

# GeneArt® Site-Directed Mutagenesis PLUS Kit

For quick, highly efficient *in vitro* site-directed mutagenesis  
of up to 3 sites on plasmids of up to 14kb

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## Kit Contents and Storage

### Kit Components

The GeneArt® Site-Directed Mutagenesis PLUS Kit is shipped on dry ice and contains the following components. The reagents supplied are sufficient to perform 10 site-directed mutagenesis reactions and one control reaction.

Component	Amount
DNA Methylase (4 units/ $\mu$ L)	12 $\mu$ L
200X SAM (S-adenosine methionine)	10 $\mu$ L
GeneArt® 2X Enzyme Mix	100 $\mu$ L
10X PCR Enhancer	100 $\mu$ L
PCR Water	1.8 mL
pMSDM-White Vector (20 ng/ $\mu$ L)	5 $\mu$ L
Control Primer Mix 1 (10 $\mu$ M)	20 $\mu$ L
Control Primer Mix 2 (10 $\mu$ M)	20 $\mu$ L
Control Primer Mix 3 (10 $\mu$ M)	20 $\mu$ L
0.5 M EDTA	500 $\mu$ L
One Shot® MAX Efficiency® DH5 $\alpha$ <sup>TM</sup> -T1 <sup>R</sup> Cells	1 box

### Storage

Store the kit components as indicated. For convenience, you may store the entire contents of the kit at  $-80^{\circ}\text{C}$ .

Store at $-20^{\circ}\text{C}$	Store at $-80^{\circ}\text{C}$
<ul style="list-style-type: none"> <li>DNA Methylase (4 units/<math>\mu</math>L)</li> <li>200X SAM (S-adenosine methionine)</li> <li>10X PCR Enhancer</li> <li>PCR Water</li> <li>pMSDM-White Vector (20 ng/<math>\mu</math>L)</li> <li>Control Primer Mix</li> <li>Control Primer Mix 1 (10 <math>\mu</math>M)</li> <li>Control Primer Mix 2 (10 <math>\mu</math>M)</li> <li>Control Primer Mix 3 (10 <math>\mu</math>M)</li> <li>0.5 M EDTA</li> </ul>	<ul style="list-style-type: none"> <li>GeneArt® 2X Enzyme Mix</li> <li>One Shot® MAX Efficiency® DH5<math>\alpha</math><sup>TM</sup>-T1<sup>R</sup></li> </ul>

### One Shot® MAX Efficiency® DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> Components

Each box of One Shot® MAX Efficiency® DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> contains the following components. The reagents provided are sufficient for 21 transformations. Store the box at  $-80^{\circ}\text{C}$ , or the individual components as indicated.

Component	Amount	Storage
Chemically Competent DH5 $\alpha$ <sup>TM</sup> -T1 <sup>R</sup> Cells	21 $\times$ 50 $\mu$ L	$-80^{\circ}\text{C}$

S.O.C. Medium	6 mL	4°C
pUC19 Control Plasmid (10 pg/μL)	50 μL	-20°C

**Genotype of  
DH5α™-T1<sup>R</sup> Cells**

F<sup>-</sup>  $\phi$ 80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>+</sup>) *phoA supE44 thi-1 gyrA96 relA1 tonA* (confers resistance to phage T1)

**Product Use**

For Research Use Only. Not for use in diagnostic procedures.

# Product Information

## Description of the System

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### GeneArt® Site-Directed Mutagenesis PLUS System

The GeneArt® Site-Directed Mutagenesis PLUS Kit provides a simple and highly efficient method for *in vitro* multi-site-directed mutagenesis in DNA plasmids of up to 14 kb from any *E. coli* plasmid, with no specialized vectors or restriction sites required.

This unique system can mutate up to three sites per vector, and generate base substitutions, deletions, or insertions of up to 3 nucleotides at each of the three sites, or up to 25 nucleotides at a single site. In addition, it can introduce up to 3 degenerated nucleotides at three sites or up to 12 nucleotides at a single site on the target plasmid. The entire procedure, including transformation, may be completed in less than 3 hours (when using a 3 kb plasmid).

In the GeneArt® Site-Directed Mutagenesis PLUS Kit, the DNA methylation and amplification steps are combined into a single reaction, and the three mutation sites are generated using three pairs of complementary mutagenic oligonucleotide primers with centrally located mutation sites. There is no requirement for an *in vitro* digestion step after the mutagenesis reaction and no purification step is required after methylation or mutagenesis.

The GeneArt® Site-Directed Mutagenesis PLUS Kit relies on the inherent properties of DNA methylase, high fidelity DNA polymerase, recombination enzymes, and *McrBC* endonuclease, as shown in the workflow diagram on page 5.

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### Applications

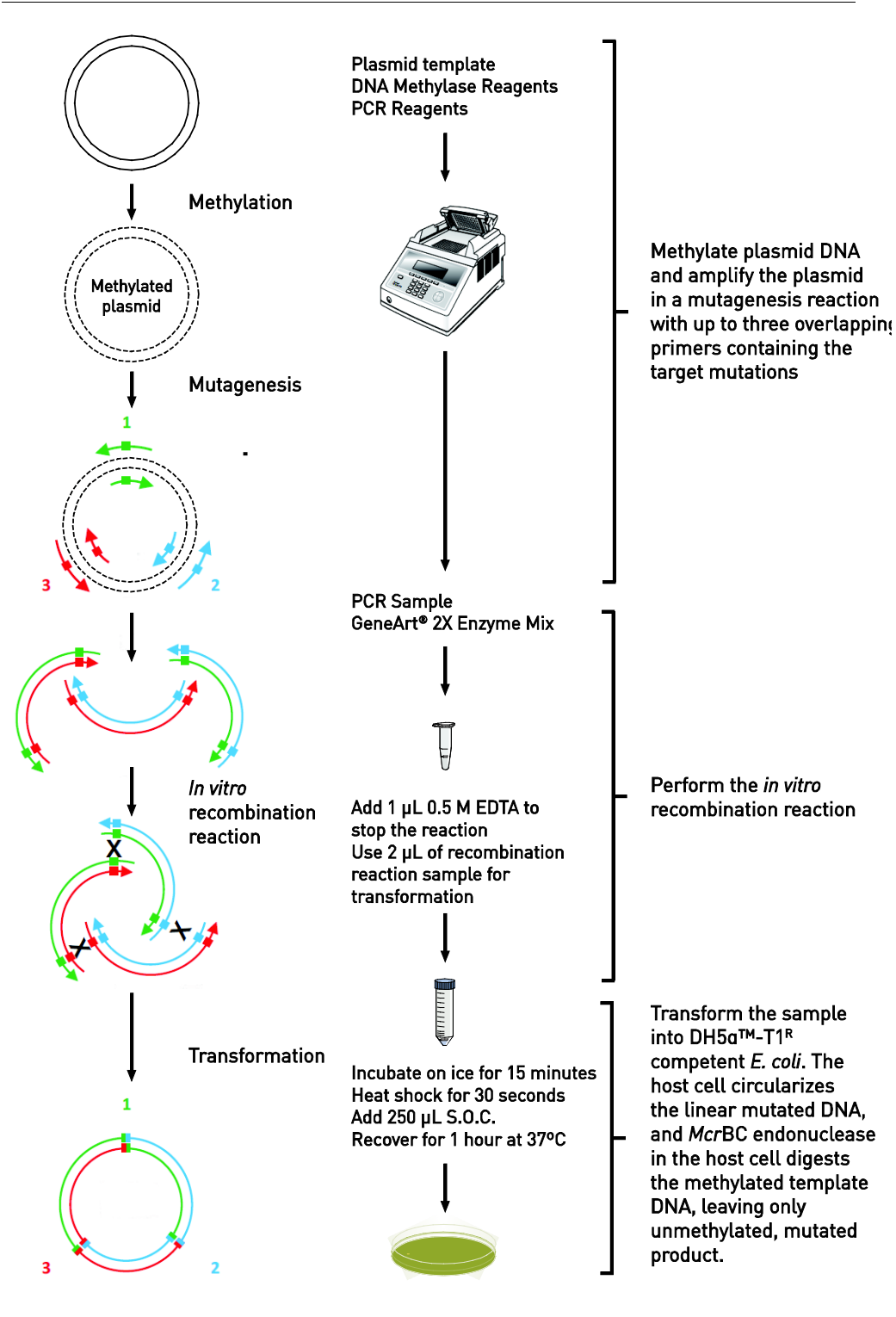
*In vitro* site-directed mutagenesis can be used to:

- Study protein function
  - Identify enzyme active sites
  - Design new proteins
- 

*Continued on next page*

# Description of the System, continued

## Multi-Site Mutagenesis Workflow



# Methods

## Plasmid and Primer Specifications

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### Target Plasmid Specifications

- This kit has been tested using plasmids ranging in size from 2.8 kb to 14 kb.
  - Plasmids may be isolated from *E. coli*.
  - No special vectors or restriction sites are required.
- 

### Primer Specifications

- You may use up to 3 pairs of mutagenic primers with the GeneArt® Site-Directed Mutagenesis PLUS Kit.
- The mutation site should be centrally located on both primers (forward and reverse) of each pair, and can contain deletions, insertions, and/or any substitutions.
- For most applications, DNA oligonucleotides purified by desalting are generally sufficient, although oligonucleotides purified by HPLC or PAGE may increase the mutagenesis efficiency.
- Prepare each primer pair at a stock concentration of 100  $\mu$ M in DNase- and RNase-free water.

#### PCR Primers for Single-Site Mutagenesis

- For single-site mutagenesis (i.e., when using a single pair of mutagenic primers), a site is defined as 25 nucleotides.
  - Mutagenic primers must have unique binding sites and complementary sequences between primers must be avoided.
  - Primers that have a similar  $T_m$  and a G or a C at their 5' end work better.
  - When using A, G, T, C for modification, up to 25 bases can be modified.
  - When using degenerate bases (see **Degenerate Bases**, page 23) for modification, up to 12 bases can be modified.
  - For single-site mutagenesis using A, G, T, C for modification:
    - If the mutation site is  $\leq 12$  nucleotides long, it should be flanked by 15 nucleotides of non-mutagenic sequences.
    - If the mutation site is between 12 and 25 nucleotides long, it should be flanked by 25 nucleotides of non-mutagenic sequences.
  - For single-site mutagenesis using degenerate bases for modification:
    - If the number of degenerate bases is  $\leq 3$ , the mutation site should be flanked by 15 nucleotides of non-mutagenic sequences starting from the outer-most degenerate base(s) (i.e., on the most 5' and 3' position).
    - If the number of degenerate bases is between 3 and 12, the mutation site should be flanked by 20 nucleotides of non-mutagenic sequences starting from the outer-most degenerate bases.
- 

*Continued on next page*



## Plasmid and Primer Specifications, continued

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### Primer Specifications continued

#### PCR Primers for Multi-Site Mutagenesis

- For multi-site mutagenesis, a site is defined as 3 nucleotides.
  - Mutagenic primers must have unique binding sites and complementary sequences between primers belonging to different mutation sites must be avoided.
  - Primers that have a similar  $T_m$  and a G or a C at their 5' end work better.
  - Each site can have an insertion (A, T, G, C, and/or degenerate bases) or a deletion of up to 3 nucleotides.
  - Each multi-site mutagenesis primer must contain 15 nucleotides of non-mutagenic sequences flanking the outer-most modification(s) (i.e., mutations on the 5' and 3' position of the mutation site).
  - If there are  $\leq 50$  nucleotides between two mutation sites, we recommend using a single mutagenic primer pair to introduce them into the target plasmid.
  - If all three sites are close enough that the mutagenic primers would overlap, we recommend designing a single primer of up to 80 nucleotides.
  - If there are  $> 50$  nucleotides between two mutation sites, we recommend using separate mutagenic primer pairs to introduce them into the target plasmid.
- 

### GeneArt® Primer and Construct Design Tool

The GeneArt® Primer and Construct Design Tool is an intuitive, web-based tool to guide you when you are designing your oligonucleotide primers for multi-site mutagenesis. The tool minimizes the planning time required for designing the PCR primers, identifies potential pitfalls linked to your specific sequences, and performs *in silico* mutagenesis using your sequences. The GeneArt® Primer and Construct Design Tool also provides you with a graphic representation of the final molecule containing the mutations as well as a downloadable GenBank file compatible with VectorNTI® and other software for molecular biology workflows (see page 21).

The GeneArt® Primer and Construct Design Tool is available at <https://rnaidesigner.invitrogen.com/oligoDesigner>.

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### Ordering Custom Primers

To order custom DNA oligonucleotides, visit [www.lifetechnologies.com/oligos](http://www.lifetechnologies.com/oligos) or contact Technical Support (see page 25). Alternatively, you can order custom DNA oligonucleotides using the GeneArt® Primer and Construct Design Tool, which allows one-click online ordering of custom primers (for countries with enabled online ordering).

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*Continued on next page*

## Plasmid and Primer Specifications, continued

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### pMSDM-White Vector

The pMSDM-White Vector, included in the kit as a control plasmid, is a 5 kb pUC19-based plasmid containing the spectinomycin-resistance gene fused to the first 1,815 bp of *E. coli*'s *lacZ* gene. The gene fusion harbors 3 point mutations that inactivate the gene's ability to perform  $\alpha$ -complementation when introduced into DH5 $\alpha$ <sup>™</sup>-T1<sup>R</sup> cells. The mutations are:

- i) a C→T replacement at position 256, which generates a premature stop codon
- ii) an A deletion at position 480, which generates a frameshift
- iii) a G insertion at position 1,062, which generates a frameshift

The mutant control plasmid produces white colonies on LB agar plates containing X-galactosidase (X-gal). For a map of the pMSDM-White Vector, see page 20.

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### Control Primer Mixes

The GeneArt<sup>®</sup> Site-Directed Mutagenesis PLUS Kit includes three separate tubes of Control Primer Mixes (Control Primer Mix 1, 2, and 3). When used together, the control primer mixes are designed to revert the point mutations on the pMSDM-White Vector back to the wild-type sequence.

Only when all three mutations are reverted to the wild-type sequences does the plasmid complement the  $\phi$ 80*lacZ* $\Delta$ M15 allele present in DH5 $\alpha$ <sup>™</sup>-T1<sup>R</sup>, and the cells produce blue colonies when growing on LB agar plates containing X-gal.

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# Methylation and Mutagenesis Reactions

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## Materials Needed

### *Components supplied with the kit*

- 10X Enhancer
- DNA Methylase (4 U/ $\mu$ L)
- 200X SAM (diluted 1:8 for a 25X SAM working solution)
- PCR Water
- *Optional:* Control Primer Mixes 1, 2, and 3 for Mutagenesis Control Reaction (see page 16)

### *Additional materials needed*

- Target plasmid DNA (20 ng/ $\mu$ L)
  - Custom primers for your target plasmid (at a stock concentration of 100  $\mu$ M in DNase- and RNase-free water; see **Primer Specifications**, pages 6–7)
  - High-fidelity DNA polymerase such as the AccuPrime™ *Pfx* DNA Polymerase (see page 24 for ordering information)
- 

## Preparing 25X SAM

Create a fresh dilution of 25X SAM from the kit-supplied 200X SAM in sterile, distilled water each time you perform the mutagenesis procedure. For example, dilute 200X SAM to 25X by adding 1  $\mu$ L 200X SAM to 7  $\mu$ L PCR Water (for 8 mutagenesis reactions). Scale up or down as needed.

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25X SAM is not stable, and loses activity within a few hours after preparation. **Do not use** 25X SAM if it is more than a few hours old.

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## DNA Polymerase

The GeneArt® Site-Directed Mutagenesis PLUS Kit can be used with any high fidelity DNA polymerase. However, the reaction mixtures and PCR protocols recommended in the user guide have been optimized using the AccuPrime™ *Pfx* DNA Polymerase, because the AccuPrime™ *Pfx* DNA Polymerase is ideal for high-fidelity, high-specificity amplification of DNA fragments (see page 24 for ordering information). When using other DNA polymerases, the reaction conditions may need to be optimized.

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## Amount of Plasmid

- Use 50 ng or less of plasmid DNA per 50  $\mu$ L of methylation reaction/PCR amplification. For best results, we recommend using 20–25 ng of the target plasmid per 50  $\mu$ L of mutagenesis reaction as a starting point.
  - Using more than 50 ng of plasmid per 50  $\mu$ L reaction volume reduces the efficiency of the methylation reaction and may decrease mutagenesis efficiency.
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## Methylation and Mutagenesis Reactions, continued

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### Overview of Multi-Site Mutagenesis Procedure

The protocols on the following pages provide instructions for performing a multi-site mutagenesis procedure for three sites using three pairs of mutagenic primers (see example image below). In this procedure, the master reaction mix is prepared and divided equally amongst three tubes, into which the mutagenic primer mixes are added. Each primer mix contains a forward and a reverse primer that corresponds to a separate mutation site to prevent the primers from annealing to each other. After the mutagenic PCR amplification, the PCR products are mixed together in the recombination reaction (see page ) and then transformed into One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>™</sup>-T1<sup>R</sup> competent *E. coli* cells, which completes the multi-site mutagenesis procedure.

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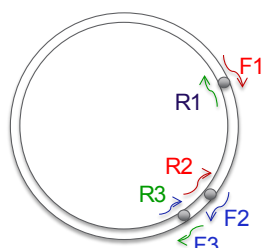


We highly recommend that you use the web-based GeneArt<sup>®</sup> Primer and Construct Design Tool to design your mutagenic primers. In addition to helping you to design the mutagenic primers, the tool gives instructions for preparing the separate mutagenic primer mixes and provides the optimum extension times for each amplification reaction. The GeneArt<sup>®</sup> Primer and Construct Design Tool is available at <https://rnaidesigner.invitrogen.com/oligoDesigner>.

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### Example for Multi-Site Mutagenesis

The following image represents a hypothetical multi-site mutagenesis procedure and it is provided as an example to illustrate the primer mixing scheme.



**F1:** Forward primer for Site 1  
**R1:** Reverse primer for Site 1

**F2:** Forward primer for Site 2  
**R2:** Reverse primer for Site 2

**F3:** Forward primer for Site 3  
**R3:** Reverse primer for Site 3

---

### Preparing Primer Mixes

1. Prepare the following primer mixes using the 100  $\mu$ M primer stocks. The final working concentration of each primer will be 10  $\mu$ M.

**Primer Mix 1:** 16  $\mu$ L H<sub>2</sub>O + 2  $\mu$ L of R1 + 2  $\mu$ L of F3

**Primer Mix 2:** 16  $\mu$ L H<sub>2</sub>O + 2  $\mu$ L F1 + 2  $\mu$ L of R2

**Primer Mix 3:** 16  $\mu$ L H<sub>2</sub>O + 2  $\mu$ L of F2 + 2  $\mu$ L of R3

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*Continued on next page*

## Methylation and Mutagenesis Reactions, continued

### Methylation and Mutagenesis Reaction

1. Prepare the following reaction mixture:

Component	Volume	Final Concentration
10X AccuPrime™ <i>Pfx</i> Reaction buffer	6 µL	1X
10X Enhancer	6 µL	1X
Plasmid DNA (20 ng/µL)	1.2 µL	24 ng
DNA Methylase (4 U/µL)	1.2 µL	4.8 units
25X SAM	2.4 µL	1X
AccuPrime™ <i>Pfx</i> (2.5 U/µL)	0.5 µL	1.5 units
PCR water	to 57 µL	—

2. Mix the reaction mixture by pipetting it up and down, and aliquot 19 µL each into three PCR tubes labeled 1, 2, and 3.
3. Add 1 µL of Primer Mix 1 into Tube 1, 1 µL of Primer Mix 2 into tube 2, and 1 µL of Primer Mix 3 to Tube 3.
4. Perform the methylation reaction and PCR using the following parameters:

Temperature	Duration	Number of Cycles
37°C	12–20 minutes*	1
94°C	2 minutes	
94°C	20 seconds	12–18 cycles†
57°C	30 seconds	
68°C	30 seconds/kb of plasmid	
68°C	5 minutes	1
4°C	As necessary	1

\*Carry out the methylation of the plasmid at 37°C for 12–20 minutes. We recommend 12–15 minutes for 2.8–5 kb plasmids and 15–20 minutes for 5–14 kb plasmids.

†The cycling parameters specify a 30-seconds extension for each 1 kb of DNA. For optimal mutagenesis efficiency, we recommend 12–15 cycles for 2.8–5 kb plasmids and 15–18 cycles for 5–14 kb plasmids.

### Analyzing the PCR Products

1. After the reaction, analyze 5 µL of the product on a 0.8% agarose gel.

**Note:** If you observe very faint bands, increase the amount of target plasmid up to 50 ng (see **Amount of Plasmid**, page 9). Note that in some cases you may obtain high mutagenesis efficiency even with multiple or faint bands on an agarose gel.

2. Proceed directly to the **Recombination Reaction**, page 12, and store excess mutagenesis reaction product at –20°C.

# Recombination Reaction

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## Introduction

The *in vitro* recombination reaction is required multi-site mutagenesis reactions. Although not essential for single-site mutagenesis, the recombination reaction boosts the mutagenesis efficiency and increases the colony yield 3- to 10-fold in single-site mutagenesis reactions.

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## Recombination Reaction

1. Set up the following 20- $\mu$ L recombination reaction using the PCR products from page 11.

Component	Volume	Final Concentration
PCR water	4 $\mu$ L	—
PCR product from Tube 1	2 $\mu$ L	—
PCR product from Tube 2	2 $\mu$ L	—
PCR product from Tube 3	2 $\mu$ L	—
GeneArt <sup>®</sup> 2X Enzyme Mix	10 $\mu$ L	1X

**Note:** Thaw the GeneArt<sup>®</sup> 2X Enzyme Mix on ice. The GeneArt<sup>®</sup> 2X Enzyme Mix might appear slightly turbid; this is normal and does not affect the enzyme activity. Do **not** centrifuge the enzyme mix to produce a clarified supernatant.

2. Mix well and incubate at room temperature for 15 minutes.
  3. Stop the reaction by adding 1  $\mu$ L 0.5 M EDTA. Mix well and place the tubes on ice.
  4. Place the tubes on ice and immediately proceed to transformation (see page 13).
-

# Transformation into DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> *E. Coli*

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## General Handling

One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> competent cells are supplied with the GeneArt<sup>®</sup> Site-Directed Mutagenesis PLUS Kit. Be extremely gentle when working with competent cells. Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Perform the transformation procedure immediately after thawing the cells on ice. **Mix by swirling or tapping the tube gently, not by pipetting or vortexing.**

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## Materials Needed

*Components supplied with the kit*

- One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> competent cells

- S.O.C. Medium

*Additional materials needed*

- 37°C shaking and non-shaking incubator
  - 10-cm diameter LB agar plates with the appropriate selection antibiotic
  - Ice bucket with ice
  - 42°C water bath
  - Test tube rack to hold all transformation tubes so that they can be put into the 42°C water bath at once
  - *Optional:* Plating beads (such as ColiRollers<sup>TM</sup> Plating Beads available from EMD Chemicals, Cat. no. 71013-3)
  - *Optional:* X-gal
- 

## Before Starting

- Equilibrate a water bath to 42°C
  - Warm a 200- $\mu$ L vial of S.O.C. medium to room temperature
  - Spread X-gal onto LB agar plates with antibiotic, if desired
- 

## Transformation Procedure

1. Thaw on ice one 50- $\mu$ L vial of One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> competent cells for each transformation. Thaw for approximately 5–7 minutes (no more than 20 minutes). For multiple reactions (vials), number the tube caps.  
**Note:** One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> cells are mcrBC+. Other seemingly similar competent cells, such as **TOP10**, **cannot be used** in place of DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> cells.
  2. Transfer 3  $\mu$ L from the recombination reaction directly into each vial of cells and mix by tapping gently. **Do not mix by pipetting up and down.**  
**Note:** To determine methylation efficiency, use 2  $\mu$ L of the methylation reaction (from Step 4, page 15) with and without SAM for transformation.
  3. Cap the vials, cover completely with ice, and incubate for 15 minutes.
  4. Transfer vials to a test tube rack and incubate the entire rack at once for exactly 30 seconds in the 42°C water bath. Do not mix or shake.
  5. Remove the rack of vials from the 42°C bath and place it on ice for 2 minutes.
- 

*Continued on next page*

## Transformation into DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> *E. Coli*, continued

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### Transformation Procedure, continued

6. Remove the vials from ice, then de-cap and add **250  $\mu$ L** of pre-warmed S.O.C. medium to each vial.  
**Note:** S.O.C. is a rich medium; sterile technique must be practiced to avoid contamination.
  7. Recap the vials and place them in a microcentrifuge rack. Secure the vials with tape and incubate them at 37°C for exactly 1 hour in a shaking incubator set to 225 rpm.  
**Note:** Incubation of the transformation reaction at 37°C for 1 hour is not necessary for ampicillin-resistant plasmids. However, the 1-hour incubation is required for kanamycin or spectinomycin-resistant plasmids.
  8. While the vials are shaking, label the LB agar plates containing the appropriate antibiotic. For blue-white screening, supplement the plates with X-Gal.
  9. Transfer 5 to 8 plating beads (such as ColiRollers<sup>TM</sup> Plating Beads, see page 13) to each agar plate and warm the plates for about 30 minutes in a 37°C incubator.
  10. Plate 30–100  $\mu$ L of the cell suspension on LB agar plates containing the appropriate antibiotics.  
**Note:** The plating volume depends on the transformation efficiency of DH5 $\alpha$ <sup>TM</sup>-T1<sup>R</sup> cells and the incubation duration at 37°C. Plate a range of volumes to ensure proper cell density. Do not allow cells to settle before aliquotting. If cells settle, mix by tapping the tube.
  11. Spread cells by shaking vigorously in a circular motion, clockwise and counter clockwise for approximately 1–2 minutes.
  12. Store the remaining transformation reaction at 4°C.
  13. Invert the plates and incubate at 37°C for 16–18 hours.
  14. Select 3 to 5 colonies and analyze by plasmid isolation, PCR, or sequencing.  
**Note:** Partially expressed *lacZ* genes containing the control mutation may result in colonies that are light blue on the inside and white on the outside. Light blue colonies should be considered mutant colonies and not wild-type.
-



# Methylation Control

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## Introduction

This optional control procedure can be used to test the methylation reaction. Note that the GeneArt® Site-Directed Mutagenesis PLUS Kit provides sufficient reactions for 10 site-directed mutagenesis reactions and 1 mutagenesis control reaction. If you wish to perform the methylation control reaction, you will have to use the reagents set aside for one of the mutagenesis reactions.

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## Methylation Control Reaction

1. To determine methylation efficiency, set up an additional control sample containing all the components except SAM during PCR sample preparation.
2. Approximately 2 minutes before the end of incubation period at 37°C, pause the PCR program.
3. Transfer 2 µL of the two PCR samples (one with SAM and one without SAM) to autoclaved tubes containing 8 µL of 20 mM EDTA.
4. Thaw on ice two 50-µL vials of One Shot® MAX Efficiency® DH5α™-T1<sup>R</sup> competent cells: one for the PCR/EDTA sample with SAM and one for the PCR/EDTA sample without SAM.
5. Proceed with the **Transformation Procedure**, as described on page 13.
6. Determine the methylation efficiency using the following formula:

$$\% \text{ methylation} = \frac{\text{Total colonies (w/o SAM)} - \text{Total colonies (w/ SAM)}}{\text{Total colonies (w/o SAM)}}$$

**Expected efficiency:** ≥90 percent.

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# Mutagenesis Control

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## Introduction

Use the pMSDM-White Vector and the Control Primer Mixes (included in the kit) as positive mutagenesis controls for The GeneArt® Site-Directed Mutagenesis PLUS Kit and for calculating the mutagenesis efficiency.

The mutant control plasmid produces white colonies on plates containing LB/Amp/X-gal. A successful methylation and mutagenesis reaction with all three control primer pairs reverts the *lacZα* gene into wild type, which produces blue colonies on plates containing LB/Amp/X-gal.

Note that the GeneArt® Site-Directed Mutagenesis PLUS Kit provides sufficient reactions for 10 site-directed mutagenesis reactions and 1 mutagenesis control reaction. If you have performed the methylation control reaction (see page 15), you will have to use the reagents set aside for one of the mutagenesis reactions to perform the mutagenesis control reaction.

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## Before Starting

For the mutagenesis control reaction, prepare LB agar plates with 100 µg/mL ampicillin and 30–100 µg/mL X-gal (see page 24 for ordering information) prior to transformation.

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## Mutagenesis Control Reactions

1. Prepare the following reaction mixture for methylation and mutagenesis:

Component	Volume
10X AccuPrime™ <i>Pfx</i> Reaction buffer	6 µL
10X Enhancer	6 µL
pMSDM-White Vector (20 ng/µL)	1.2 µL
DNA Methylase (4 U/µL)	1.2 µL
25X SAM	2.4 µL
AccuPrime™ <i>Pfx</i> (2.5 U/µL)	0.5 µL
PCR water	to 57 µL

2. Mix and aliquot the PCR mixture into three PCR tubes (Tubes 1, 2, and 3), each containing approximately 19 µL of the mix.
- 

*Continued on next page*

## Mutagenesis Control, continued

### Mutagenesis Control Reactions, continued

- Add 1  $\mu\text{L}$  of Control Primer Mix 1 into Tube 1, 1  $\mu\text{L}$  of Control Primer Mix 2 into Tube 2, and 1  $\mu\text{L}$  of Control Primer Mix 3 into Tube 3.
- Perform PCR using the following parameters.

Temperature	Duration	Number of Cycles
37°C	15–20 minutes	1
94°C	2 minutes	
94°C	20 seconds	16 cycles
57°C	30 seconds	
68°C	30 seconds–2.5 minutes*	
68°C	1–5 minutes <sup>†</sup>	1
4°C	As necessary	1

\*2.5 minutes for Tube 1, 30 seconds for Tubes 2 and 3.

<sup>†</sup>5 minutes for Tube 1, 1 minute for Tubes 2 and 3.

- Set up the following 20- $\mu\text{L}$  recombination reaction using the PCR products from page 16.

Component	Volume
PCR water	6 $\mu\text{L}$
PCR product from Tube 1	2 $\mu\text{L}$
PCR product from Tube 2	2 $\mu\text{L}$
PCR product from Tube 3	2 $\mu\text{L}$
2X GeneArt <sup>®</sup> Enzyme Mix	10 $\mu\text{L}$

**Note:** Thaw the 2X GeneArt<sup>®</sup> Enzyme Mix on ice. The 2X enzyme mix may appear turbid. Mix the solution by briefly vortexing, and then very briefly centrifuging it. Do not centrifuge the solution at high speed.

- Mix the reaction mixture well and incubate at room temperature for 15 minutes.
- Stop the reaction by adding 1  $\mu\text{L}$  0.5 M EDTA. Mix well and place the tubes on ice.
- Place the tubes on ice and immediately proceed to transformation (see page 13).
- After transformation and incubation, count blue and white colonies on the plates.

**Results:** The percentage of blue colonies (wild-type) should be  $\geq 85\%$ .

# Transformation Control

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## Introduction

Use the pUC19 plasmid supplied with the competent cells in the  $-80^{\circ}\text{C}$  module to test the transformation efficiency.

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## Transformation Control Reaction

If you do not obtain the expected number of colonies, we recommend that you test the transformation efficiency of the competent cells. Transform One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ <sup>™</sup>-T1<sup>R</sup> competent cells with the supercoiled pUC19 plasmid included in the kit as described below.

1. Prepare LB agar plates containing 100  $\mu\text{g}/\text{mL}$  ampicillin.
  2. Transform 5  $\mu\text{L}$  (50 pg) of pUC19 into 50  $\mu\text{L}$  of competent cells.
  3. Incubate the vial on ice for 30 minutes.
  4. Incubate the vial for exactly 30 seconds in the  $42^{\circ}\text{C}$  water bath. Do not mix or shake.
  5. Remove the vial from the  $42^{\circ}\text{C}$  bath and place on ice.
  6. Add **250  $\mu\text{L}$**  of pre-warmed S.O.C. medium to each vial.  
**Note:** S.O.C. is a rich medium; sterile technique must be practiced to avoid contamination.
  7. Place the vial in a microcentrifuge rack on its side and secure with tape to avoid loss of the vial. Incubate the vial at  $37^{\circ}\text{C}$  for exactly 1 hour in a shaking incubator set to 225 rpm.
  8. Dilute the transformation reaction 1:100 and plate 30  $\mu\text{L}$  on LB/Amp plates.
  9. Incubate the plates overnight at  $37^{\circ}\text{C}$  and count colonies. Calculate transformation efficiency using the formula below.
- 

## Calculation

Calculate the transformation efficiency as transformants per 1  $\mu\text{g}$  of plasmid DNA. **Use the formula below to calculate transformation efficiency:**

$$\frac{\# \text{ of colonies}}{50 \text{ pg plasmid DNA}} \times \frac{10^6 \text{ pg}}{\mu\text{g}} \times \frac{300 \mu\text{L (total transformation volume)}}{30 \mu\text{L (plated)}} \times 100 \text{ (dil'n factor)} = \frac{\# \text{ of colonies}}{\mu\text{g plasmid DNA}}$$

**Expected transformation efficiency:**  $\geq 1 \times 10^9$  cfu/ $\mu\text{g}$  supercoiled plasmid

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## Troubleshooting

Problem	Cause	Solution
PCR product not visible	Too little DNA	Determine DNA concentration. Use up to 50 ng DNA per 50 $\mu$ L reaction.
	Poor quality of DNA	Purify new plasmid. We recommend using the PureLink <sup>®</sup> Quick Plasmid Miniprep Kit or the PureLink <sup>®</sup> Hi Pure Plasmid Maxiprep Kit (see page 24 for ordering information).
	DNA polymerase	AccuPrime <sup>™</sup> Pfx DNA Polymerase is recommended for The GeneArt <sup>®</sup> Site-Directed Mutagenesis PLUS Kit. Use 1 unit of AccuPrime <sup>™</sup> Pfx DNA Polymerase for amplification.
	PCR conditions	Optimize annealing temperature and extension time. We recommend an extension time of 30 seconds per 1 kb. However, you should experiment with different extension times.
	Poor primer design	Use the GeneArt <sup>®</sup> Primer and Construct Design Tool to reduce potential secondary structure or increase primer length. The tool is available at <a href="https://rnaidesigner.invitrogen.com/oligoDesigner">https://rnaidesigner.invitrogen.com/oligoDesigner</a> .
High background when testing mutagenesis reaction product	Inactive DNA methylase or inactive SAM	Test the activity of the DNA methylase and 25X SAM using the methylation control reaction on page 15. <b>Note:</b> The GeneArt <sup>®</sup> Site-Directed Mutagenesis PLUS Kit provides sufficient reactions for 10 site-directed mutagenesis reactions and 1 mutagenesis control reaction. If you wish to perform the methylation control reaction, you will have to use the reagents set aside for one of the mutagenesis reactions.
	Too much DNA	Use no more than 50 ng of DNA per 50 $\mu$ L of methylation reaction.
	Over-amplification	Reduce PCR cycles to 12 for small plasmids and 15 for intermediate size plasmids.
Too few colonies	Dilution for plating is too high	Decrease dilution of transformants for plating.
No blue colonies on control plate <b>Note:</b> pMSDM-White Control Vector should render blue colonies when transformed into DH5 $\alpha$ <sup>™</sup> -T1 <sup>R</sup> cells	Inactive X-gal or insufficient amount of X-gal added to plate	Use a fresh stock of X-gal. Spread 100 $\mu$ g/mL X-gal on LB plates.

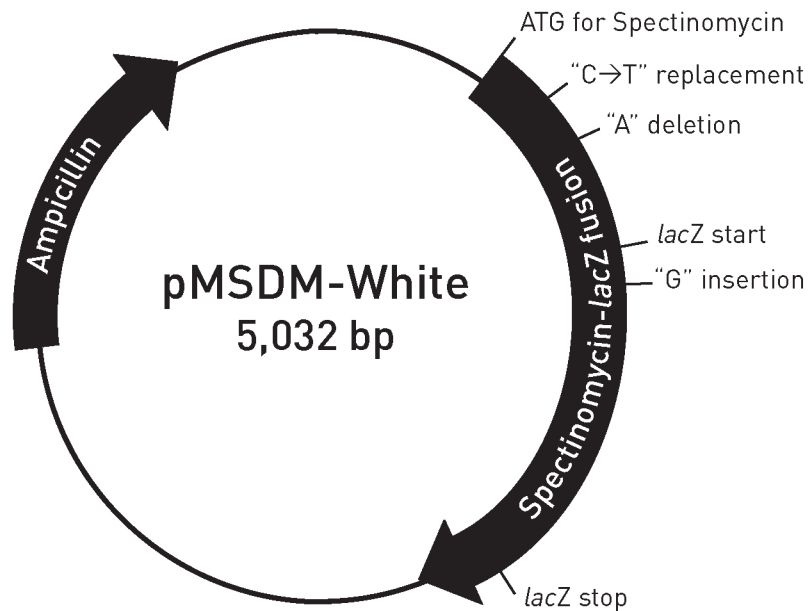
## Appendix A: Vectors

### pMSDM-White Vector

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#### Map of pMSDM-White

The figure below shows the features of the pMSDM-White Vector. The sequence is available at [www.lifetechnologies.com](http://www.lifetechnologies.com) or by contacting Technical Support (see page 25).



#### Comments for pMSDM-White 5,032 nucleotides

Spectinomycin-*lacZ* fusion: bases 1–2,829

*lacZ* gene: bases 1,015–2,741

Ampicillin (*bla*) resistance gene: bases 3,143–4,000

#### Mutations:

"C→T" replacement: position 256

"A" deletion: position 480

"G" insertion: position 1,063

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## Appendix B: Tools for Primer Design

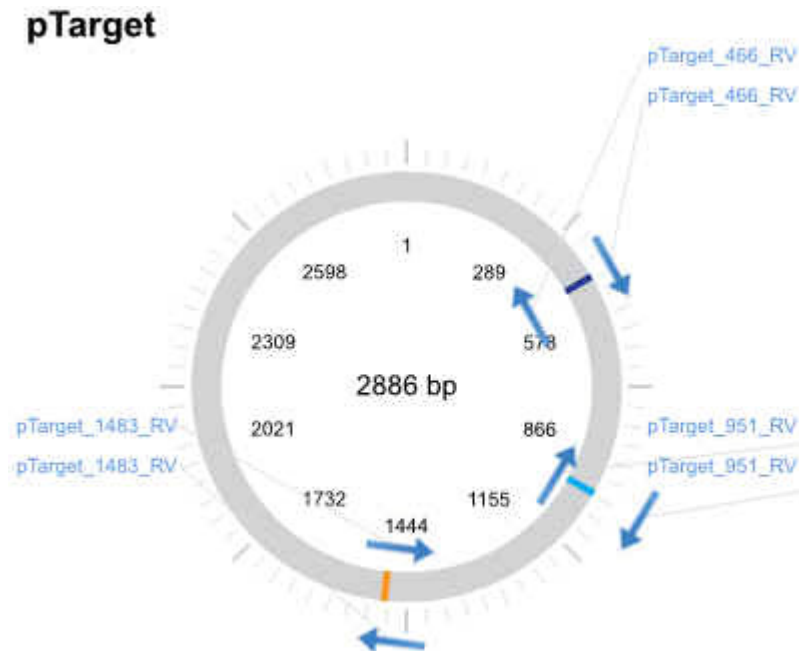
### GeneArt® Primer and Construct Design Tool

#### Introduction

The GeneArt® Primer and Construct Design Tool is an intuitive, web-based tool to guide you when you are designing your oligonucleotide primers for multi-site mutagenesis. The tool minimizes the planning time required for designing the PCR primers, identifies potential pitfalls linked to your specific sequences, performs *in silico* mutagenesis using your sequences, and allows one-click online ordering for custom primers (for countries with enabled online ordering). The GeneArt® Primer and Construct Design Tool also provides you with a graphic representation of the final molecule containing the mutations as well as a downloadable GenBank file compatible with VectorNTI® and other software for molecular biology workflows. The GeneArt® Primer and Construct Design Tool is available at <https://rnaidesigner.invitrogen.com/oligoDesigner>.

#### Output example from GeneArt® Primer and Construct Design Tool

The example below shows the output from GeneArt® Primer and Construct Design Tool for a multi-site mutagenesis experiment. In this example, three mutations are introduced into the pTarget plasmid using three separate mutagenic primer pairs, because the mutations are placed sufficiently apart (see **GeneArt® Mutagenic Primer Design Rules**, see page 22). In addition to the graphical representation and sequences of the mutagenic primer pairs and the target plasmid, the tool also provides detailed instructions for performing the site-directed mutagenesis reaction.



Continued on next page

## GeneArt® Primer and Construct Design Tool, continued

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### GeneArt® Mutagenic Primer Design Rules

The GeneArt® Primer and Construct Design Tool uses the following rules when designing oligonucleotide primers for multi-site mutagenesis.

- The tool restricts the size of the plasmid or gene to be mutagenized to 14 kb.
  - For single-site mutagenesis using canonical bases (i.e., A, C, G, T), the tool places the following constraints:
    - If the mutated bases span a region of up to 12 bp, then 15 nt on each side of the most external mutations are non-editable.
    - If the mutated bases span a region between 12 and 25 bases, then 25 nt on each side of the most external mutations are non-editable.
    - Mutations spanning a region of >25 bp are not allowed.
  - For single-site mutagenesis using degenerate bases (see page 23), the tool places the following constraints:
    - If the number of degenerate bases is  $\leq 3$ , the mutation site is flanked by 15 nt of non-editable (i.e., non-mutagenic) sequences starting from the outer-most degenerate base(s) (i.e., on the most 5' and 3' position).
    - If the number of degenerate bases is between 3 and 12, the mutation site is flanked by 20 nt of non-editable (i.e., non-mutagenic) sequences starting from the outer-most degenerate bases.
    - Mutations spanning a region of >12 bp are not allowed.
  - For single-site mutagenesis using degenerate bases, the tool allows up to 12 nt to be modified.
  - For multi-site mutagenesis, 15 nt on each side of the most external mutations are non-editable.
  - For multi-site mutagenesis, the tool designs a single primer of up to 80 nt if all three mutation sites are close enough that separate mutagenic primers would overlap.
  - For multi-site mutagenesis, if there are more than 50 bases between two site modifications, the tool designs separate primers to capture these mutations.
  - When possible, the tool designs primers with a sequence-specific partial melting temperature ( $T_m$ ) of 60–68°C.
-



## Degenerate Bases

### IUPAC Notation for Degenerate Bases

Degenerate bases represent a position on a DNA sequence that can have multiple possible alternatives. Degenerate bases should not be confused with non-canonical bases because each particular degenerate base is in fact one of the four nucleotides commonly found in DNA (i.e., A, C, G, T).

Under the commonly used IUPAC (International Union of Pure and Applied Chemistry) system, nucleobases are represented by the first letters of their chemical names: Adenine, Cytosine, Guanine, and Thymine. This shorthand also includes eleven "ambiguity" or "degenerate" characters associated with every possible combination of the four DNA bases. The degenerate characters were designed to encode positional variations found among families of related genes. The IUPAC notation, including degenerate characters and suggested mnemonics, is shown in the table below.

Symbol	Description	Bases represented				
<b>A</b>	Adenine	A				1
<b>C</b>	Cytosine		C			
<b>G</b>	Guanine			G		
<b>T</b>	Thymine				T	
<b>U</b>	Uracil				U	
<b>W</b>	Weak	A			T	2
<b>S</b>	Strong		C	G		
<b>M</b>	Amino	A	C			
<b>K</b>	Keto			G	T	
<b>R</b>	PuRine	A		G		
<b>Y</b>	PYrimidine		C		T	3
<b>B</b>	Not A ( <b>B</b> comes after A)		C	G	T	
<b>D</b>	Not C ( <b>D</b> comes after C)	A		G	T	
<b>H</b>	Not G ( <b>H</b> comes after G)	A	C		T	
<b>V</b>	Not T ( <b>V</b> comes after T and U)	A	C	G		4
<b>N</b>	ANy base (not a gap)	A	C	G	T	

## Appendix C: Ordering Information

### Accessory Products

**Additional Products** The following additional products may be used with The GeneArt® Site-Directed Mutagenesis PLUS Kit. For more information, visit [www.lifetechnologies.com](http://www.lifetechnologies.com) or contact Technical Support (see page 25).

Product	Amount	Cat. no.
One Shot® MAX Efficiency® DH5α™-T1 <sup>R</sup>	1 box (21 × 50 µL)	12297-016
Ampicillin	200 mg	11593-027
X-gal	1 g	15520-018
AccuPrime™ Pfx DNA Polymerase*	200 reactions	12344-024
	1,000 reactions	12344-032
PureLink® Quick Plasmid Miniprep Kit	50 preps	K2100-10
PureLink® Hi Pure Plasmid Maxiprep Kit	10 preps	K2100-06

# Documentation and Support

## Obtaining Support

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### Technical Support

For the latest services and support information for all locations, go to [www.lifetechnologies.com](http://www.lifetechnologies.com).

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
  - Search through frequently asked questions (FAQs)
  - Submit a question directly to Technical Support ([techsupport@lifetech.com](mailto:techsupport@lifetech.com))
  - Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
  - Obtain information about customer training
  - Download software updates and patches
- 

### Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at [www.lifetechnologies.com/sds](http://www.lifetechnologies.com/sds).

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### Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at [www.lifetechnologies.com/termsandconditions](http://www.lifetechnologies.com/termsandconditions). If you have any questions, please contact Life Technologies at [www.lifetechnologies.com/support](http://www.lifetechnologies.com/support).

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