DESCRIPTION

There are many parameters affecting the efficiency, specificity and fidelity of an amplification reaction. These include the nature of the template DNA, primers, concentration of dNTPs, $MgCl_2$, and *Taq* DNA Polymerase. Since there are many parameters and concentrations to be optimized, finding the optimized condition is a time-consuming process. Therefore the 2X Ampli-Optimization Kit is designed to provide an easy and convenient way to optimize reaction conditions specific for your amplicon. This kit contains the **2X *Taq* Master Mix** with a range of $MgCl_2$ concentration (2-7mM). All the other reaction components in **2X *Taq* Master Mix** such as dNTPs, reaction buffer and *Taq* DNA Polymerase are provided at the optimized concentration. Using this kit, the user would be able to determine the specific $MgCl_2$ concentration for their amplification reaction.

KIT COMPONENTS

The kit contains 6 tubes of **2X *Taq* Master Mix** with different $MgCl_2$ concentrations. Each **2X *Taq* Master Mix** consists of *Taq* DNA Polymerase (0.05U/µl), 0.4mM dNTPs and 2X ViBuffer A [20mM Tris-HCl (pH9.1 at 20°C), 100mM KCl, 0.02% Triton X-100]. The concentration of $MgCl_2$ in each tube (A-F) is stated in **Table A**.

Table A: Kit components

2X *Taq* Master Mix	$MgCl_2$ concentration	Amount	Cap Color
A	2mM	0.625ml	Yellow
B	3mM	0.625ml	White
C	4mM	0.625ml	Orange
D	5mM	0.625ml	Red
E	6mM	0.625ml	Blue
F	7mM	0.625ml	Green

STORAGE & STABILITY

- Kit components are stable at -20°C for one year or at 4°C for 6 months if properly stored.
- All components are stable for twenty freeze-thaw cycles. To avoid frequent freeze-thaw, keeping small aliquot at -20°C is recommended.
- For daily use, keeping an aliquot at 4°C is recommended.

FEATURES

- Reaction buffer, dNTPs and *Taq* DNA Polymerase are provided in an optimized concentration.
- Easy and convenient for optimization of $MgCl_2$ concentration.
- Color caps are provided for convenient recognition of $MgCl_2$ concentration within the **2X *Taq* Master Mix**.
- Saves time and reduces contamination due to reduced number of pipetting steps.
- Stable at 4°C for 6 months, allowing immediate reaction setup without the time-consuming thawing of reagents.
- Suitable for all routine DNA amplification amplifications.

QUALITY CONTROL

All preparations are assayed for contaminating endonuclease, exonuclease, and non-specific DNase activities. Functionally tested in DNA amplification.

PRELIMINARY CONSIDERATIONS

Primers

Guideline on designing primers

- Usually the lengths of primers are 18-28 nucleotides long.
- GC content of the primer should be 45-60%. The G and C nucleotides should have a balanced distribution within the full length of the primer. Avoid more than three G or C nucleotides at the 3' end to lower the risk of non-specific priming.
- The primer is not self-complementary or complementary to other primers in the reaction mixture to avoid internal secondary structure or the formation of primer dimers.
- Melting temperature ™ of the primer should be 50-70°C. Melting temperature of flanking primer should not differ more than 5°C.
- If degenerate primer is used, avoid degeneracy for the last 3 nucleotides at the 3'end.
- The designed primers should be checked for possible complementary sites within the template DNA. Primer with more than one complementary site within the template DNA should be avoided, as non-specific priming will occur.

Standard concentration of primer in the reaction mixture is 0.2µM. Increasing the primers concentration may increase the possibility of non-specific priming. However for degenerate primers, the concentration may increase to 0.5µM in order to increase the amount of "correct" primer; thus, increasing the yield of the expended product.

Template DNA

The template DNA is likely the largest variable in PCR. This is because the same amount of DNA from different organisms represents different copy numbers. In general, optimal amount of template for plasmid, cDNA and phage DNA is in the range of 0.01-10ng and for genomic DNA is in the range of 0.05 – 1µg per 50µl reaction volume.

$MgCl_2$ Concentration

Specific $MgCl_2$ concentration is essential for each amplification reaction. $MgCl_2$ forms a complex with dNTPs, primers and template DNA. In addition, *Taq* DNA Polymerase requires free $MgCl_2$ as its cofactor, therefore $MgCl_2$ affects strand dissociation, primer annealing, enzyme activity and fidelity. Thus the optimal concentration of $MgCl_2$ is very crucial for each amplification reaction. The recommended concentration range is 1 – 3.5mM $MgCl_2$.

Too low $MgCl_2$ concentration results in low yield of desired product while too high $MgCl_2$ concentration increases the yield of non-specific products and decreases the fidelity of the reaction. Lower $MgCl_2$ concentration is preferred when fidelity of DNA synthesis is crucial.

Taq DNA Polymerase

Most amplification reactions use 1 to 2.5 units of *Taq* DNA Polymerase for a 50µl total reaction volume. Generally, high concentration of *Taq* DNA Polymerase produces more products as the efficiency is better. However, non-specific products may appear at higher concentration of *Taq* DNA Polymerase. 2X Ampli-Optimization Kit have fixed 1.25 units of *Taq* DNA Polymerase per 50µl total reaction volume. Users may optimize the unit of *Taq* DNA Polymerase after the specific $MgCl_2$ concentration has been determined in order to increase the yield of amplification products or to avoid non-specific amplification. In addition, *Taq* DNA Polymerase from different manufacturers may have different amplification efficiencies. Therefore, users may need to adjust the amount experimentally when using *Taq* DNA Polymerase from different manufacturers.

Reaction Buffer

The pH and salt (KCl) concentration of the buffer affects the stability and strand dissociation of the template DNA. At high salt concentration, the binding strength of the double stranded DNA is stronger and therefore, the melting temperature of the template and primers is increased. Long amplicons are not recommended to be amplified at high salt concentrations as the template DNA is more difficult to denature. It is not worthy that $MgCl_2$ can also affect strand dissociation.

CYCLING CONDITION

Amplification reactions are generally cycled along three steps: denaturation of template DNA, annealing of primers and elongation. Optimum cycling conditions vary according to the template, primers, size of amplicon, reaction buffer, total reaction volume and thermocycler used.

Initial Denaturation

A complete denaturation of DNA template at the beginning of the amplification reaction is essential as incomplete denaturation of DNA will result in low efficiency in the first amplification cycle, leading to poor yield of the amplified product. For most applications, the initial denaturation of 2 to 5 minutes at 94°C is usually sufficient. For GC rich templates, the addition of DMSO between 3-10% (5% is recommended as a starting point) may improve the amplification efficiency.

Denaturation

Since the amplified product in the first cycle is significantly shorter than the template DNA, denaturation at 94°C for 30 second is usually sufficient. For amplicon with high GC content the denaturation time may be increased to 2-4 minutes.

Annealing

Annealing temperature is a vital parameter of an amplification reaction. The annealing temperature is usually chosen based on the length and GC content of the oligonucleotide primers. Annealing temperature is often 1-5°C below the primer's melting temperature (Tm). It is recommended that the sequences of both primers are chosen such that the Tm of both primers does not differ more than 5°C. If the Tm of the flanking primers is different, use 1-5°C below the lowest Tm of primers in the reaction mixture as the annealing temperature. Annealing time for 30 seconds to 2 minutes is usually sufficient. In general, higher annealing temperature will increase primer-template specificity and result in less non-specific amplification. Lower annealing temperature will improve the binding of primers and amplification efficiency, but may result in non-specific amplifications.

Elongation

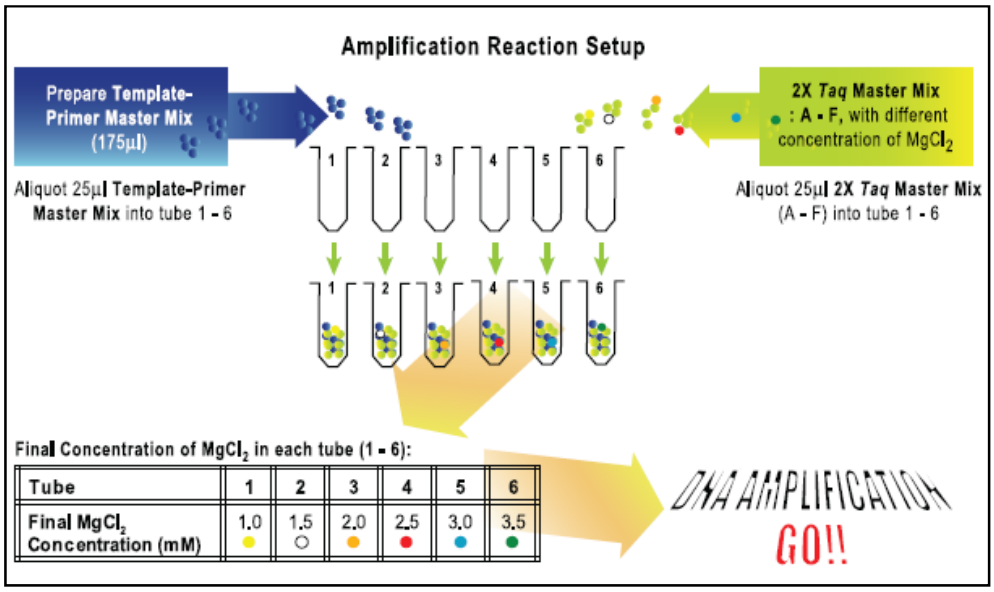
Usually the extending step is performed at 72°C, which is the optimal temperature for primer extension by *Taq* DNA Polymerase. Recommended elongation time is 30-60 seconds for each kilo base pairs of product to be amplified.

Number of Cycles

The choice for the number of amplification cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the amplification. If quantity of the DNA template is higher than 100 copies, 25 to 35 cycles are usually sufficient. If fidelity of synthesis is crucial, maximum allowable amount of template with minimum amplification cycles is recommended.

Final Elongation

Upon the end of the last cycle, reaction mixtures are usually incubated at 72°C for 2 to 5 minutes in order to let the *Taq* DNA Polymerase to add extra adenine nucleotides to the 3'-ends of the amplified products. If amplified fragments are to be used for TA cloning purposes, this step can be extended up to 30 minutes.



AMPLIFICATION PROTOCOL

Optimization of $MgCl_2$ concentrations

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Dilute your forward and reverse primer to 10µM working stock solutions.
3. Label a set of six 0.2ml microcentrifuge tubes as 1-6.
4. Use a sterile microcentrifuge tubes to prepare a **Template-Primer Master Mix** for seven reactions as follows:

Reagent	Volume (For 7 reactions)	Final Concentration (in each of 50ul reaction)
Forward Primer (10µM)	Variable (3.5-35µl)	0.1-1µM (recommended 0.2µM)
Reverse Primer (!0µM)	Variable (3.5-35µl)	0.1-1µM (recommended 0.2µM)
Template DNA	Variable	0.01-10ng for plasmid 0.05-1µg for genomic DNA
Water, nuclease-free	to 175µl	

5. Mix thoroughly, centrifuge briefly and aliquot 25µl of **Template-Primer Master Mix** to tube 1-6.
6. Add 25ul of **2X *Taq* Master Mix** (A-F) to tubes 1-6 respectively.
7. Mix thoroughly and centrifuge briefly. Place the reaction tubes in a thermocycler and start the amplification program.

Recommended program for most common amplifications:

Step		Temperature	Time	Number of Cycles
1	Initial Denaturation	94°C	2 mins	1
2	Denaturation	94°C	30 secs	25 – 35
	*Annealing	50 – 68°C	30 secs	
	Extension	72°C	30 secs/kb	
3	Final Extension	72°C	5 mins	1

* Recommended annealing temperature is 1-5C below the lowest melting temperature of the primers in the reaction mixture.

TROUBLESHOOTING

Problem	Possibility	Recommended Solution
Low yield or no PCR product	Missing component in reaction mixture	Check the reaction components and repeat the reaction.
	Insufficient template	Use more templates or increase cycle number.
	Insufficient cycles	Increase the number of cycles by 3 to 5 cycles at a time.
	Annealing temperature too high	Decrease annealing temperature by 2°C at a time.
	Annealing time too short	Increase annealing time by 1 minute at a time.
	GC-rich template	Increase denaturing temperature to 97-99°C and denaturing time to 5-10 minutes or add GC destabilizing co-solvent such as DMSO (3-10%) to the reaction mixture.
	Denaturation temperature too high	Decrease denaturing temperature by 2°C at a time.
	Denaturation time too long	Decrease denaturing time by 10s at a time.
	Elongation time too short	Increase the elongation time by 1 minute at a time.
Smearing	Agarose gel was not fresh	Repeat electrophoresis with fresh agarose gel.
	Too many cycles	Reduce the number of cycles by 3 to 5 cycles at a time.
	Too much template	Reduce amount of template by 10 to 1000 times dilution.
	Degraded template	Check and confirm template integrity by agarose gel electrophoresis. If necessary, repurify template using methods that minimizes shearing and nicking. Resuspend template in sterile TE buffer, pH 8.0 or water.
	Denaturation temperature too high	Decrease denaturing temperature by 2°C at a time.
	Denaturation time too long	Decrease denaturing time by 10s at a time.
	Elongation time too short	Increase the elongation time by 1 minute at a time.
Non-specific bands	Too many cycles	Reduce the number of cycles by 3 to 5 cycles at a time.
	Too much template	Reduce amount of template by 10 to 1000 times dilution.
	Annealing temperature is too low	Increase the annealing temperature by 3°C at a time.
	Elongation time too long	Reduce elongation time by 30s at a time.
	Cross contamination	Use a separate workplace, pipettes and filter tips. Wear gloves at all times.