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DNA AmpliTools Green Master Mix

REF.	FORMAT	CONTENT
10.431	250 rxns of 25 µl	DNA AmpliTools Green Master Mix
10.432	500 rxns of 25 µl	DNA AmpliTools Green Master Mix

Store at -20ºC

Research Use Only. Not for use in diagnosis procedures

Notice to users: Some of the applications which may be performed with this product are covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application.

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1. PRODUCT DESCRIPTION

The new developed **DNA AmpliTools Green Master Mix (2X)** is a ready-to-use solution for performing PCR amplifications of a wide range of templates (including genomic DNAs and templates rich in CG-content).

The DNA AmpliTools Green Master Mix includes a density reagent and two tracking dyes that allow direct loading onto a gel and monitoring of progress during electrophoresis.

The ready to use format dramatically reduces set up time, liquid handling steps, thereby minimising the risk of contamination. With the exception of primers and template, the convenient DNA AmpliTools Green Master Mix provides all the necessary reagents at optimal concentrations to perform PCRs for targets up to ***10 kb in length***. The DNA AmpliTools Green Master Mix (2X) works with different reaction volumes between **20-50 µl**.

The DNA AmpliTools Green Master Mixes of Biotools allow for convenient and easy to use PCRs, and they are ideally suited to routine PCR applications with minimal optimisation.

Applications of *DNA AmpliTools Green Master Mix*:

- √ *Routine PCR (up to 10 kb)*
- √ *High throughput PCR*
- √ *Primer extension*
- √ *Generation of PCR products for TA cloning*
- √ *Gene sequencing*
- √ *RT-PCR*

Note: *This master mix is not recommended for certain experiments dealing with amplification of sequences homologous to those found in E. coli.*

Components: The DNA AmpliTools Green Master Mix (2X) contains recombinant Biotools DNA Polymerase; dNTPs; $MgCl_2$; Reaction Buffer; stabilisers; and a proprietary buffer containing a compound that increases sample density, and two dyes, which function as loading dyes when reaction products are analised by agarose gel electrophoresis. The blue dye migrates with 3-5 kb DNA fragments, and the orange dye migrates faster than 100 bp DNA fragments in 1% agarose gel.

2. STORAGE AND HANDLING INSTRUCTIONS

Store DNA AmpliTools Master Mix tubes at **-20ºC** in a constant temperature freezer (frost-free freezers are not recommended). The mix must be thawed and handled on ice. For frequent use, divide in aliquots to avoid multiple freeze-thaw cycles.

For short-term storage and frequent use, the DNA AmpliTools Green Master Mix, may be kept at 2-8ºC for up to 3 months, depending on the expiration date.

If stored under the recommended conditions, the product will maintain performance through the indicated date on the label.

3. GENERAL CONSIDERATIONS

Template: DNA condition is a key point to obtain optimal results. DNA prepared using standard isolation techniques is a suitable substrate for amplification. Nevertheless, even trace amounts of certain agents used in DNA purification procedures (phenol, EDTA, proteinase K, ionic detergents, silica particles, etc.) often inhibit amplification. We recommend the use of our Speedtools line for extraction of genomic DNA from blood (*Speedtools DNA Extraction*); from tissue (*Speedtools Tissue DNA Extraction kit*); from food (*Speedtools Food DNA Extraction kit*); and from plant material (*Speedtools Plant DNA Extraction kit*).

Samples should be transported and stored frozen. In samples that have been stored without refrigeration, DNA can be degraded. In case of working with clinical samples, handle them as if they are capable of transmitting infectious agents.

The quantity of template DNA to be added in each reaction depends on the source and quality of the template. For the DNA AmpliTools Green Master Mix we recommend **0.1 pg-10 ng** for a simple template such as plasmid and phage DNA, and between **0.1-500 ng** for complex templates such as genomic DNA. Higher amounts of template increase the formation of non-specific products and may reduce PCR yield.

$MgCl_2$ Concentration: Magnesium ion concentration affects primer annealing and template denaturation, as well as DNA polymerase activity and fidelity. Generally, excess Mg^{2+} results in accumulation of non-specific products, whereas insufficient Mg^{2+} results in reduced yield of the desired PCR product. The final $MgCl_2$ concentration in 1X DNA AmpliTools Green Master Mix is **2 mM**. This supports acceptable amplification of most amplicons.

Primer Design: Primers typically are 15-30 bases in length and contain approximately 40-60% GC-content: the annealing temperatures of primer pairs should be nearly identical.

Care must be taken to design primers that do not form hairpin loop structures or are self-complementary. The 5´ end of a primer may contain mismatches between the primer and template, whereas this is not recommended at the 3´ end. Avoid placing more than three G or C nucleotides at the 3' end to lower the risk of non-specific priming.

The optimal quantity of template and primers must be determined empirically for each combination of template and primer. The recommended concentration range of each primer is 0.1-1.0 µM. The range **0.2 µM-0.5 µM** works for most amplification; use 0.2 µM of each primer as starting point of optimisation.

Additives: Amplification of some difficult targets, like GC- rich sequences, may improve with additives, such as DMSO, formamide, or betaine. The DNA AmpliTools Green Master Mix is compatible with common PCR additives.

Cycling Parameters: Many parameters influence both the specificity and efficiency of amplification including the temperature and duration of denaturation, annealing and elongation; and total cycle number. Variations to the program may be required depending on the amplicon size, the template, etc.

When primers with annealing temperatures above 60 °C are used, a two-step thermocycling protocol is possible.

-Denaturation:

- Initial denaturation: 1-5 min at 94-96 °C for template DNA with ≤ 50% in GC-content and 5-10 min for genomic DNA or DNA with > 50% in GC-content.
- Subsequent denaturation steps will be between 15-60 seconds at 94-96 °C.

-Annealing:

- Annealing temperature should be 5 °C lower than the melting temperature of primers.
- The annealing step is between 15-60 seconds.

-Extension:

- The optimal extension temperature for Biotools DNA Polymerase is 72°C. For larger products, the extension temperature should be reduced at 68 °C.
- The recommended extension step is 1 min/kb of the expected amplicon.
- A final extension of 3-10 min at 72°C is recommended to fill-in any possible incomplete reaction products.

-Cycle Number:

- Generally, 25-35 cycles result in optimal amplification of desired products. Occasionally, up to 40 cycles may be performed, especially for detection of low-copy targets.

Reaction Conditions: The DNA AmpliTools Green Master Mix (2X) should be used at 1X concentration with template and primers, in a final reaction volume of **20**, **25**, or **50 µl**.

4. STANDARD PROTOCOL

These guidelines cover routine PCR reactions. Amplification of templates with high GC content, high secondary structure, low template concentrations, or amplicons greater than 5 kb may require further optimisation.

Materials to be supplied by user:

- Downstream oligonucleotide primer
- Upstream oligonucleotide primer
- Nuclease-free water
- Template DNA

Laboratory workflow must be unidirectional, from pre-amplification to amplification areas. Specific equipment for each working area must be used, in order to avoid cross contaminations.

1. Thaw DNA AmpliTools Green Master Mix; primers; and template DNA on ice. Mix the master mix before use in order to prevent localised concentrations of salts; then spin it briefly.

2. Calculate the number of needed reactions; do not forget to include a no template control (NTC) reaction to check for contamination.

3. Prepare one of the following mixes on ice:

COMPONENT	Final Concentration	Volume for 20 µl rxn	Volume for 25 µl rxn	Volume for 50 µl rxn
DNA AmpliTools Green Master Mix (2X)	1 X	**10 µl**	**12.5 µl**	**25 µl**
Forward primer	0.1-1 µM	2.0-20 pmol (x µl)	2.5-25 pmol (x µl)	5.0-50 pmol (x µl)
Reverse primer	0.1-1 µM	2.0-20 pmol (x µl)	2.5-25 pmol (x µl)	5.0-50 pmol (x µl)
Nuclease free water	-	to 20 µl	to 25 µl	to 50 µl
Template DNA*	variable	x µl	x µl	x µl

Template DNA*	Plasmid: 0.1 pg-10 ng gDNA: 0.1-500 ng

***Start with 10 pg for a simple template such as plasmid and phage DNA, or 10 ng for complex templates such as gDNA.*

Note: *The optimal quantity of template and primers must be determined empirically for each new combination of template and primer. The reaction conditions described in this protocol are general recommendations only.*

4. Mix the reagents by vortexing. Spin tubes briefly in a microcentrifuge to collect the material at the bottom of the tube.

5. For thermal cycler without heated lid overlay add a mineral oil layer.

6. Transfer PCR tubes from ice to a thermal cycler with the block preheated to 95 °C and perform the selected PCR program.

Standard Amplification Program

Cycle Step	Nº Cycles	Temperature	Time
Initial Denaturation	1	95 ºC	1-5 min
Denaturation	25-35	95 ºC	15-60 sec
Annealing		T_m-5 °C	15-60 sec
Extension		68-72 ºC	60 sec/kb
Final Extension (optional)	1	68-72 ºC	5 min
Cooling	∞	4 ºC	∞

7. Load directly 20-50% of PCR mixture directly on a gel to analyse the PCR product. *There is no need to add loading buffer as the mix is sufficiently high density to sink to the bottom of the gel.*

5. TROUBLESHOOTING

Little or no amplification detected

1. **Check template quality and quantity.** Check the quality of your template DNA by agarose gel electrophoresis or fluorimetry. Some DNA purification procedures, particularly genomic DNA isolation, can result in the purification of inhibitors. Reduce the volume of template DNA in reaction or dilute the template prior to adding to reaction. Ethanol precipitation of DNA and repetitive treatments of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the sample.
   Sample DNA damaged or degraded. Use sample that has been processed and stored properly to minimise shearing and nicking. Use of excess template can also reduce PCR yield.
2. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer-dimers.
3. **Optimise primer concentration.** Although lower primer concentration can prevent primer-dimer formation, sufficient primers are needed for successful PCR. Increase primer concentration in increments of 0.1 µM.
4. **Increase initial template denaturation.** Increase the length of initial denaturation. It is very important in the early cycles to make sure that the template is completely denatured. Templates rich in GC-content or with secondary structures often require longer initial denaturation (≤ 10 min).
5. **Lower annealing temperature.** Lower the annealing temperature in 2 °C decrements. Remember that PCR additives and enhancing agents usually affect the annealing temperature of primers.
6. **Increase number of cycles.** Perform additional cycles in increments of 5 cycles. Occasionally, up to 40 cycles may be performed, especially for detection of low-copy targets.
7. **Change extension phase conditions.** Increase the extension time by increments of 30 sec. For large products reduce the extension temperature up to 68 °C.
8. **Add additives to the reaction mixture.** Adding PCR enhancing agents (e.g. DMSO or betaine) may improve yield. General estabilising agents (e.g. albumin) also may help to overcome amplification failure.

Multiple products or a smear detected

1. **Decrease concentration of reaction components.** Check the concentration of template DNA by agarose gel electrophoresis or fluorimetry. Decrease the amount of DNA and/or primer added to the reaction.
2. **Sample DNA damaged or degraded.** Use sample that has been processed and stored properly to minimise shearing and nicking. Low template quality templates or higher amounts of template increase the risk of generation of non-specific PCR products.
3. **Check the primers for degradation.** Check by electrophoresis in a denaturing acrylamide gel.
4. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer-dimers.
5. **Increase annealing temperature.** Increase the annealing temperature in 2°C increments.
6. **Decrease number of cycles.** Decrease number of cycles in decrements of 5 cycles.
7. **Carryover contamination.** If the no template control (NTC) shows PCR product or a smear, exchange all reagents.

6. ORDERING INFORMATION

DESCRIPTION	Format	Reference
DNA AmpliTools Green Master Mix	250 rxns of 25 µl	10.431
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