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DNA AmpliTools Complex Master Mix

REF.	FORMAT	CONTENTS
10.451	250 rxn of 25 µL	DNA AmpliTools Complex Master Mix
10.452	500 rxn of 25 µL	DNA AmpliTools Complex Master Mix

Store at -20ºC

Warning for users: Some of the applications that can be performed with this product are protected by patents applicable in some countries. Purchase of this product does not include or provide a licence to perform patented applications. In some cases, depending on the country and/or application, users are required to purchase a licence.

Ed 03 – March 19

1. PRODUCT DESCRIPTION

The **DNA AmpliTools Complex Master Mix** from Biotools is a 2X master mix optimised to achieve maximum yield and specificity in amplification reactions using complex (genomic DNA, GC-rich templates, templates with secondary structures) and/or scarce DNA templates.

Genomic DNA, GC-rich templates or those with a secondary structure are difficult to amplify. The main reason for this difficulty is that conventional polymerases introduce errors during DNA replication that can stop the amplification reaction, thereby resulting in truncated products and/or error accumulation in the amplified sequence and compromising the yield and fidelity of the PCR.

The DNA AmpliTools Complex Master Mix overcomes these difficulties by incorporating a mix of highly efficient enzymes, including Biotools HotSplit DNA Polymerase, which has a hot start effect, and Biotools *Pfu* DNA Polymerase, which has proofreading activity. The combination of these enzymes, together with the special reaction buffer and cofactors included in the mix, results in high yield and copy fidelity in complex amplification reactions. The hot start nature of the HotSplit DNA Polymerase contributes significantly to the specificity achieved with the DNA AmpliTools Complex Master Mix by reducing the formation of primer dimers and nonspecific amplification products.

Applications of DNA AmpliTools Complex Master Mix:

- ✓ *Amplification of GC-rich DNA templates*
- ✓ *Amplification of DNA templates rich in secondary structures*
- ✓ *Amplifications using tiny amount of template (detection limit of 0.1 pg)*
- ✓ *Amplification of fragments up to 10 kb (genomic DNA)*
- ✓ *Amplification of fragments up to 30 kb (lambda DNA)*

Components: The DNA AmpliTools Complex Master Mix contains Biotools HotSplit DNA Polymerase; Biotools *Pfu* DNA Polymerase; dNTPs: $MgCl_2$; reaction buffer; adjuvants and stabilisers at concentrations suitable for performing a wide range of DNA amplification reactions.

2. STORAGE CONDITIONS

Store vials of DNA AmpliTools Complex Master Mix at **-20ºC** in a freezer that ensures a constant temperature (frost-free freezers are not recommended). If the kit is to be used frequently, we recommend to prepare aliquots to avoid frequent freeze/thaw cycles.

If handled and stored according to these recommendations, the stability of this product will be as indicated on the corresponding label.

3. GUIDELINES FOR USING THIS PRODUCT

Template: The quality of the DNA template is key to obtaining optimal results in amplification reactions. Although conventional extraction methods produce templates with sufficient quality for PCR, some of the reagents used during purification (phenol, EDTA, proteinase K, ionic detergents, silica particles, etc.) often inhibit amplification. Biotools recommends its Speedtools product range for the extraction and purification of genomic DNA from blood (*Speedtools DNA Extraction*); tissue (*Speedtools Tissue DNA Extraction kit*); food (*Speedtools Food DNA Extraction kit*); and from plants (*Speedtools Plant DNA Extraction kit*).

Samples should be transported cold as a lack of refrigeration may lead to degradation of the DNA. All clinical samples must be handled as though they were potentially infectious.

The quantity of DNA to be included in the amplification reaction will depend on the source and quality of the template to be used. We recommend **0.2-10 ng** for low-complexity DNA (e.g. plasmid, lambda or bacterial DNA) and **10-250 ng** for complex templates (human genomic DNA); use 1 ng and 50 ng, respectively, for initial optimisation. An excess of template increases the formation of nonspecific amplification products and decreases the yield of the reaction.

$MgCl_2$ concentration: The concentration of magnesium ions affects primer annealing and denaturation of the template, as well as polymerase activity and fidelity. High $MgCl_2$ concentrations can result in the formation of nonspecific amplification products and low concentrations can lower the yield of the reaction. The final $MgCl_2$ concentration in the DNA AmpliTools Complex Master Mix 1X is **2 mM,** an ideal concentration for the majority of amplicons.

Primer design: The primers used in the amplification reaction are usually around 15-30 bases long and have a GC content of 40-60%. Moreover, the annealing temperatures for both primers should be practically identical.

When performing the design, remember that the primers should not form forks or be mutually complementary. The absence of complete homology with the DNA template at the 5' end of primers is not as critical as the lack of complementarity at the 3' end. Avoid including more than three G or C nucleotides at the 3' end of the primers in order to reduce nonspecific annealing.

The optimal quantity of DNA and primers in the PCR must be determined experimentally for each new template/primer combination. The optimal concentration for complex amplifications using DNA AmpliTools Complex Master Mix is in the range **0.3-0.7 µM**; use 0.5 µM of each primer for initial optimisation.

PCR program: Some program parameters, including the denaturation, annealing and elongation temperature and time and the number of cycles, affect both the amplification specificity and efficiency. Variations in the size of the amplification product, the source of the template and primer sequence usually require changes in the amplification program.

A two-step amplification protocol can be applied when primers with an annealing temperature higher than 60 ºC are used.

- Denaturation:

- Initial denaturation: *The HotSplit DNA Polymerase* is activated during this phase of the amplification cycle. 5 min at 94-96 °C is sufficient for templates with a GC content ≤ 50% and 8 min for genomic DNA or templates with a GC content > 50%.
- Subsequent denaturations: 25-30 s at 94-96 °C.

- Annealing

- Optimal annealing temperature: approximately 5 °C below the melting temperature of the primers.
- Extension of the annealing step: 10-15 s.

- Extension:

- The optimal extension temperature for the enzyme mixture included in the DNA AmpliTools Complex Master Mix is 72°C. For long amplicons (>3 kb), we recommend to reduce this temperature to 68 °C.
- Extension period: 50 s/kb expected amplification product.
- A final extension phase of 3-10 min at 68-72°C is recommended to complete the extension of incomplete reaction products.

- Number of amplification cycles:

- Optimal number of cycles: 30-35 cycles; this can occasionally be increased to 40, especially when templates with a low number of copies are used.

Reaction conditions: The DNA AmpliTools Complex Master Mix (2X) should be used at a final concentration of 1X in the reaction mixture that will include the primers and the template. The recommended reaction volume is **25 µL**.

4. STANDARD PROTOCOL

Materials to be provided by the user:

- Downstream Primer
- Upstream Primer
- Nuclease-free water
- Template

The work flow in the laboratory must be unidirectional, from the pre-amplification zone to the amplification zone. To avoid cross-contamination, we recommend using specific equipment for each working zone.

1. Thaw the DNA AmpliTools Complex Master Mix, primers and DNA template in ice. Keep the reagents cold while preparing the reaction mixture.

2. Calculate the number of reactions necessary, remembering to include at least one template-free control reaction to rule out contaminants in the mixture.

3. **Mix the DNA AmpliTools Complex Master Mix** prior to use in order to prevent localised concentration of salts.

> ***Note:*** *The HotSplit DNA Polymerase included in the mixture is activated during the initial denaturation phase.*

4. Prepare the reaction mixtures according to the instructions in Table 1.

Table 1. Reaction Mixture Preparation

COMPONENT	Final Concentration	25 µl rxn
DNA AmpliTools Complex Master Mix (2X)	1 X	**12.5 µl**
Forward primer	0.5 µM	12.5 pmol (x µl)
Reverse primer	0.5 µM	12.5 pmol (x µl)
Nuclease-free water	-	to 25 µl
Template DNA*	variable	x µl

Template DNA* | Low complexity DNA: 0.2-10 ng
High complexity gDNA: 10-250 ng

Start with **1ng for low complexity templates, or **50 ng** for high complexity templates.*

> ***Note:*** *The quantity of DNA and primers must be optimised experimentally for each new template/primer combination. The reaction conditions described in this protocol are only general recommendations.*

5. Mix the reaction components on a vortex and apply a spin prior to commencing cycling.

6. Place the vials in the pre-heated thermal cycler and run the amplification program selected.

Table 2. Standard Amplification Program

Steps	Nº of Cycles	Temperature	Time
Initial Denaturation	1	94-96 ºC	5-8 min
Denaturation	25-35	94-96 ºC	20-30 s
Annealing		T_m-5 °C	10-15 s
Extension		68-72 ºC	50 s/kb
Final Extension	1	68-72 ºC	5-10 min

7. Load 20-40% of the reaction mixture onto an agarose gel to analyse the amplification products.

5. TROUBLESHOOTING

Lack of amplification or low reaction efficiency

1. **Check the quantity and quality of the DNA template.** Check the quality and quantity of the template by electrophoresis on an agarose gel or by fluorimetry. Some DNA purification protocols may co-purify amplification inhibitors. Reduce the volume of template in the reaction or dilute it prior to addition to the reaction mixture. Precipitation of DNA with ethanol, followed by washing the pellet several times with 70% ethanol, is usually sufficient for removing trace quantities of contaminants present in the sample.
   Template DNA damaged or degraded. Store the DNA under appropriate conditions to prevent degradation. An excess of template or a poor quality template reduce the yield of the amplification reaction.
2. **Redesign the primers.** Design primers with a higher annealing temperature and which do not form forks and/or dimers with each other.
3. **Optimise the primer concentration.** Although a low primer concentration can prevent dimer formation, a sufficient concentration thereof is necessary for correct performance of the PCR. Start optimisation with 0.5 µM of each primer and, if necessary, increase its concentration in steps of 0.1 µM.
4. **Mix the DNA AmpliTools Complex Master Mix.** The 2X master mix must be exhaustively mixed prior to pipetting the required volume in order to ensure homogeneity.
5. **HotSplit DNA Polymerase has not been activated.** Check that the conditions applied during the initial denaturation phase are those recommended: 5-8 min at 94-96 °C.
6. **Increase the initial denaturation time.** During the initial amplification cycles it is very important to ensure that the template is completely denatured. For templates with a high GC content or those rich in secondary structures, we recommend to prolong the initial denaturation to 8 min.
7. **Reduce the annealing temperature.** Reduce the annealing temperature in decrements of 2 °C.
8. **Increase the number of cycles.** Include an additional number of amplification cycles, in increments of 5 cycles. Occasionally, a maximum number of 45 cycles often improves the yield of those PCRs intended to amplify templates with a low number of copies or complex templates.
9. **Changes in the extension step.** Increase the extension time in increments of 30 s. For long amplification products (>3 kb), we recommend to decrease the extension temperature to 68 °C

Non-specific or smeared amplification products

1. **Reduce the concentration of the reaction components.** Check the concentration of the DNA template by electrophoresis on an agarose gel or by fluorimetry. Reduce the quantity of template and/or primers in the reaction mixture.
2. **Template DNA damaged or degraded.** Store the DNA under appropriate conditions to prevent degradation. Degraded templates favour the generation of nonspecific or smeared amplification products.
3. **Check the state of the primers.** Check that the primers are not degraded by performing an electrophoresis on denaturing acrylamide gel.
4. **Redesign the primers.** Design primers with a higher Tm and which do not form forks and/or dimers with each other.
5. **Increase the annealing temperature.** Increase the temperature of the annealing step in increments of 2 °C.
6. **Reduce the annealing time.** The recommended annealing time to be used with the DNA Amplitools Complex Master Mix is 10 s and never more than 15 s.
7. **Reduce the extension time.** The extension time for simple templates can be reduced to 30-40 s/kb.
8. **Reduce the number of amplification cycles.** Decrease the number of cycles in decrements of 5 cycles.
9. **Contaminants in the reaction mixture.** If amplification bands are observed in the negative control, change all the reagents included in the mixture.

6. ORDERING INFORMATION

DESCRIPTION	Format	Size	Reference
DNA AmpliTools Complex Master Mix	250 rxns of 25 µl	1 x 3.4 mL	10.451
DNA AmpliTools Complex Master Mix	500 rxns of 25 µl	2 x 3.4 mL	10.452