TagMaster® Site-Directed Mutagenesis Kit

For 1-60 bp Substitution/Insertion & 1-660bp Deletion

INSTRUCTION MANUAL

Catalog # 7001 (20 reactions) and Catalog # 7002 (6 reactions)

Revision A

For Research Use Only. Not for Use in Diagnostic Procedures

GM Biosciences www.gmbiosciences.com

TagMaster® Site-Directed Mutagenesis Kit Catalog # 7001, # 7002

QUICK-REFERENCE PROTOCOL

1. Prepare sample reaction(s) and control reactions as indicated below:

Sample Reactions	Point Mutation (1-3bp)	Other Mutation*
10X Reaction Buffer	5 µl	5 µl
dsDNA template plasmid (20-100ng)	*X µl*	*X µl*
dNTP Mixture (2mM each)	5 µl	5 µl
$MgSO_4$ (25mM)	4 µl	3 µl
Forward Mutagenic Primer (20µM)	1 µl	1 µl
Reverse Mutagenic Primer (20µM)	1 µl	1 µl
50x TagMaster Supplement	----	1 µl
ddH_2O to a final volume of 49µl	*Y ul*	*Y ul*
TagMaster Enzyme (1.0U/µl)	1 µl	1 µl

Control Reactions	Negative Control	Substitute 2bp	Insert 6xHis Tag
10X Reaction Buffer	5 µl	5 µl	5 µl
pUC19 Control Template (50ng/µl)	1 µl	1 µl	1 µl
dNTP Mixture (2mM each)	5 µl	5 µl	5 µl
$MgSO_4$ (25mM)	4 µl	4 µl	3 µl
Control Primer #1 (20µM)	----	1 µl	----
Control Primer #2 (20µM)	----	1 µl	----
Control Primer #3 (20µM)	----	----	1 µl
Control Primer #4 (20µM)	----	----	1 µl
50x TagMaster Supplement	----	----	1 µl
ddH_2O to a final volume of 49µl	34 µl	32 µl	32 µl
TagMaster Enzyme (1.0U/µl)	1 µl	1 µl	1 µl

*** Long-Range Mutation (4-60bp), 4-660bp Deletion, Multiple Sites Mutation (2 sites)**

2. Perform reaction using the following parameters:

	Temperature	Time
1 cycle	95°C	2 minutes
20 cycles	95°C	20 seconds
	60°C	10 seconds
	68°C	30 seconds/ kb of plasmid length
1 cycle	68°C	5 minutes
1 cycle	4°C	forever

3. Directly transform 5 µl of each reaction into 50 µl of TagMaster Competent cells:

Note *Any enzymatic treatment of the reaction mixture is unnecessary. The reaction mixture should be directly transformed into TagMaster Competent cells.*

- ► Add 5 µl of ice-chilled each reaction into 50 µl TagMaster Competent cells, gently swirl the tube
- ► Incubate on ice for 30min
- ► Heat-shock the tube in a 42°C water bath for 30 seconds, put on ice for 2 minutes
- ► Add 250 µl of SOC medium (pre-warm to room temperature or to 37°C) to each tube
- ► Shake at 250rpm for 1 hour. Spread appropriate volume on agar plate, and culture at 37°C overnight

TagMaster® Site-Directed Mutagenesis Kit

CONTENTS

TagMaster® Site-Directed Mutagenesis Kit

KIT CONTENTS

	Catalog # 7001[a]	Catlog # 7002[b]
TagMaster Enzyme (1.0U/µl) [d]	20 reactions	6 reactions
10x Reaction Buffer [d]	100 µl	30 µl
dNTP Mixture (2mM each) [c d]	100 µl	30 µl
$MgSO_4$ (25mM)	80 µl	24 µl
Control Primer #1 (20µM)	10 µl	10 µl
Control Primer #2 (20µM)	10 µl	10 µl
Control Primer #3 (20µM)	10 µl	10 µl
Control Primer #4 (20µM)	10 µl	10 µl
50x TagMaster Supplement [d]	20 µl	10 µl
TagMaster Competent Cells [d]	4 x 300 µl	1 x 300 µl
pUC19 Control Plasmid (50ng/µl)	10 µl	10 µl

[a] The TagMaster Site-Directed Mutagenesis Kit (Catlog# 7001) contains enough reagents for 20 total reactions, which includes 3 control reactions.

[b] The TagMaster Site-Directed Mutagenesis Kit (Catlog# 7002) contains enough reagents for 6 total reactions, which includes 3 control reactions.

[c] Thaw the dNTP mixture once, prepare single-use aliquots, and store the aliquots at –20C. **Do not subject dNTP mixture to multiple freeze-thaw cycles.**

[d] The composition of TagMaster Enzyme, 10x Reaction Buffer, dNTP mixture, 50x TagMaster Supplement, and TagMaster Competent Cells are proprietary. TagMaster is a trademark of GM Biosciences,Inc.

STORAGE CONDITIONS

TagMaster Competent cells: -80°C

All other Components: -20°C

MATERIALS NEEDED BUT NOT PROVIDED

S.O.C. Medium: see *Preparation of Media*

INTRODUCTION

The TagMaster Site-directed Mutagenesis Kit* provides a rapid, efficient and accurate mutagenesis method to generate mutant plasmid. It has been optimized for mutagenesis of plasmids of up to 10kb. In addition to point mutation, the kit allows the highest accuracy and efficiency to introduce a variety of mutations: insertion (~60bp), substitutions (~60bp), deletion (~660bp) and multiple sites mutation (two sites). It is easy to introduce a commonly used tag such as 6His, Myc, Flag, HA, V5 into a gene by a single mutagenesis reaction. Exclusive to the TagMaster Site-Directed Mutagenesis kit is the proprietary high fidelity enzyme recipe and the most advanced competent cells, which together allow for mutagenesis in approximately 1~2 hour, plus an overnight transformation.

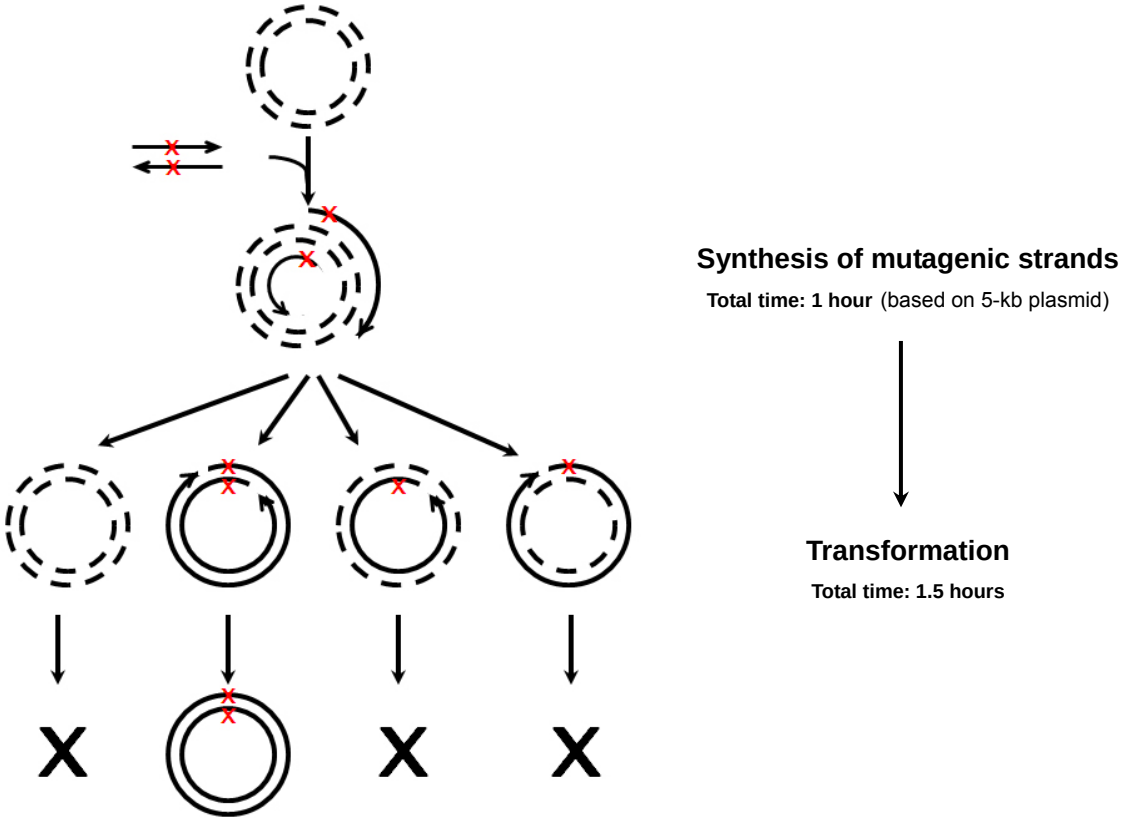

Fig 1. Flowchart of TagMaster Site-Directed Mutagenesis

Site-directed mutagenesis is one of the most commonly used techniques in biology for studying gene function, protein expression, protein modification, and for manipulation of vector. Several approaches to this technique have been available[1-8], but these approaches require single-stranded DNA (ssDNA), specialized vector, unique restriction sites, multiple transformation, in vitro methylation treatment steps, or in vitro DpnI treatment steps. The TagMaster Site-Directed Mutagenesis

kit does not require all the above steps. The kit consists of only two-step procedures (mutagenesis reaction and transformation).

The TagMaster Enzyme is a novel proprietary blend that has a comparable fidelity as *pfu* DNA polymerase or *Phusion* DNA polymerase, which ensures that the mutagenesis reaction has the highest fidelity. The basic procedure utilizes a dsDNA plasmid as template and two synthetic reverse complementary primers. The design of the complete reverse complementary primers ensures that only original parent plasmid would be used as template in each reaction cycle, which dramatically improves the accuracy of the mutagenesis reaction. The mutagenesis reaction containing both the parent template and mutagenized daughter DNA is then transformed into TagMaster Competent Cell. The TagMaster Cells would discriminate and eliminate parent plasmid, and thus specifically allow mutagenized daughter DNA to replicate and to be enriched thereafter.

Note *Plasmid DNA isolated from almost all of the commonly used E.coli strain (dam+) is suitable for mutagenesis, whereas plasmid DNA isolated from dam- E.coli strain, such as JM110 and SCS110, is not suitable.*

The TagMaster Site-Directed Mutagenesis Kit may be used to make substitution/insertion for up to 60bp. It can introduce any commonly known tag such as 6His(18bp), Flag(24bp), Myc tag(30bp), HA tag (27bp), V5 tag(42bp) into any location of a plasmid by a single mutagenesis reaction. Protein targeting signals, such as nuclear localization signal, secretion signal peptide, can be inserted into a gene as well, as long as the peptide signal is less than 20 amino acids (60bp) long.

The kit is also powerful in deleting fragment from short to long. A GST gene (660bp) has been successfully deleted from a GST expression plasmid by a single mutagenesis reaction. It is also capable of performing multiple sites mutation. Two separate sites can be mutated simultaneously by a single mutagenesis reaction.

Combination of the simplicity, unparallel efficiency and accuracy, this kit represents the most powerful *in vitro* site-directed mutagenesis kit in the world.

* Patent Pending

TagMaster MUTAGENESIS CONTROL

The pUC19 is a commonly used plasmid with 2686bp in length. The pUC19 plasmid is purified from DH5a and is used as a control to test the mutagenesis efficiency of the TagMaster Mutagenesis kit. The kit provides both control primers (#1, #2) for introducing point mutation and control primers (#3, #4) for inserting a 6His tag.

The control primer #1 and #2 are reverse complement of each other. They are designed for 2bp substitution in LacZ gene in pUC19.

Control primer #1:

*5'-gtacccggggatcctct**CA**agtcgacctgcaggcat-3'*

Control primer #2:

*5'-atgcctgcaggtcgact**TG**agaggatccccgggtac-3'*

The control primer #3 and #4 are reverse complement of each other. They are designed for inserting a 6His tag (18bp) into LacZ gene in pUC19.

Control primer #3:

*5'-gcctgcaggtcgact**CACCACCACCACCACCAC**tagaggatccccggg-3'*

Control primer #4:

*5'-cccggggatcctcta**GTGGTGGTGGTGGTGTG**agtcgacctgcaggc-3'*

Note *The β-galactosidase activity in TagMaster Competent cell has not been abolished, therefore TagMaster cell is not suitable for blue-white selection. All the clones growing in TagMaster Competent cell should be blue (in the presence of X-gal and IPTG) no matter whether LacZ gene in plasmid is mutated or not.*

MUTAGENIC PRIMER DESIGN

Careful primers design is critical for successful mutagenesis reaction. Please follow the following guidelines when designing your primers.

General Guidelines:

- Both primers (forward and reverse) should contain the desired mutation.
- The desired mutation should be in the middle of the primers. Primers are composed of DNA sequences before mutation site, mutated sequences, and DNA sequences after mutation sites.
- Forward primer and reverse primer should be completely reverse complement of each other except for multiple sites mutagenesis.

Note *The completely reverse complement of forward and reverse primers ensures that novel synthesized daughter chains from previous cycle cannot be used as template for DNA synthesis in next round cycle. In each cycle of DNA synthesis, only the original plasmid templates are used as template. Therefore, potential erroneous DNA synthesis in previous cycle cannot be amplified and accumulated in next cycle. It ensures the accuracy of DNA synthesis during the mutagenesis reaction. Combined with our highest fidelity TagMaster Enzyme, the TagMaster mutagenesis kit has the unparalleled accuracy of DNA synthesis.*

The amount of DNA synthesis in this mutagenesis reaction increase linearly, which is in contrast to traditional PCR (the amount of DNA synthesized increase exponentially because of daughter DNA can be used as template in next round DNA synthesis).

Note *For multiple sites mutagenesis, forward primer should not be reverse complement to the reverse primer. Please see below for multiple sites mutagenesis primers design.*

- Primers do not need to be 5'-phosphorylated.
- For most applications, primers purified by desalting are generally sufficient, although purification by HPLC or PAGE may increase the mutagenesis efficiency.

Point Mutation (1-3bp):

In addition to the *General Guidelines* mentioned above, primers design for point mutation has the following considerations. Point mutation means mutation (substitution, or insertion, or deletion) of a short-range (1~3bp).

- Primers should be between 25 and 45 bases in length.

- Primers should have a melting temperature (Tm) of ≥78°C, following the formula below:

  Tm=81.5 + 0.41(%GC) – 675/ N – % mismatch

  ("*N*" is the primer length in bases, value for "*%GC*" and "*% mismatch*" are whole numbers)

Example: *substitution of 2bp (from AG to CA) in LacZ gene in pUC19*

Original sequences:	*5'……aattcgagctcggtacccggggatcctct**AG**agtcgacctgcaggcat gcaagcttgg……3'*
Desired sequences:	*5'……aattcgagctcggtacccggggatcctct**CA**agtcgacctgcaggcat gcaagcttgg……3'*

The mutagenic forward primer (Control Primer#1) is chosen from Desired sequences with 36 bases in length:

*5'-gtacccggggatcctct**CA**agtcgacctgcaggcat-3'*

The Tm value of the forward primer is: 82°C (81.5+ 0.41x (22/36) x 100 – 675/36 – (2/36) x 100 = 82°C).

Reverse and complement the forward primer to get the reverse primer (Control Primer #2):

*5'-atgcctgcaggtcgact**TG**agaggatccccgggtac-3'*

Long-Range Mutation (4-60bp), and Deletion of Long Fragment (~660bp):

Long-range mutation means mutation (substitution, or insertion, or deletion) of a fragment with length of 4~60bp. It can be achieved in a single mutagenesis reaction. In addition, the kit has successfully deleted GST gene (660bp) from a GST expression plasmid. Deletion of longer fragment may work as well, although it has not been tested yet.

The unique enzyme blend and its optimized supplement allow the system to have a much broader tolerance to primers with a broad range of Tm values. The system makes the annealing of template and primers feasible when the primers have low Tm value, while the annealing of template and primers with normal Tm value keeps intact. Furthermore, the potential secondary structure of long primers is prevented in the reaction system.

In addition to the *General Guidelines* mentioned above, primers design for long-range mutation and deletion of long fragment has the following considerations:

- Primers should be between 30 and 100 bases in length.

- Tm calculation of mutagenic primers is not necessary.

- The DNA length before and after the mutation site is critical. The suitable DNA length is determined by its amount of A and T. Please follow the following table to choose the DNA length before and after mutation site.

Suitable DNA Length before or after mutation site	Total number of A and T
14-15 bases	0-5
15-18 bases	6-9
17-20 bases	10-13
21-22 bases	14-22

Note *1. Never count the mutated sequences when determine the DNA length*
2. Never count the A and T from mutated sequences

Example: *Inset a 6 His tag into LacZ gene in pUC19*

Original Sequences:	*5'……ccaagcttgcatgcctgcaggtcgacttagaggatccccgggtaccga gctcgaatt……3'*
Desired Sequences after insertion 6His:	*5' ……ccaagcttgcatgcctgcaggtcgact**CACCACCACCACCA CCAC**tagaggatccccgggtaccgagctcgaatt……3'*

Forward primer design:

Step 1: Determine the DNA sequences before mutation site (before 6x His tag)

All the suitable DNA sequences are listed below:

	DNA length before mutation site	Total number of A and T
cctgcaggtcgact	14 bases	5 *(2 of A, 3 of T)*
gcctgcaggtcgact	15 bases	5 *(2 of A, 3 of T)*
tgcctgcaggtcgact	16 bases	6 *(2 of A, 4 of T)*
atgcctgcaggtcgact	17 bases	7 *(3 of A, 4 of T)*
catgcctgcaggtcgact	18 bases	7 *(3 of A, 4 of T)*

Step 2: Determine the DNA sequences after mutation site (after 6x His tag):

All the suitable DNA sequences are listed below:

	DNA length after mutation site	Total number of A and T
tagaggatccccgg	14 bases	5 *(3 of A, 2 of T)*
tagaggatccccggg	15 bases	5 *(3 of A, 2 of T)*
tagaggatccccgggt	16 bases	6 *(3 of A, 3 of T)*
tagaggatccccgggta	17 bases	7 *(4 of A, 3 of T)*
tagaggatccccgggtac	18 bases	7 *(3 of A, 2 of T)*

Step 3: Concatenate the DNA sequences before mutation site, mutation sequences, and DNA sequences after mutation site to form the complete forward primer

Let's say, both of 15 bases sequences from Step 1 and Step 2 (bold sequences) are chosen, the final complete forward primer for inserting 6xHis tag is:

5'-gcctgcaggtcgactCACCACCACCACCACCAC tagaggatccccggg-3'

This primer is actually the Control Primer #3 provided in the kit.

Reverse primer design:

Reverse complement the forward primer (Control Primer #3) to give the sequences of reverse primer (Control Primer #4):

*5'-cccggggatcctcta**GTGGTGGTGGTGGTGGTG**agtcgacctgcaggc-3'*

Multiple Sites Mutations (2 sites):

The TagMaster Site-Directed Mutagenesis Kit can mutate two separate sites simultaneously in a single reaction. Two mutation sites separated from each other at least 44 bases (≥44) are viewed as two separate sites.

Note *If two mutation sites are separated less than 44 bases, they are viewed as a single mutation site. The total length of the sequences of these two mutation sites and between them should be less than 60bp(see Example 2 and Fig 3).*

In addition to the *General Guidelines* mentioned above, primers design for *Multiple Sites Mutations* has the following considerations:

- Each primer contains one of two mutation sequences respectively. Forward Primer contains the upstream mutation, and the Reverse Primer contains the downstream mutation (see Example 1 and Fig 2).

- Forward Primer and Reverse Primer have to be chosen from two different chains of the dsDNA. The Forward Primer is chosen from the sense strand and complementary to the anti-sense strand, whereas the Reverse Primer is chosen from anti-sense strand and complementary to the sense strand (see Example 1 and Fig 2).

- Design Forward Primer and Reverse Primer according to their different mutation type: *Point Mutation* or *Long-range mutation*, or *Deletion of Long fragment.* Please see their corresponding section for detail primer design.

Example 1:

Two mutation sites (site x, and site xx) are separated by 100bp. The design of Forward Primer and Reverse Primer are illustrated below:

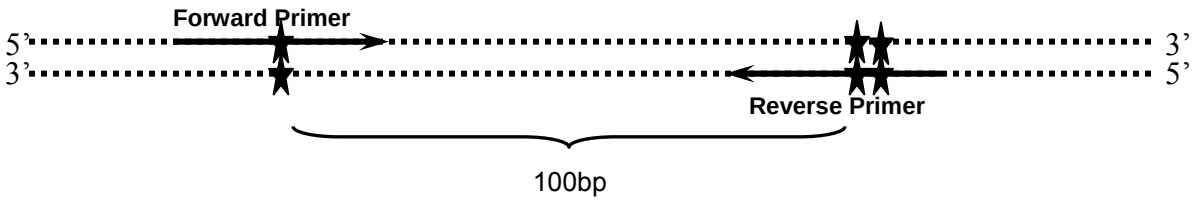

Fig 2. Location and Direction of Forward and Reverse Primers

Example 2:

Two substitution mutation sites (site xxxx, and site xxx) are separated by 40bp, and the total length of the two mutation sequences and the sequences in between is 60bp. They are viewed as a single site. Please see Long-Range Mutation (4-60bp) for primer design. The design of Forward Primer and Reverse Primer illustrated below:

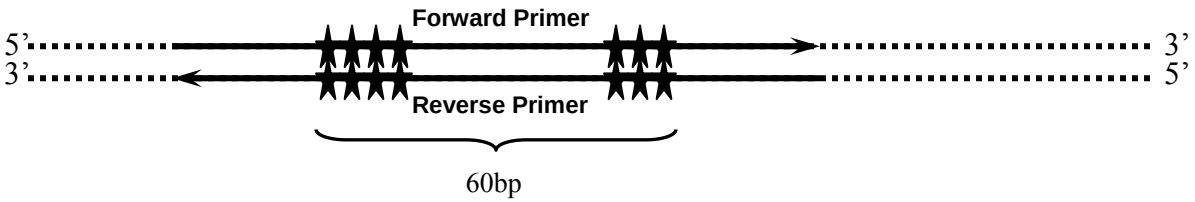

Fig 3. Location and Direction of Forward and Reverse Primers

Mutagenesis reaction

Note *Ensure that the plasmid DNA template is isolated from a dam+ E.coli strain. The majority of the commonly used E.coli strains are dam+. Commonly used dam+ E.coli strains include DH5α, TOP10, XL-1 Blue, BL21.* ***Plasmid DNA isolated from dam- E.coli strains (e.g. JM110 and SCS110) is not suitable for mutagenesis reaction.***

Note *Thin-walled PCR tubes are strongly recommended to ensure ideal result. A PCR machine with a hot-top cap is also recommended.*

Note *Plasmid DNA isolated from TagMaster cells cannot be used as template for mutagenesis reaction, because TagMaster competent cells cannot discriminate and eliminate plasmid DNA originally purified from themselves. To overcome this problem, transform the plasmid into DH5α and extract the plasmid from DH5α.*

1. Synthesize two primers containing the desired mutation according to *Mutagenic Primer Design.* Dissolve the primers in ddH_2O to give 20µM solution.

2. Three control reactions are suggested: negative control, 2bp substitution control, 6His insertion control. Prepare the control reactions as indicated below:

Control Reactions	Negative Control	Substitute 2bp	Insert 6His Tag
10X Reaction Buffer	5 µl	5 µl	5 µl
pUC19 Control Template (50ng/µl)	1 µl	1 µl	1 µl
dNTP Mixture (2mM each)	5 µl	5 µl	5 µl
$MgSO_4$ (25mM)	4 µl	4 µl	3 µl
Control Primer #1 (20µM)	----	1 µl	----
Control Primer #2 (20µM)	----	1 µl	----
Control Primer #3 (20µM)	----	----	1 µl
Control Primer #4 (20µM)	----	----	1 µl
50x TagMaster Supplement	----	----	1 µl
ddH_2O to a final volume of 49µl	34 µl	32 µl	32 µl
TagMaster Enzyme (1.0U/µl)	1 µl	1 µl	1 µl

3. Prepare the sample reactions as indicated below:

Note *Set up a series of sample reaction using various amounts of dsDNA plasmid template (e.g. 20ng, 50ng, 100ng).*

Note *If primers and/or template DNA is in TE buffer (Tris-EDTA), the EDTA will chelate Mg^{2+} in the mutagenesis reaction. Each molecule of EDTA will chelate one molecule of Mg^{2+}. Increase the Mg2+ accordingly.*

Sample Reactions	Point Mutation (1-3bp)	Other Mutations
10X Reaction Buffer	5 µl	5 µl
dsDNA template plasmid (20-100ng)	*X µl*	*X µl*
dNTP Mixture (2mM each)	5 µl	5 µl
$MgSO_4$ (25mM)	4 µl	3 µl
Forward Mutagenic Primer (20µM)	1 µl	1 µl
Reverse Mutagenic Primer (20µM)	1 µl	1 µl
50x TagMaster Supplement	----	1 µl
ddH_2O to a final volume of 49µl	*Y ul*	*Y ul*
TagMaster Enzyme (1.0U/µl)	1 µl	1 µl

Note *Other Mutations include: Long-Range Mutation (4-60bp), up to 660bp Deletion, and Multiple Site Mutation. For these reactions, 50X TagMaster Supplement is necessary.*

4. Perform mutagenesis reaction using the following parameters:

Note *Perform control and sample reactions simultaneously. Determine the 68°C extension time according to the sample's plasmid length.*

	Temperature	Time
1 cycle	95°C	2 minutes
20 cycles	95°C	20 seconds
	60°C	10 seconds
	68°C	30 seconds/ kb of plasmid length
1 cycle	68°C	5 minutes
1 cycle	4°C	forever

5. Directly perform the transformation step. Any treatment of the PCR reaction is unnecessary.

Transformation of TagMaster Competent Cells

Note *TagMaster Competent cell can discriminate parent DNA and novel synthesized daughter DNA. Therefore, parent plasmid template DNA would be eliminated and only daughter DNA synthesized in mutagenesis reaction can be enriched.*

Note *TagMaster cells are resistant to chloramphenicol. The mutagenized plasmid has to contain resistance marker (e.g. Ampicilline, Kanamycine, tetracyclines) other than chloramphenicol.*

Note *TagMaster cells are not suitable for blue-white selection. The strain has been modified for dramatically improving plasmid yield.*

1. Pre-warm SOC medium to room temperature or to 37°C.

2. Gently thaw TagMaster Competent cells on ice. Aliquote 50ul into each ice-chilled eppendorf tube. The competent cell should not leave on ice longer than 20 minutes. Thaw the competent cell only ONCE, prepare single-use aliquote and store the aliquots at -80°C. Aoid multiple freeze-thaw cycles.

3. Transfer 5 µl of mutagenesis reaction directly into 50 µl TagMaster Competent cells and incubate on ice for 30min. Mix by swirling or tapping the tube gently. Do not mix by vortexing or pipetting up and down.

4. Heat-shock the tube in a 42°C water bath for 30 seconds, put on ice for 2 minutes.

5. Add 250 µl of SOC medium to each tube. Shake at 37°C, 250rpm for 1 hour.

6. Spread appropriate volume on agar plate. It is generally plate the entire transformation mixture. Incubate the plate at 37°C for 16-24 hours.

7. Select 3 to 5 clones for plasmid isolation, PCR, sequencing to verify the desired mutation.

Note *Plasmid DNA isolated from TagMaster Cell cannot be directly used as template for next round mutagenesis reaction. To overcome this, transform the plasmid into DH5a and extract the plasmid from DH5a.*

Expected Results for the Control Transformations

If the entire transformation mixture was plated, the Negative Control would give less than 10 colonies, the 2bp mutation control would give several hundreds of colonies, and 6His insertion control would give more than 100 colonies. Greater than 95% of the colonies should contain the mutation.

Expected Results for Sample Transformations

The expected colony number depends on the DNA sequence, the length of the plasmid template, and the mutation type. Longer plasmid template would generally give less colony number. Difficult mutation (*Long-Range Mutation*, *Deletion of Long Fragment*, and *Multiple Site Mutation*) would give much less colony number than *Point Mutation*.

Multiple Site Mutation would give colonies containing both 2 sites mutations and single site mutation. The colonies containing 2 sites mutations would be up to 50% of all the mutated colonies.

If only a few colonies are obtained, go ahead to pick them to verify the mutation. TagMaster Competent cell can efficiently eliminate parent plasmid template, it is common that the several colonies would contain the desired mutation.

If tens of colonies are obtained, it is believe that the mutagenesis reaction succeeds. The majority of the colony would contain the desired mutagenized plasmid.

TROUBLESHOOTING

Variations in the DNA sequence composition, the length of the plasmid template, the mutation type, and the PCR machine performance may contribute to differences in the mutagenesis efficiency. The following guidelines are provided for troubleshooting these variations.

Observation	Suggestions
Absence of PCR product on agrose gel	Although it is not part of the standard protocol, some researchers choose to verify amplification by gel electrophoresis (typically, 10 µl of the PCR product is analyzed on a 1% agarose gel). While a positive result verifies successful synthesis, a negative result does not indicate failure of the reaction. Successful mutagenesis may or may not result from a visible band of mutagenesis reaction product. In some cases, even multiple bands or faint bands or smear bands may give successful mutagenesis. We recommend proceeding transformation when gel electrophoresis analysis yields negative results. Multiple Sites Mutation will give a strong shorter band that is the amplified fragment between the two primers. It is not necessary to remove it before performing transformation.
No colony obtained for sample reaction	If possible, try not to do difficult mutations (Long-Range Mutation, Deletion of Long Fragment, and Multiple Site Mutation) in a large plasmid (>8kb). Difficult mutations in a large plasmid would decrease the efficiency synergistically. Do difficult mutation in a short plasmid (less than 8kb), or do a Point Mutation in a large plasmid.
	For Point Mutation, increase the annealing temperature from 60°C to 68°C. Increase the amount of the plasmid template to 100ng would also be helpful in some cases.
	Some plasmid template (vector backbone and/or the gene insert) forms strong secondary structure, which would prevent successful mutagenesis reaction. Try to use a different vector backbone.
	Ensure the plasmid template in good purity and quantity. Too many nicked or linearized plasmid DNA will lead to failure of the mutagenesis reaction. Verify that the template DNA is at least 80% supercoiled by run the plasmid on a gel.
	If using a mineral oil overlay during thermal cycling, ensure that excess mineral oil is not transferred into the transformation reaction. Using the smallest pipet tips available, insert the pipet tip completely below the mineral layer overlay to collect the sample.
	Confirm the LB plate with the correct antibiotics.
Low colony number for sample reaction	When performing difficult mutations (Long-Range Mutation, Deletion of Long Fragment, and Multiple Site Mutation) or performing reaction for a large plasmid, it is common to observe low numbers of colonies. Most of the colonies, however, will contain desired mutagenized plasmid.
Low colony number with control reaction	Three control reactions are suggested in the kit: negative control, 2bp mutation control, 6His insertion control. The negative control is expected to give only a few colonies. The 2bp mutation control and 6His insertion control are expected to give hundreds of colonies.
	Ensure the TagMaster Competent cells are stored at -80°C immediately upon arrival. Thaw the competent cell only one time, prepare single-use aliquote and store the aliquots at -80°C. Aoid multiple freeze-thaw cycles.
	Avoid multiple freeze-thaw cycles for the dNTP mixture. Thaw the dNTP only one time, prepare single-use aliquote and store the aliquots at -20°C.

	Different PCR machine may contribute to variations in amplification efficiencies. Optimize the cycling parameters for the control reaction and then repeat the protocol for the sample reactions using the adjusted parameters.
Erroneous mutation in the primer region	Poor quality primers can lead to erroneous mutations. Pick and screen more colonies for the desired mutation.

PREPARATION OF MEDIA

LB Agar

1% NaCl
1% tryptone
0.5% of yeast extract
2% of agar
Adjust pH to 7.0 with 5 N NaOH
Autoclave, Cool to 55°C and add appropriate antibiotics.
Pour into petri dishes

S.O.C. medium

2% Tryptone
0.5% Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM MgCl2
10 mM MgSO4
20 mM glucose
Sterilize by autoclaving

REFERENCES

1. Taylor, J. W., Ott, J. and Eckstein, F. (1985) *Nucleic Acids Res* 13(24):8765-85.
2. Kunkel, T. A. (1985) *Proc Natl Acad Sci U S A* 82(2):488-92.
3. Vandeyar, M. A., Weiner, M. P., Hutton, C. J. and Batt, C. A. (1988) *Gene* 65(1):129-33
4. Nelson,R.M., Long,G.L. (1989) Ana Biochem 180(1):147-51
5. Landt,O., Grunert,H.P., Hahn,U. (1990) Gene 96(1): 125-8
6. Hall, L., Emery, D.C. (1991) Protein Eng. 4(5):601
7. Deng W.P., Nickoloff, J.A.(1992) Ana Biochem 200(1): 81-8
8. Weiner,M.P., Felts,K.A., Simcox,T.G., Braman,J.C. Gene (1993) 126(1):35-41

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information is available on the web at *http://www.gmbiosciences.com*. MSDS documents are not included with product shipments.

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by GM Biosciences. GM Biosciences shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SUPPORT

GM Biosciences, Inc.
4539 Metropolitan CT.
Frederick, MD 21704
USA
www.gmbiosciences.com

Ordering:

Email: ordering@gmbiosciences.com
Tel: 240-595-9177
Fax: 301-378-2862

Technical Support:

Email: support@gmbiosciences.com
Tel: 240-595-9177