Thermo Scientific Phire Plant Direct PCR Master Mix

#F-160
Store at -20 °C

Thermo SCIENTIFIC

1. Introduction

Thermo Scientific™ Phire™ Plant Direct PCR Master Mix is designed to perform PCR directly from plant leaves and seeds without prior DNA purification. Fresh plants, plant material stored at +4 °C or frozen are all suitable templates for this Master Mix, as well as plant material stored on commercially available cards such as Whatman 903 and FTA Cards. A list of plants tested with this Master Mix is available at www.thermoscientific.com/directpcr.

The Master Mix employs Phire Hot Start II DNA Polymerase, a specially engineered enzyme with a DNA-binding domain that enhances the processivity of the polymerase. Phire Hot Start II DNA Polymerase also exhibits extremely high resistance to many PCR inhibitors found in plants. The Phire Plant Direct PCR Master Mix contains reagents for two alternative methods: Direct and Dilution & Storage protocols. Dilution Buffer is included for optional sample treatment before PCR (see 'Dilution & Storage protocol' in Section 5). It can be used to treat larger/more difficult samples (e.g. more fibrous or latex-containing samples), or when multiple PCR reactions are performed from a single sample. It is also a useful choice when longer DNA fragments (> 1 kb) are amplified. Dilution & Storage protocol is also useful when retesting possibility is needed. Samples in Dilution Buffer can be stored for up to 8 weeks in different temperatures (-20 °C, +4 °C or room temperature) before using in PCR. In addition the kit includes a pair of control primers for amplification of a highly conserved region of chloroplast DNA[1]. The Master Mix is recommended for end point PCR protocols and it contains premixed gel loading dye which allows direct sample loading on the gel. The loading dye in the Master Mix does not interfere with PCR performance and is compatible with downstream applications such as DNA sequencing, ligation and restriction digestion. For applications that require PCR product analysis by absorbance or fluorescence excitation, we recommend using the Phire Plant Direct PCR Kit (F-130) or purifying the PCR product prior to analysis.

2. Package information

Component	#F-160S 100 rxns	#F-160L 500 rxns
2X Phire Plant Direct PCR Master Mix	2 × 1.25 mL	10 × 1.25 mL
Dilution Buffer	5 mL	2 × 12.5 mL
Control Primer Mix (25 µM each)	40 µL	40 µL
Water, nuclease- free	2 x 1.25 mL	10 x 1.25 mL
O'GeneRuler Express DNA Ladder	100 applications (50 µg)	

3. Important notes

- Primer annealing temperatures for Phire are different from many common DNA polymerases (such as *Taq* DNA polymerases). Read Section 7.3 carefully.
- Use 98 °C for denaturation.
- Add the sample directly into a PCR reaction instead of an empty tube.
- Use the Dilution & Storage protocol for difficult samples, for long amplicons or for performing multiple reactions from the same sample.

4. Guidelines for PCR

Carefully mix and spin down all tubes before opening to ensure homogeneity and improve recovery. The PCR setup can be performed at room temperature. **Always add the sample last to the reaction.** Read Section 5 carefully for sampling guidelines.

4.1 Positive control reaction with purified DNA

When optimizing the reactions, it is recommended to perform a positive control with purified DNA to ensure that the PCR conditions are optimal. If the positive control with purified DNA fails, the PCR conditions should be optimized until the control PCR gives a desired result.

4.2 Negative control

It is recommended to add a no-template control to all Direct PCR assays.

Table 1. Pipetting instructions (add items in this order)

Component	20 µL rxn	50 µL rxn*	Final conc.
H_2O	add to 20 µL	add to 50 µL	-
2X Phire Plant Direct PCR Master Mix	10 µL	25 µL	1X
Primer A	X µL	X µL	0.5 µM
Primer B	X µL	X µL	0.5 µM
Plant tissue (see Section 5) Direct protocol	-	0.5 mm punch/small sample of seed	-
Dilution & Storage protocol	0.5 µL	1.25 µL	

*50 µL reaction volume is recommended for the direct protocol.

Table 2. Cycling protocol

Cycle step	2-step protocol		3-step protocol		Cycles
	Temp.	Time	Temp.	Time	
Initial denaturation	98 °C	5 min	98 °C	5 min	1
Denaturation	98 °C	5 s	98 °C	5 s	35-40
Annealing (see 7.3)	-	-	X °C	5 s	
Extension (see 7.4)	72 °C	20 s ≤1 kb 20 s/kb >1 kb	72 °C	20 s ≤1 kb 20 s/kb >1 kb	
Final Extension	72 °C 4 °C	1 min hold	72 °C 4 °C	1 min hold	1

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5. Guidelines for sample handling

To obtain small and uniform samples, we recommend using the Harris tools. The Harris Uni-Core™ may be disposed of after use or cleaned and reused up to 500 times, depending on the thickness and firmness of the sample material. If the puncher is to be reused, it is very important to clean the cutting edge properly to prevent cross-contamination between samples. The Harris Cutting Mat provides the best possible cutting surface for Harris Uni-Core. It is made of inert self-healing material and has two cutting surfaces. The cutting mat can be reused several hundred times. Other ways to take a sample is by cutting with scalpel to obtain 0.50 mm sample. Scalpel and cutting mat must be cleaned properly to prevent cross-contamination between samples.

5.1 Plant leaves

Direct protocol:
Take a sample from the plant leaf using the 0.50 mm Haris Uni-Core puncher supported by the Cutting Mat or cut a sample of approximately this size (•). Place the punch disc directly into the PCR reaction (50 µL in volume). It is recommended to eject the disc into a liquid, rather than onto the wall of an empty tube. Make sure that you see the sample disc in the solution. We recommend using young leaves. Fresh plant material is usually the best choice, even though plant material stored at +4 °C, frozen or on commercially available cards such as Whatman® 903 and FTA® cards can also be used (see Section 5.3). For amplifying long fragments or difficult samples using the direct protocol, a 0.35 mm punch disc may give more robust results.

Dilution & Storage protocol:
As with the direct protocol, young leaves are recommended. Take one small leaf or a piece of leaf (e.g. a punch approximately 2 mm in diameter) and place it in 20 µL of Dilution Buffer. Crush the leaf sample with a 100 µL pipette tip by pressing it briefly against the tube wall. If larger amount of leaf tissue is used (do not exceed 1 mg), increase the volume of the Dilution Buffer to 50 µL. After crushing the leaf, the solution should be greenish in colour. Spin the plant material down, and use 0.5 µL of the supernatant as a template for a 20 µL PCR reaction. The required volume of the supernatant may vary depending on the plant material used and the volume used for the dilution.

5.2 Plant seeds

Direct protocol:
Using a clean scalpel, remove the seed coat and cut a small sample of the seed (approximately the size of this dot •). Place the sample directly into the PCR reaction (50 µL in volume). Note that it is recommended to use dehulled seeds. For very small seeds (such as *Arabidopsis*), use 1–2 whole seeds and place them directly into the PCR reaction.

Dilution & Storage protocol:
Cut a small sample of the dehulled seed by using a scalpel (approximately the size of this dot •) and place it directly into 20 µL of Dilution Buffer. Briefly vortex the tube and incubate at room temperature for 3 min. Make sure that the seed sample is covered with Dilution Buffer. Spin briefly and use 0.5 µL of the supernatant as a template for a 20 µL PCR reaction.

5.3 Plant material stored on commercially available storage cards, e.g. Whatman 903 and FTA Cards

Direct protocol:
Use the 0.50 mm Harris Uni-Core to cut a disc from the sample in the storage card or cut a sample of approximately this size (•). Place the punch disc directly into a 50 µL PCR reaction. For amplifying long fragments or difficult samples, a 0.35 mm punch disc may give more robust results.

6. Notes about reaction components

6.1 2X Phire Plant Direct PCR Master Mix

2X Phire Plant Direct PCR Master Mix has been optimized for Direct PCR from plant material. It contains the dNTPs and provides 1.5 mM $MgCl_2$ concentration in the final reaction. It also includes a density reagent and two tracking dyes for direct loading of PCR product on a gel. The Master Mix employes Phire Hot Start II DNA Polymerase, that possesses the following activities: 5'→3' DNA polymerase activity and a weak 3'→5' exonuclease activity. When cloning fragments amplified with Phire Hot Start II DNA Polymerase blunt end cloning is recommended. If TA cloning is required, it can be performed by adding A overhangs to the blunt PCR product with Thermo Scientific *Taq* DNA Polymerase, for example (protocol available at www.thermoscientific.com/pcrcloning).

6.2 Dilution buffer

The Dilution Buffer has been optimized to release DNA from a wide variety of different sample materials such as plant leaves and seeds. This buffer is also suitable for storing the DNA sample for short periods of time at +4 °C or room temperature. For long term storage store at –20 °C. Before storage it is recommended to transfer the supernatant into a new tube.

6.3 Primers

The recommendation for final primer concentration is 0.5 µM. The results from primer Tm calculations can vary significantly depending on the method used. Always use the Tm calculator and instructions at www.thermoscientific.com/pcrwebtools to determine the Tm values of primers and optimal annealing temperature.

7. Notes about cycling conditions

7.1 Initial denaturation

In Direct PCR protocols, the initial denaturation step is extended to 5 minutes to allow the lysis of cells, making genomic DNA available for PCR.

7.2 Denaturation

Keep the denaturation time as short as possible. Usually 5 seconds at 98 °C is enough for most templates. Note that the denaturation time and temperature may vary depending on the ramp rate and temperature control mode of the thermal cycler.

7.3 Primer annealing

Note that the optimal annealing temperature for Phire Hot Start II DNA Polymerase may differ significantly from that of *Taq*-based polymerases. **Always use the Tm calculator and instructions at www.thermoscientific.com/pcrwebtools to determine the Tm values of primers and optimal annealing temperature.** As a basic rule, for primers > 20 nt, anneal for 5 seconds at a Tm +3 °C of the lower Tm primer. For primers ≤ 20 nt, use annealing temperature equal to the Tm of the lower Tm primer. In some cases, it may be helpful to use a temperature gradient to find the optimal annealing temperature for each template-primer pair combination. The annealing gradient should extend up to the extension temperature (two-step PCR). Two-step cycling without an annealing step is recommended for high-Tm primer pairs (Tm at least 69–72 °C).

7.4 Extension

The extension is performed at 72 °C. The recommended extension time is 20 seconds for amplicons ≤1 kb, and 20 s/kb for amplicons >1 kb.

8. Control reactions

We recommend performing direct PCR control reactions with both Direct and Dilution & Storage protocols using the control primers supplied with this Master Mix. As a template, use the same plant material as in the actual experiment. If the PCR using control primer mix is not working, the plant sample may not be suitable for direct PCR. Control primers are supplied as a mix of primers in H_2O that amplify a 297 bp fragment of a highly conserved region of chloroplast DNA[1]. The control primer mix has been validated with a large number of species (refer to the list of tested plants at www.thermoscientific.com/directpcr). Each primer concentration is 25 µM.

Primer #1 (20-mer)
5'- AGTTCGAGCCTGATTATCCC -3'
Melting point: 62.4 °C

Primer #2 (20-mer)
5'- GCATGCCGCCAGCGTTCATC -3'
Melting point: 75.5 °C

Table 3. Pipetting instructions for control reactions.

Component	20 µL rxn	Final conc.
H_2O	add to 20 µL	-
2X Phire Plant Direct PCR Master Mix	10 µL	1X
Control primer mix	0.4 µL	0.5 µM
Plant tissue (see Section 5) Direct protocol: Dilution & Storage protocol	0.5 mm punch/small sample of seed 0.5 µL	-

Table 4. Cycling instructions for control reactions.

Cycle step	Temp.	Time	Cycles
Initial denaturation	98 °C	5 min	1
Denaturation Annealing Extension	98 °C 62 °C 72 °C	5 s 5 s 20 s	40
Final Extension	72 °C 4 °C	1 min hold	1

9. References

1. Demesure B. *et al.* (1995) *Molecular Ecology* 4: 129–131.

11. Troubleshooting

No product at all or low yield	
Inhibition (positive control works with custom and control primers from the kit)	**Direct Protocol:** Try to titrate sample amount. If not successful, try Dilution & Storage protocol.
	Dilution & Storage Protocol: Dilute supernatant (from the sample treated with Dilution Buffer) 1:10 and/or 1:100 with H_2O or TE buffer, and use 1 µL as a template in PCR.
Not enough template	Try to titrate sample amount. If seeds are used, do not forget to store the sample in Dilution Buffer in room temperature for 3 minutes before PCR.
Problems with primer design	Positive control with purified DNA and custom primers fails, whereas there is product with control primers. Assess your primer design.
Incorrect cycling parameters	Make sure to follow the cycling parameters recommended in the Product Information.
Incorrect annealing temperature	Optimize annealing temperature (run a temperature gradient). The optimal annealing temperature for Phire Hot Start II DNA Polymerase may differ significantly from that of *Taq*-based polymerases. Always use the Tm calculator and instructions on our website: www.thermoscientific.com/pcrwebtools.
Too short extension time	The recommended extension time is 20 seconds for amplicons ≤1 kb, and 20 s/kb for amplicons >1 kb.
Too less cycles	For difficult amplicons try using 40 cycles.
Non-specific products	
Problems with primers	Decrease primer concentration to. Design new primers. Make sure these are not primer dimmers (when short amplicons are amplified).
Too low annealing temperature	Increase the annealing temperature or perform temperature gradient.
Adjust cycling times	Reduce the total number of cycles. Shorten extension time.

10. Shipping and storage

Upon arrival, store the components at –20 °C. The Dilution Buffer can also be stored at +4 °C once it is thawed.

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