Antibody Design LABORATORIES

FAST-Licase™

INSTRUCTION MANUAL

FAST-Licase™

Catalog #: MB101S, MB101L

Version: A1.3 – January 2017

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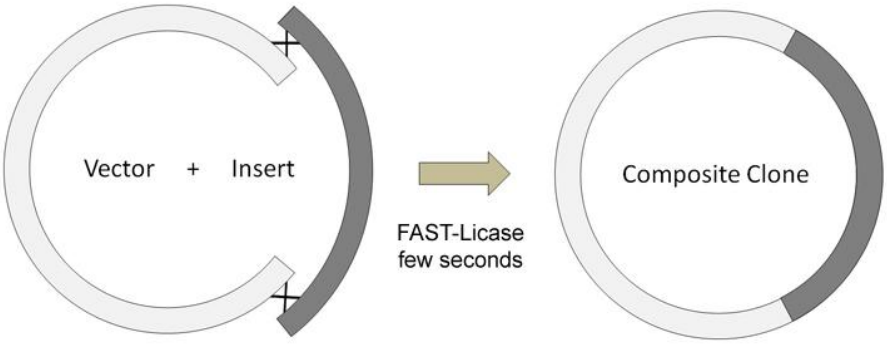
Description

Introduction

FAST-Licase™ is designed for the ultra-rapid seamless cloning of two or more DNA fragments. This cloning method is based on the homologous recombination of short, 15 to 20 bp-long overlaps between fragments resulting in precise and unique cloning events. The **licase™** complex comprises several DNA modifying enzymes that are sequentially activated to achieve an extremely high cloning efficiency in less than 5 minutes, sometimes in no more than a few seconds, saving time at the bench. Competent bacteria with medium transformation efficiency in the range 1 x 10^8 transformants/µg DNA are usually sufficient to complete the cloning projects, thus saving additional expenses on high efficiency competent cells.



Content, Shipping & Storage

Content

COMPONENT	COMPOSITION	AMOUNT
Licase™	**Licase™** complex in glycerol 50% v/v	20 µl (MB101S), 100 µl (MB101L)
Buffer	5x buffer	200 µl (MB101S), 1000 µl (MB101L)

Shipping & Storage

FAST-Licase™ is shipped on wet ice. Upon receipt, store at -20°C.

Required Materials Not included

The following materials are necessary to complete the cloning process: DNA polymerases for generating PCR products, a high-fidelity bacterial polymerase with proof-reading activity is recommended; restriction enzymes to cut the vector, if necessary; the DpnI restriction enzyme to cut the template DNA after PCR; a thermal cycler; a water bath; competent bacterial cells; and LB (Luria-Bertani) or 2xYT agar plates supplemented with appropriate antibiotics.

For research use only; not intended for any animal or human therapeutic or diagnostic use.

Cloning with FAST-Licase™

Overview

A New Way of Cloning

The **Licase™** cloning method virtually eliminates the ligation step from molecular biology, thus converting cloning into a seamless ultra-fast procedure, obviating negative controls and use of expensive cloning kits and ultra-competent cells. **Licase™** cloning not only saves both time and money, but, in conjunction with the availability of highly processive DNA polymerases and synthetic DNA fragments, changes the traditional way of designing cloning experiments. **Licase™** represents a versatile toolbox to create clones in a simple, rapid and efficient manner at the bench.

How it Works

The **Licase™** complex uses a thermal switch to toggle 3′→5′ exonuclease activity and annealing activity resulting in ultrafast homologous recombination between DNA fragments bearing sequence homology at their ends.

A Method Inscribed in History

From a historical perspective, cloning went from the serendipity of phenotypic selection in the 1970s to the more controlled use of restriction enzymes, rapidly followed by the advent of PCR in the 1980s. Unfortunately, restriction enzyme cloning is extremely laborious for a number of reasons. The method is sequence-dependent as restriction sites must be present. Both vector and insert must be cut and purified; cut vectors can re-ligate, leading to high backgrounds; overhangs are often palindromic, leading to fragment polymerization and inefficiency. Moreover, unexpected ligation events between non-complementary overhangs can lead to errors that are difficult to troubleshoot. Recently, recombination of DNA fragments bearing sequence homology has established itself as an efficient and reliable method to clone DNA. The method is seamless, leaving no scar on the sequence. The DNA linking event is primarily based on sequence homology, making it highly specific. **FAST-Licase™** goes one step further by making the reaction quasi-instantaneous, markedly changing the speed of molecular cloning at the bench.

Applications

To help identify the best strategy to achieve a particular cloning project, several individualized applications enabled by **FAST-Licase™** are presented. Within the traditional cloning concept of vector plus insert, **Licase™** cloning can work with a cut vector on one side, and an insert on the other side, which can be either a PCR product or a synthetic DNA fragment. Alternatively, short overlapping oligonucleotides can be assembled at the cloning site. Because of the availability of high-fidelity DNA polymerases, the vector can also be amplified by PCR. This strategy enables the rapid combination of vector fragments with the possibility of mutagenesis at the junctions.

There is no limit to the number of fragments that can be assembled, but since **FAST-Licase™** is focused on speed, limiting the reaction to two to three fragments is recommended.

Cloning of DNA Fragments

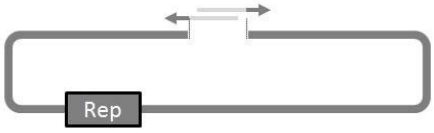
In this application, the DNA fragment to clone is amplified by PCR and assembled into a vector that includes an origin of replication (“Rep”). The vector is linearized by restriction enzyme digestion. Each of the two primers contain two parts; the 5’ region includes 15 to 20 nucleotides that are homologous to one end of the cut vector and the 3’ segment allows for

amplification of the cloning fragment. Together, the two primers are designed to PCR amplify the fragment to clone and mediate recombination to the ends of the linearized vector.

Alternatively, the DNA to be inserted into the vector is a double-stranded DNA fragment obtained by direct synthesis. 5' phosphorylation of the synthetic DNA is not necessary. However, as with the insertion of a PCR fragment, areas of sequence homology with the vector termini, 15 to 20 nucleotides long, must be added on each side of the DNA fragment.

Insertion of Oligonucleotides

Sometimes the sequence that is to be inserted may be too small and therefore precludes the use of a synthetic double-stranded DNA fragment. Instead, the use of two partially overlapping oligonucleotides, creating two natural recesses after annealing, is preferable. In this application, the **FAST-Licase™** reaction is conducted twice. During the first reaction, only the cut or amplified vector is added to the mixture; the overlapping oligonucleotides are added during the second reaction (see Experimental Procedures).

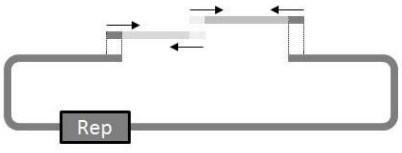


Mutagenesis & Vector Engineering

In this application, two DNA fragments are amplified by PCR with primers creating regions of homology 15 to 20 nucleotides long on each end. One of the two fragments contains a suitable origin of replication to allow survival and amplification of the recombinant DNA in the bacterial host. This application is particularly suitable for creating or modifying vectors and for mutagenesis, which is easily achieved by incorporating mutations in one or more of the recombining overlaps.

Cloning of Multiple DNA Fragments

In this application, multiple DNA fragments sharing short stretches of homologous sequences are cloned at once into a linearized vector or into a PCR product bearing an origin of replication for plasmid maintenance in the bacterial host.



Experimental Procedures

Thermal Control

Manual Reaction

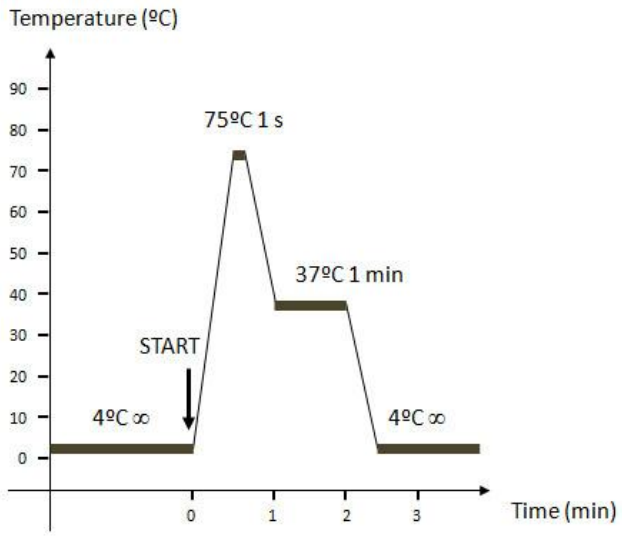
There are two ways to clone using **FAST-Licase™**. DNA recombination can be achieved by manually incubating the reaction mixture between 2 s and 5 s at 75°C. Alternatively, a thermal cycler can be used to control the states of activation of the **Licase™** complex.

Using Your Thermocycler

The use of a thermal cycler results in a two times higher cloning efficiency and is recommended for difficult cloning procedures, when more than two fragments need to be assembled, for the insertion of oligonucleotides, and for automated applications.

The following program should result in a successful **Licase™** reaction. . Any conventional thermal cycler will be suitable for **FAST-Licase™** reactions.

CYCLE	TEMPERATURE	DURATION
Cycle 1	4°C	Infinite loop
Cycle 2	75°C 1 s followed by 37°C 1 min	Plateau at 75°C 1 s followed by plateau at 37°C 1 min
Cycle 3	4°C	Infinite loop

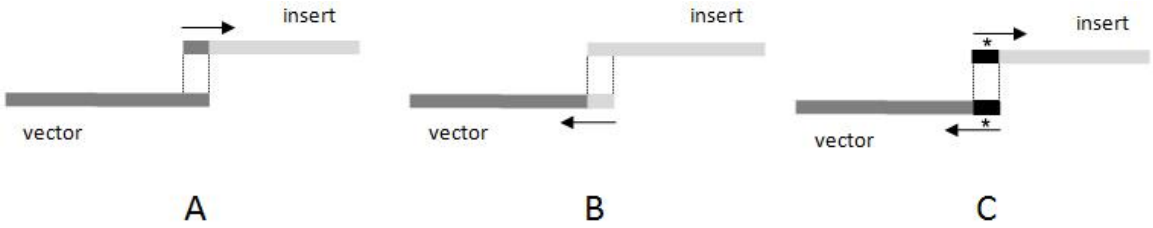


Preparation of DNA Fragments

Designing Homologous Overlaps

Homologous overlaps are essential to a successful **FAST-Licase™** cloning reaction. Design rules are less stringent than for PCR primers. Overlaps will be shared between DNA fragments; they must be unique and free of secondary structures such as hairpins or tandem repeats. They should be 15 bp to 100 bp long, but in most cases 15 to 20 bp homologous overlaps will be sufficient. Longer overlaps of 30 bp to 40 bp or more will improve success for longer inserts (> 5 kb) or when more than two DNA fragments are joined together.

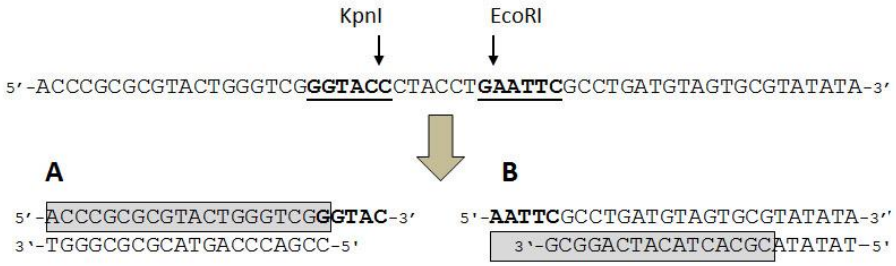
Overlaps can be generated on the vector or the insert side of the DNA junction and created by PCR or by direct DNA synthesis. When cloning different inserts into a vector, it is beneficial to add the overlap to the inserts using the vector sequence (junction A); if the restriction sites are conserved, then the sites must be added to the overlaps. Alternatively, if the insert is too long for PCR amplification, the vector may be PCR amplified instead, and insert sequence can be included in the overlap (junction B). Finally, for mutagenesis or insertion of a short sequence between the two fragments, the overlap can be a different sequence that is included in both fragments (junction C).



Primer Design

Each primer is made of two components, a 5’ homology region and a 3’ priming region. The 3’ priming region is used for PCR amplification. Standard primer design criteria for template annealing during PCR amplification apply to this component. The 5’ homology region is usually a 15 to 20 nucleotides long sequence that is homologous to the 3’-end of the sense strand of the pairing DNA fragment; absence of hairpin structures or repeated sequences in this region is essential. As noted above, the homology region can be included on the vector- or the insert-side. Therefore, a primer may or not include a homology section. As illustrated in example (B), above, the primer to amplify the insert will only have a priming region while the primer to amplify the vector will include a homology region based on the insert sequence, and possibly a few extra nucleotides to complete the restriction site, if needed.

The 3’->5’-exonuclease activity digests the DNA from a 3’ end and exposes the complementary strand as single stranded DNA. Therefore, when the fragment terminus has been generated with a restriction enzyme, the homologous region starts at the actual cut on the complementary strand, which is the strand left after exonuclease digestion. The following schema illustrates a hypothetical DNA sequence containing a KpnI site and an EcoRI site with the goal of cloning a PCR fragment between the two sites. After digestion by the two enzymes and removal of the small region between the two restriction sites, the two homologous 20-bp regions to be picked for primers are highlighted. Fragment A is based on the sense upper strand DNA and ends at the KpnI cut on the other strand. Fragment B is based on the anti-sense strand and ends at the EcoRI cut on the sense strand:



When there is no restriction on primer length, the homology region can simply be counted immediately downstream from the restriction sites. To restore restriction sites after cloning, the necessary nucleotides must be added between the homology region and the priming region.

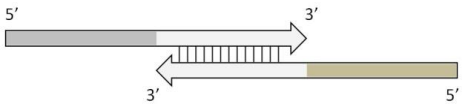
It is highly recommended to build an *in silico* assembly of the final DNA sequence and verify the presence of each of the primers. Alternatively, starting a cloning project by creating an *in silico* template sequence will guarantee the proper design of error-free primers. For primer design, use of the GenBeans software is recommended, which has advanced sequence editing capabilities (www.genbeans.org).

Gel-purification of the PCR products is usually not necessary when a single product of amplified DNA at the expected molecular size emerges after PCR on an agarose gel during DNA electrophoresis. Use of a standard PCR cleanup kit followed by DNA concentration determination by UV spectrophotometry normally suffices. Treatment by DpnI is recommended when the PCR template can produce background colonies. To do this, add 1 µl (20 U) of DpnI restriction enzyme to the completed PCR reaction and incubate between 1 h to overnight at 37°C before PCR clean-up.

Preparing Plasmid Fragments by Restriction Digest

After digestion, restriction enzymes can be inactivated by heat or removed with a PCR cleanup kit. There is no need to dephosphorylate the vector. Isolation of the properly cut fragment by agarose gel electrophoresis and removal of the agarose by a cleanup kit is recommended. If only one restriction enzyme is used to cut the vector, the vector may re-ligate inside the competent bacteria after transformation and generate background clones. In that case, dephosphorylation may decrease the background.

Using Overlapping Oligonucleotides



Single oligonucleotides may be used to introduce a short stretch of nucleotides using **FAST-Licase™**. In this case, a pair of oligonucleotides is designed where the 3’ ends are self-complementary and the 5’ ends are homologous to the respective DNA ends to be linked. The homology between the two oligonucleotides must be long enough to sustain formation of double-stranded DNA at 37°C. In theory, more than two oligonucleotides may be used in a single reaction, but the use of a synthetic DNA fragment always offers a better option for cloning longer stretches of DNA.

This method may be used to introduce a foreign stretch of nucleotides between two fragments or link two fragments that are difficult to amplify by PCR or harvest from bacteria.

Protocols

Homologous Overlap Design

Length

- 15 bp to 20 bp.
- 30 bp to 40 bp for inserts > 5 kb, more than two inserts, or for mutagenesis over large areas.

Sequence

- Taken on either end or shared between the ends of the two DNA fragments.
- For novel DNA insertions, mutagenesis or other engineering applications: *de novo* sequence is added at both ends of the DNA fragments and participates to the overlap.

Structure

- No stable hairpin.
- No DNA repeats.

Summary of DNA Fragment Preparation

Preparing PCR Fragments

1. Create an *in silico* template sequence and design primers.
2. Amplify DNA fragments by PCR.
3. Check amplification by agarose gel electrophoresis and verify molecular weight of the amplified DNA.
4. Treat PCR reaction by DpnI (1 µl = 20 U of enzyme per 50 µl reaction, 1 h to overnight at 37°C). *DpnI cuts methylated sites and decreases the transformation efficiency of template plasmid DNA prepared in* dam^+ *bacterial strains.*
5. Optional: Gel-purify the desired PCR product, if multiple products are obtained during PCR.
6. Purify the desired DNA fragment, using standard spin column technology (such as NucleoSpin®, Macherey-Nagel) and elute in a small volume (< 50 µl) of pure water.
7. Measure DNA concentration by UV spectrophotometry.

Preparing DNA Fragments by Restriction Digest

1. Digest the plasmid DNA with restriction enzymes.
2. Optional: Heat-inactivate restriction enzymes.
3. Optional: Gel-purify the cut vector DNA. *Background may be increased if this step is omitted.*
4. Purify the DNA fragment on a standard spin column (such as NucleoSpin®, Macherey-Nagel) and elute in a small volume (< 50 µl) of pure water.

5. Measure DNA concentration by UV spectrophotometry.

Preparing Synthetic DNA Fragments

1. Resuspend dry synthetic DNA fragment in pure water, at appropriate concentrations, such as 5 ng/µl.

Preparing Oligonucleotides

1. Dilute the oligonucleotides in pure water at 0.1 µM.

Setting Up the FAST-Licase™ Reaction

Materials & Reagents

- Thermocycler or water bath at 75°C
- **Licase™** complex & 5x buffer
- Purified and quantified DNA fragments

Calculating DNA amounts

Choosing the amount of vector

By convention, the vector is the DNA fragment that contains the origin of replication. Use the following table to determine the amount of vector to add to the reaction:

VECTOR LENGTH	DNA AMOUNT
< 4,000 bp	50 ng
4,000 - 6,000 bp	75 ng
6000 - 10,000 bp	100 ng
> 10,000 bp	150 ng

Choosing the insert to vector molar ratio

For assembly with a vector linearized by restriction enzymes, use a insert/vector molar ratio of 2. If the vector was amplified by PCR, use a ratio of 1.

For insertion of small DNA fragments, use a ratio of 4 (< 400 bp), 6 (< 300 bp) or 10 (< 200 bp).

Calculating the amount of insert

Use the following equation:

insert (ng) = vector (ng) x [insert length/vector length] x ratio

For example, cloning a synthetic DNA fragment of 500 bp into a plasmid of 3.5 kb will require 50 ng of cut, linearized plasmid, an insert to vector ratio of 2 and 50 x (500/3500) x 2 = 14 ng of synthetic DNA fragment.

Assembly and Operation of the **FAST-Licase™** Reaction

REAGENT	AMOUNT / VOLUME
Pure water	To 20 µl final volume
5x buffer	4 µl
Vector DNA	As calculated
Insert DNA	As calculated
Licase™ Complex	1 µl

All reagents must be added successively in a single PCR tube on ice to a final volume of 20 µl. The **licase™** is added last.

Start the thermal cycler before adding the **licase™**; when the temperature reaches the 4°C plateau, add the **licase™**, mix briefly using the tip f the finger, insert the tube in the cycler and un-pause the thermal cycler to engage the next cycle and start the reaction. The **FAST-Licase™** cycle will complete in a few minutes. After completion, transfer the PCR tube on ice and proceed to the bacterial transformation. **FAST-Licase™** reactions can be stored at -20°C, if necessary, before transformation.

If the manual method is used, mix briefly after adding the **licase™**, hold the tube between 2 s and 5 s in a water bath set to 75°C. If a microfuge tube is used instead of a PCR tube, incubate for 5 s at 75°C. Keep on ice or at room temperature on the bench before bacterial transformation. For long term storage before bacterial transformation, keep at -20°C.

Running a FAST-Licase™ Reaction with Oligonucleotides

1. Run the **FAST-Licase™** reaction with the vector and without the oligonucleotides.
2. Add 0.5 µl of each oligonucleotide (0.1 µM in water).
3. Run the **FAST-Licase™** a second time using the same tube.
4. Proceed to the bacterial transformation.

Analyzing Transformants

Bacterial Transformation

Follow the manufacturer's recommendations for the transformation protocol. Use of chemically competent cells with medium efficiency in the range of 1 x 10^8 cfu/µg DNA is recommended. Mix 1 µl of the **FAST-Licase™** reaction per 25 µl of cells. The volume of SOC can be limited to 200 µl per reaction.

Clone Analysis

1. Pick 4 colonies and grow overnight at 37°C with agitation at 250 rpm in 3 ml of 2xYT medium supplemented with the proper antibiotic in 14 ml Falcon® tubes or equivalents.
2. The next day, spin all 4 tubes at 5000 rpm for 10 min and discard the supernatant.
3. Freeze two tubes at -20°C for backup.
4. Prepare the plasmids from the two other tubes and analyze the DNA for proper assembly.

Frequently Asked Questions (FAQs)

What is the success rate of FAST-Licase™ ?

The success rate of **FAST-Licase™** is around 90% of proper assembly of DNA fragments among bacterial transformants. Insertion of oligonucleotides is lower, between 25% and 50% success rate.

Do I need to add a negative control?

There is no need to use a negative control with **FAST-Licase™**. If no recombinant clones are found after analysis of the four colonies, you can either use brute force by colony PCR to identify a proper recombinant, or modify the **FAST-Licase™** reaction as follows: if the background is high, decrease the amount of vector by a factor of two, or increase the amount of insert by a factor of two to four. These simple modifications usually lead to the isolation of the proper clone. Always verify the design of the homologous regions.

Can I clone repetitive DNA fragments?

DNA fragments with repetitive sequences can anneal unpredictably and lead to clones with unintended assemblies. Unfortunately, such unexpected cloning events are usually discovered very late. However, the competition between several annealing options usually leads to a mosaic of correct and incorrect clones.

Can I use non-matching 5'-ends?

When the annealing process creates non-matching 5' overhangs, cloning by **FAST-Licase™** is still possible, because the bacterial machinery will remove these overhangs. This is the reason why repetitive DNA fragments can generate incorrect clones after annealing at sequence positions other than the expected terminal homologous region.

Can I use shorter homology regions?

Although cloning by homologous recombination has been reported for overlaps as short as 12 bp, using at least 15 bp overlaps is strongly recommended. Longer overlaps of 20 bp or more increase the success rate of the **FAST-Licase™** reaction significantly, especially with very long fragments or when more than two fragments are assembled.

Can I use longer homology regions?

Yes. Overlaps of up to 100 bp and more have successfully been used in **FAST-Licase™** reactions.

Do I need to purify the PCR product?

The use of small spin columns combined with elution in small volumes of pure water to prepare DNA fragments is key to successful molecular cloning of small amounts of material. Gel-purification of PCR products may prove necessary when multiple products are visible on an agarose gel.

Can I assemble more than two DNA fragments?

FAST-Licase™ is designed for speed and efficiency. There is a decrease of cloning efficiency as the number of fragments increases. However, there is no theoretical limit to the number of fragments that can be assembled at once. Designing longer overlaps between 20 bp and 40 bp and using the thermal cycler method with a longer plateau at 37°C may improve efficiency when using multiple DNA fragments.

Troubleshooting

The FAST-Licase™ reaction yields no colonies after bacterial transformation.

Background colonies from the vector should always occur to some extent. Using higher efficiency competent cells or increasing the amount of vector will help solve the issue in most cases.

Only background colonies are found.

Increasing the amount of insert fragment usually solves the issue. This is particularly true when using small (< 200 bp) DNA fragments.

When using restriction enzymes, make sure the vector is cut properly. Molecular cloning using a single restriction site is prone to high background and gel-purification of the linearized form of the vector and dephosphorylation may prove necessary. Alternatively, the vector can also be amplified by PCR and *in-situ* digestion of the PCR reaction by DpnI to limit background deriving from the template DNA may further reduce the number of background colonies.

Appendix

MSDS Information

MSDSs (Material Safety Data Sheets) are available on the **Antibody Design Labs** website at the corresponding product page.

Quality Control

Specifications and quality control are detailed on the online product page. **Antibody Design Labs** certifies that the product will perform according to these specifications.

Technical Support

Visit the **Antibody Design Labs** website at www.abdesignlabs.com for technical resources, including manuals, vector maps and sequences, application notes, FAQs, etc.

For more information or technical assistance, call, write, fax, or email us at:

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