For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.

ISO 9001/14001 Certified Company

Muta-Direct™
Site-Directed Mutagenesis Kit

RUO | REF 15071 | Σ 15 | -18℃ / -22℃

DESCRIPTION

In vitro site-directed mutagenesis is an useful technique for studying structural function relationships of protein and for identifying amino acids. The Muta-Direct™ Site-Directed Mutagenesis Kit can be used for creating a mutation at a defined site in a plasmid. The Muta-Direct™ Site-Directed Mutagenesis Kit has convenient and simple three steps for all experimental procedures.

The Muta-Direct™ Site-Directed Mutagenesis Kit is used to make point mutations, substitute amino acids, and delete or insert nucleotide sequence(s). The Muta- Direct™ Site-Directed Mutagenesis Kit „s characteristics enable the kit to be applicable to protein engineering including the improvement of protein function or protein productivity as well as analysis of gene function.

The creation of a mutation is possibly to complete through just simple three-steps including performance of PCR using the prepared mutagenic primers and use of the Muta-direct™ enzyme which has a very low error rate with proof-reading function; digestion of non-mutated parental DNA template (contained with methylated and hemimethylated DNA sequence) by treatment with Mutazyme™ Enzyme; and transformation of the mutated plasmid (Fig. 1). It can be checked whether mutagenesis is completed or not by sequencing of mutated plasmid if necessary.

The Muta-Direct™ Site-Directed Mutagenesis Kit provides a simplified user-friendly protocol for the convenient use of those who are unfamiliar with the Muta-Direct™ Site- Directed Mutagenesis Kit or new to site-directed mutagenesis.

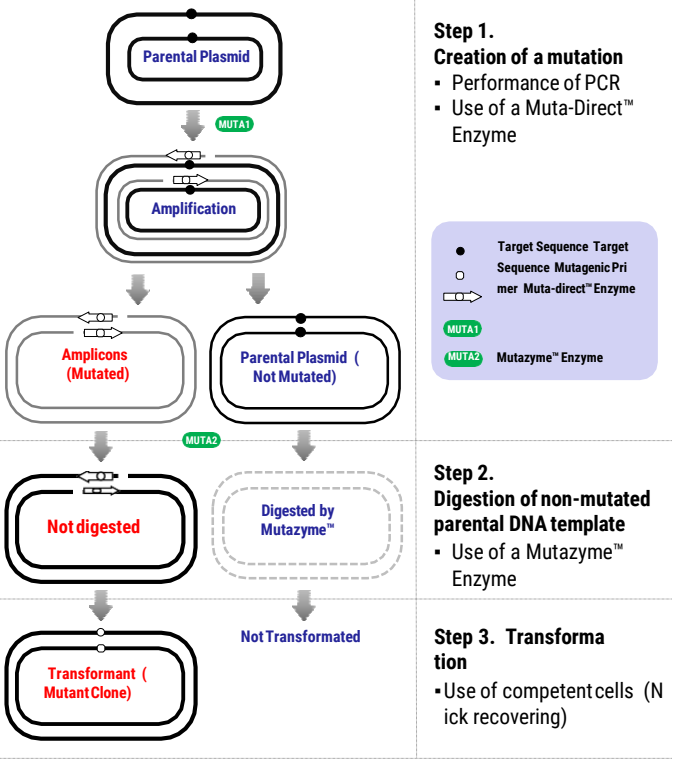

Fig 1. Overview of the Muta-Direct™ Site-Directed Mutagenesis kit

CHARACTERISTICS

- Easy to use : No special skills are required
- Simple : Three steps with two days
- High mutation efficiency : The success rate is 99%

APPLICATION

- Codon switch
- Re-mutation to wild type of plasmid
- Functional analysis of a gene or protein
- Protein engineering

ADDITIONAL REQUIRED EQUIPMENT

- PCR machine
- Pipette set
- Standard tabletop micro-centrifuge
- Incubator

KIT CONTENTS AND STORAGE

Label[1,2]	Contents
Muta-Direct™ Enzyme (2.5 U/μl)	15 μl
Muta-Direct™ Reaction Buffer (10×)	100 μl
dNTP Mixture	30 μl
Mutazyme™ Enzyme (10 U/μl)	15 μl
pUC18 Control Plasmid (10ng/μl)[3]	10 μl
Control Primer Mix (20 pmole/μl)[3]	10 μl

[1] All components should be stored at -20°C.
[2] Muta-Direct™ Site-Directed Mutagenesis Kit contains sufficient contents to perform approximately 15 × 50 μl mutagenesis reactions.
[3] pUC18 Control Plasmid and Control Primer Mix contained in this kit are sufficient to perform 5 control reactions.

PRODUCT WARRANTY AND SATISFACTION GUARANTEE

All products undergo extensive quality control test and are warranted to perform as described when used correctly. Immediately any problems should be reported. Satisfaction guarantee is conditional upon the customer providing full details of the problem to iNtRON within 60 days, and returning the product to iNtRON for examination.

IMPORTANT POINTS BEFORE STARTING

◈ Muta-Direct™ Control Reaction

The pUC18 Control Plasmid and Control Primer Mix contained in the **Muta-Direct™ Site-Directed Mutagenesis Kit** are provided to check whether the mutagenesis experiment is performed completely. The pUC18 Control Plasmid and Control Primer Mix should be used in the Muta-Direct™ Control Reaction. Through the Muta-Direct™ Control Reaction, a translational termination codon is introduced into the *lacZ* gene contained in pUC18 Control Plasmid. The change from serine (TCG) to translational termination codon (TAA) can block the expression of protein product of the *lacZ* gene. Only white colonies are formed after transformation when the experiment completes properly.

◈ Primer Design

At first, design of the mutagenic primers is required for the use of the Muta-Direct™ Site- Directed Mutagenesis Kit.

It is generally accepted that the length of mutagenic primers is 25~45 bp. We recommend the use of the mutagenic primer which is 30-35 bp in length. It is important that the nucleotide desired to be mutated should be settled in the middle of the mutagenic primers.

Design a primer of 30 bp in length and then need to estimate a melting temperature (Tm) with Tm formula. Tm of the mutagenic primers should be greater than or equal to 78°C (At least more than 40% of GC ratio). If the Tm is under 78°C, the change of the primer length is necessary.

Note : Check points below for the design of primer.

1) Design forward and reverse primers which are 30 bp each in length. In this step, locate the nucleotide desired to be mutated in the middle of the mutagenic primers.
2) Estimate the Tm of the mutagenic primers. If the Tm is under 78℃, adjust the length of primers for 78°C (At least more than 40% of GC ratio).
3) Avoid using desalting-grade primers. It is recommended to use HPLC or PAGE grade of primers. Most companies commonly provide HPLC grade primer but customer are required to check this point.

The following formula is commonly used for estimating the Tm of mutagenic primers

▪ **Tm formula :** **Tm=0.41(% GC) – 675/L+81.5**
(**L**: Primer length in base pairs; **% GC**: GC % of primer)

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PROTOCOLS

Step 1. Creation of a mutation

In this step, you may create a mutation at a defined site in a plasmid performing PCR with the mutagenic primers and Muta-direct™ Enzyme containing proof-reading activity

1. Prepare plasmids for using as a PCR template.

Note : Use *dam+ E. coli* strains such as DH5α strain as host strain. Most of *E. coli* strains commonly used for molecular biology work are *dam+* strains except JM110 and SCS110 strains. For *end+* strains, low number of colonies may be obtained because of activity of endonuclease. But this may not affect the mutation efficiency. It is recommended to use DNA-spin™ Plasmid DNA Extraction Kit when you prepare the plasmids.

2. [Optional] Prepare the control reaction as described below.

Muta-Direct™ Control Reaction (50 µl reaction volume)	
Muta-Direct™ Reaction Buffer (10×)	5 µl
pUC18 Control Plasmid (10 ng/µl)	2 µl
Control Primer Mix (20 pmole/µl)	2 µl
dNTP mixture	2 µl
dH_2O	38 µl
Muta-Direct™ Enzyme (2.5 U/µl)	1 µl

3. Prepare the Sample reaction(s) as described below.

Muta-Direct™ Control Reaction (50 µl reaction volume)	
Muta-Direct™ Reaction Buffer (10×)	5 µl
Sample plasmid (10 ng/µl, total 20 ng)	2 µl
Sample forward primer (10 pmole/µl)	1 µl
Sample reverse primer (10 pmole/µl)	1 µl
dNTP mixture	2 µl
dH_2O	38 µl
Muta-Direct™ Enzyme (2.5 U/µl)	1 µl

4. Perform the PCR according to the following PCR condition and a final extension step may be skipped.

Segment	Cycle	Temperature	Time
1	1 cycle	95°C	30 sec
2	12 ~ 18 cycles	95°C 55°C 72°C	30 sec 1 min 1-2 min/ kbp of plasmid length

Note : In the PCR condition described above, the number of PCR cycle of segment 2 can be adjusted according to the following table. In case more than 4 nucleotides are mutated, mutation efficiency may be decreased.

Mutation	Cycles
Point Mutation	12 cycles
Single amino acid changes	16 cycles
Multiple amino acid deletion or insertion	18 cycles

5. After PCR, place the PCR tube in ice for 5 minutes.

Note : To check the amount of mutated plasmid, electrophorese 10 µl of Mutazyme™ Enzyme– treated PCR mixture on a 1% agarose gel.

Step 2. Digestion of non-mutated parental DNA template

In this step, you may digest the non-mutated parental DNA template (methylated or hemimethylated plasmid) by treatment with the Mutazyme™ Enzyme after the completion of PCR.

1. Add 1 µl of Mutazyme™ Enzyme (10 U/µl) to PCR mixture.

2. After mixing shortly, incubate the mixture at 37°C for 1hour.

Note : In case of using significantly excess amount of PCR template plasmid, the treatment with the Mutazyme™ Enzyme may be incomplete. This may cause difficulty in the correct selection of mutated clones. Therefore, proper amount of PCR template plasmid should be used.

Step 3. Transformation

This step is to recover the nick on the mutated plasmid by transforming *E. coli* with the PCR mixture treated with Mutazyme™ Enzyme. Transformation can be performed according to conventional methods.

iNtRON offers ready-to-use competent cells for optimized transformation. (refer to related products)

1. Use directly 1-10 µl of Mutazyme™ Enzyme–treated PCR mixture for the transformation of aliquot 50 µl of the competent cells.

Note : To check the amount of mutated plasmid, electrophorese 10 µl of Mutazyme™ Enzyme – treated PCR mixture on a 1% agarose gel.

Note : The Muta-Direct™ Site-Directed Mutagenesis Kit is compatible with a wide range of chemically modified competent cells which are available from iNtRON. In particular, DH5α, JM109 and TOP10 competent cells are appropriate to blue/white colony selection and exhibit the high transformation efficiency. Use 3-10 µl aliquot of ligation mixture to transform competent cells.

2. Thaw the competent cells on ice. When thawed, gently mix and aliquot 50 µl of competent cells into each of pre-chilled tube.

3. Add a Mutazyme™ Enzyme–treated PCR mixture (<2.5µl) directly to the competent cells. Stir gently to mix and return the tube to the ice, making sure that the tube is immersed on ice except for the cap. Repeat for additional samples.

Note : Transformation efficiencies can be increased several fold by diluting the ligated products 5-fold with TE or water prior to adding the DNA to the competent cells.

Note : We recommend the adding volume of Mutazyme™ Enzyme–treated PCR to below 2.5 µl when you use 50 µl of competent cells. If you need to use much more volume (~10 µl), you should increase the volume of competent cell up to 100 µl.

4. Incubate the tubes on ice for 30 min.

5. Heat the tubes for exactly 90 sec. (DH5α = 90sec, XLI-BLUE = 45 sec, JM109/TOP10 = 30 sec) in a 42℃ water bath or heat block; do not shake.

Note : This "Heatshock" step is most easily accomplished if the tubes are in a rack that leaves the lower halves of the tubes exposed. Hold the rack in the water bath so that the lower halves of the tubes are submerged for 90sec, and then replace the rack on ice.

6. Place the tubes on ice for 5 min.

7. Add 250 µl of pre-heated SOC medium (provided) or 950 µl LB medium (not provided) (w/o antibiotics) to each tube.

8. Incubate for 1hr at 37℃ at the shaking incubator.

9. During incubation, spread 40 µl of X-Gal Soln. (40mg/ml) and 4 µl of IPTG Soln .(400mg/ml) of α-Complementation Solution (iNtRON, Cat.No. 15032) onto LB agar plate (containing appropriates elective marker; antibiotics) and allow these components to absorb for 30 minutes at 37℃ prior to plating cells.

Note : Selection for transformants are accomplished by plating on medium containing antibiotic(s) for the plasmid-encoded drug resistance(s).

10. After incubation, harvest incubated cell mixture. Then 150 µl of pellet and media leave the tube and remove supernatant.

11. Resuspend cell pellet, and spread on dried LB agar plate (w/antibiotics). 12

. Incubate the plate at 37℃ for overnight(14~16hr) then select a mutant clone.

◈ *Expected Results for the Control Transformations*

1. The expected colony number should be between 50 and 800 colonies. Greater than 80% of the mutagenized control colonies should contain the mutation and appear as blue colonies on agar plates containing IPTG and X-gal.

Note : The mutagenesis efficiency (ME) for the control plasmid is calculated by the following formula:

$$ME = \frac{\textit{Number of mutant colony forming units (cfu)}}{\textit{Total number of colony forming units (cfu)}} \times 100\%$$

2. If transformation of the pUC18 control plasmid was performed, the transformation efficiency should be >250 colonies (>10^8 cfu) with >98% having the blue phenotype.

Note : The insert of interest should be sequenced prior to further experimentation to ensure the most accurate results.

TROUBLESHOOTING GUIDE

Observation	Possible cause(s)	Suggestion(s)
Low transformation efficiency or low colony number	• Insufficient amount of DNA template used in the reaction	• Visualize the DNA template on a gel to verify the quantity and quality
	• Too much mineral oil pipetted with the Mutazyme™ Enzyme- treated DNA while transferring to the transformation reaction	• Using the smallest pipet tips available, insert the pipet tip completely below the mineral layer overlay and clear the pipet tip while submerged beneath the mineral oil overlay before collecting the sample
	• Insufficient amount of mutant DNA synthesized in the reaction	• Increase the amount of the Mutazyme™ Enzyme treated DNA used in the transformation reaction to 4 µl
Low mutagenesis efficiency or low colony number with the control reaction	• Little or no linear amplification products	• Following temperature cycling, resolve a sample of the control reaction by electrophoresis on an agarose gel; if no product is observed at 4.5 kb, adjust the cycling parameters for the control reaction
	• Insufficient amounts of X-gal and IPTG on the agar plates	• Prepare the LB–ampicillin agar plates for the transformed control cells by pipetting 20 µl of 10% (w/v) X-gal (prepared in DMF) and 20 µl of 100 mM IPTG (prepared in filter-sterilized dH2O) into a 100-µl pool of NZY+ broth and then spreading the mixture across the plate
	• competent cells stored at n improper temperature	• Store the competent cells immediately at the a bottom of a –80°C freezer
	• Differences in thermal cyclers may contribute to variations in ramping efficiencies	• Adjust the cycling parameters for the control reaction and repeat the protocol for the sample reactions. For the best visualization of the blue (β- gal+) phenotype, the control plates must be incubated for at least 16 hours at 37 °C
	• Subjecting the dNTP mix to multiple freeze-thaw cycles	• Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C. Do not subject the dNTP mix to multiple freeze-thaw cycles
Low mutagenesis efficiency with the sample reaction(s)	• Differences in thermal cyclers may contribute to variations in ramping efficiencies	• Adjust the cycling parameters for the sample reaction
	• Improper mixing of reagents	• Add the Mutazyme™ Enzyme restriction enzyme below the mineral oil overlay in the digestion step and ensure proper mixing of all components in the reaction especially the Mutazyme™ Enzyme
	• The amplification reaction contains too many DNA template	• The Mutazyme™ Enzyme must be able to completely digest the parental template in the time allotted for the digestion; repeat the digestion if necessary
	• Subjecting the dNTP mix to multiple freeze-thaw cycles	• Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C. Do not subject the dNTP mix to multiple freeze-thaw cycles
	• The parental plasmid DNA was not methylated	• Check the Host E. coli Strain. If the host strain has dcm or dam gene, the parental plasmid DNA will not be methylated. Therefore the Mutazyme can not be activated to digest parental DNA.
False positives	• Quality of the primers is poor	• Radiolabel the primers and check for degradation on an acrylamide gel or re- synthesize the primers
	• False priming	• Increase the stringency of the reaction by increasing the annealing temperature to within 5°C of the melting temperature of the mutation primers

EXPERIMENTAL INFORMATION

◈ Primer design example

The example below is for a primer design with a mutation of **G**CG→**A**CG.

5' CCTCCTTCAGTATGTAG**G**CGACTTACTTATTGCGG-3'

1) At the first step, design the forward and reverse primers which are 30 bp each in length with locating A (or T) in the middle of the primer.

Primer #1: 5'-CCTTCAGTATGTAG**A**CGACTTACTTATTGC-3'
Primer #2: 5'-GCAATAAGTAAGTCG**T**CTACATACTGAAGG-3'

2) This primer has 40% of GC and 30 of L value. With these data, Tm is calculated to be 75.4°C (**Tm**=0.41×40-675/30+81.5). If Tm is under 78°C, that is not an appropriate primer.

3) In this case, it is necessary to adjust the length of primer.

Primer #1: 5'-*CCT*CCTTCAGTATGTAG**A**CGACTTACTTATTGC*GG*-3'
Primer #2: 5'-*CC*GCAATAAGTAAGTCG**T**CTACATACTGAAGG*AGG*-3'

The case above with adding 5 nucleotides to original primers (italic and underlined), the primers has 45.7% of GC and 35 of L value. With these data, Tm is re-calculated to be 80.952°C (**Tm**=0.41×45.7-675/35+81.5). This designed primer may be used.

◈ Mutagenesis example

The example below is for mutagenesis with a mutation of **G**CG→**A**CG.

[Reaction Mixture]

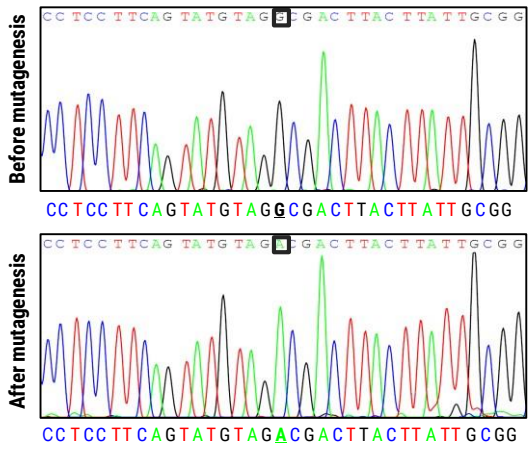
Muta-Direct™ Control Reaction (50 µl reaction volume)	
Muta-Direct™ Reaction Buffer (10×)	5 µl
Sample plasmid (6.3kb, 10 ng/µl, total 20 ng)	2 µl
Sample forward primer (10 pmole/µl)	1 µl
Sample reverse primer (10 pmole/µl)	1 µl
dNTP mixture	2 µl
dH_2O	38 µl
Muta-Direct™ Enzyme (2.5 U/µl)	1 µl

[PCR Condition]

Segment	Cycle	Temperature	Time
1	1 cycle	95°C	30 sec
2	15 cycles	95°C 55°C 72°C	30 sec 1 min 6 min 30 sec (plasmid length 6.3 kb)

[Sequencing Analysis]

Sequencing result of the mutated plasmid is as follows.



◈ Preparation of media and reagents

LB Agar (per Liter)

- 10 g of NaCl
- 10 g of tryptone
- 5 g of yeast extract
- 20 g of agar
- Add deionized H2O to a final volume of 1 liter
- Adjust pH to 7.0 with 5 N NaOH
- Autoclave
- Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin Agar (per Liter)

(Use for reduced satellite colony formation)

- 1 liter of LB agar
- Autoclave
- Cool to 55°C
- Add 50 mg of filter-sterilized ampicillin
- Pour into petri dishes (~25 ml/100-mm plate)

NZY+ Broth (per Liter)

- 10 g of NZ amine (casein hydrolysate)
- 5 g of yeast extract
- 5 g of NaCl
- Adjust to pH 7.5 using NaOH
- Autoclave
- Add the following supplement prior to use
- 12.5 ml of 1 M MgCl2 and 12.5 ml of 1 M MgSO4
- 10 ml of a 2 M filter-sterilized glucose solution or 20 ml of
- 20% (w/v) glucose
- Filter sterilize

Preparing the Agar Plates for Color Screening

Prepare the LB-ampicillin agar plates for blue–white color screening 30 minutes prior to plating the transformations. Pipet 20 µl of 10% (w/v) X-gal and 20 µl of 100 mM IPTG into a 100-µl pool of NZY+ broth, and then spread the mixture across the plate. Prepare the X-gal in dimethylformamide (DMF). Prepare the IPTG in sterile DDW. Do not mix the IPTG and the X-gal before pipetting them into the pool of NZY+ broth because these chemicals may precipitate.

◈ Symbols

RUO Research Use Only

REF Catalog Number

Σ 15 Sufficient for 15 Tests

-18℃ -22℃ Store at -22 ~ -18℃

RELATED PRODUCTS

Product Name	Cat. No.
MacCell™-DH5α	15052/15053/15054
MacCell™-Top 10	15055/15056/15057
DNA-spin™ Plasmid DNA Extraction Kit	17096/17097/17098
LINKeed®Rapid DNA Ligation Kit (Version 2.0)	15023
α-Complementation Solution	15032
pLUG ® TA-Cloning Vector Kit	11041