepared with or without antibiotics.

	Agar	X-Gal	TB
Base	√		√
Ampicillin	√	√	√
Blasticidin	√	√	√
Hygromycin	√	√	√
Kanamycin	√	√	√
Puromycin	√		√
Zeocin	√	√	√

SPECIAL HANDLING

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

METHOD

For customer convenience, 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- 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.
4- Swirl gently to mix the preparation and re-heat for 30 seconds. Swirl gently again.
5- Repeat step 4 if necessary until the medium is completely dissolved. Do not overboil.
6- Allow the medium to cool to 50-55 °C, use directly for liquid medium, or pour plates for solid medium.

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.*

For preparation of Fast-Media Base.

- Follow the instructions above and when media has cooled to 50-55 °C add the antibiotic at the appropriate concentration for selection of *E. coli.*

TECHNICAL SUPPORT
Toll free (US): 888-457-5873
Outside US: (+1) 858-457-5873
E-mail: info@invivogen.com
Website: www.invivogen.com

InvivoGen™
3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA