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Phusion® Human Specimen Direct PCR Kit

Product code:
F-150, 200 PCR reactions 20 µl each

Stable for one year from the packaging date. Store at –20°C. The Dilution Buffer can also be stored at +4°C once it is thawed.

1.Introduction

Finnzymes' Phusion® Human Specimen Direct PCR Kit is designed to perform PCR directly from various human samples with no prior DNA purification. Samples such as buccal swabs, saliva, fingernails, teeth, hair, amniotic fluid and skin biopsies are suitable starting materials. The kit also works with formalin-fixed paraffin-embedded (FFPE) tissues. Note that the kit is for research use only.

The Phusion Human Specimen Direct PCR Kit employs a modified Phusion® Hot Start II High-Fidelity DNA Polymerase, a specially engineered enzyme with a DNA-binding domain that enhances the processivity of the polymerase and exhibits extremely high resistance to many PCR inhibitors found in human samples.

The Phusion Human Specimen Direct PCR Kit contains reagents for two alternative methods: direct and dilution protocols. Specific guidelines for different starting materials are given (see Chapter 4 for information about protocol options). When using new primer pairs or sample materials, it is recommended to start with the dilution protocol.

The kit includes a universal control primer mix for amplification of a 237 bp fragment from human samples. The kit is recommended for end-point PCR protocols.

Important notes

- Use 98°C for denaturation.
- The annealing rules are different from many common DNA polymerases (such as *Taq* DNA polymerases). Read Section 6.3 carefully.
- For extension, use 15 s for amplicons ≤ 1 kb or 15 s/kb for amplicons >1 kb.
- Add the sample directly into the PCR reaction instead of an empty tube.
- Phusion DNA Polymerases produce blunt end PCR products.

2. Package information

Component	
Phusion® Human Specimen DNA Polymerase	80 µl
2x Phusion® Human Specimen PCR Buffer (includes dNTPs and $MgCl_2$)	2 x 1 ml
Universal control primer mix (25 µM each)	40 µl
Dilution Buffer	2 x 5 ml
DNARelease™ Additive	3 x 100 µl

Material safety data sheet (MSDS) is available at www.finnzymes.com.

3. 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 Chapter 4 carefully for sampling guidelines.

Table 1. Pipetting instructions.

Components	20 µl reaction	50 µl reaction	Final conc.
H_2O	add to 20 µl	add to 50 µl	
2x Phusion® Human Specimen PCR Buffer	10 µl	25 µl	1x
primer A	x µl	x µl	0.5 µM
primer B	x µl	x µl	0.5 µM
Phusion® Human Specimen DNA Polymerase	0.4 µl	1 µl	
Sample (see Chapter 4) Direct protocol: Dilution protocol:	Amount depends on the sample 0.5 µl	Amount depends on the sample 1.25 µl	

Table 2. Recommended 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	1 s	98°C	1 s	35–40
Annealing (see 6.3)	-	-	X°C	5 s	
Extension (see 6.4)	72°C	15 s ≤ 1 kb 15 s/kb > 1 kb	72°C	15 s ≤ 1 kb 15 s/kb > 1 kb	
Final extension	72°C 4°C	1 min hold	72°C 4°C	1 min hold	1

4. Guidelines for sample handling

4.1 Sample types and protocols

This kit is optimized for various human samples. Please refer to Section 4.2 to see a list of validated human samples. Each sample material has its own specific protocol.

4.2 Solid samples

4.2.1 Buccal swabs (e.g. Nylon flocked swab)

a) Direct protocol: Using the Harris™ Uni-Core sampling tools (available separately from Finnzymes), take a 0.5 mm punch (or similar) and place it directly into a 20 µl PCR reaction. In some cases, increasing the reaction volume to 50 µl or reducing the punch size to 0.35 mm may improve the results.

b) Dilution protocol: Place the buccal swab tip into a 1.5 ml tube containing 50 µl Dilution Buffer, 1.5 µl DNARelease Additive and 250 µl TE, pH 8. Rotate the swab 5–10 times before removing it from the tube by gently pressing the brush against the side of the tube. Mix by vortexing and spin down. Incubate at 98°C for 2 minutes, spin down again, and use 0.5 µl of the supernatant as a template for a 20 µl PCR reaction.

Note: Swabbing technique and storage conditions (not thoroughly dried) may cause yield variation.

4.2.2 Hair

a) Direct protocol: Take a 1–2 mm region of hair including the bulb, and place it directly into a 20 µl PCR reaction. In some cases, increasing the reaction volume to 50 µl may improve the results.

b) Dilution protocol: Place a 2–3 mm region of hair including the bulb in a tube that contains 20 µl Dilution Buffer and 0.5 µl DNARelease Additive. Make sure the sample is covered with the solution. Mix by vortexing and spin down. Incubate for 2–5 minutes at room temperature followed by 2 minutes at 98°C. Spin down again, and use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

4.2.3 Teeth

a) Direct protocol: Place a sample of tooth approximately this size (●) directly into a 20 µl PCR reaction.

Note: When amplifying longer DNA fragments (> 1 kb), the dilution protocol is recommended.

b) Dilution protocol: Place an approximately 13–15 mg sample of tooth in a tube that contains 50 µl Dilution Buffer and 1.5 µl DNARelease Additive. Make sure the sample is covered with the solution. Mix by vortexing and spin down. Incubate for 2–5 minutes at room temperature followed by 2 minutes at 98°C. Spin down again, and use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

Note: Using finely crushed sample may yield better results, e.g. improved sensitivity. The sample can be crushed for example by grinding in liquid nitrogen with a mortar and pestle or a homogenizer.

4.2.4 Skin biopsies (non-fixed)

a) Direct protocol: Using the Harris Uni-Core sampling tools (available separately from Finnzymes), take a 0.5 mm sample and place it directly into a 20 µl PCR reaction. In some cases, increasing the reaction volume to 50 µl or reducing the punch size to 0.35 mm may improve the results.

b) Dilution protocol: Place a 2 mm punch sample into a tube containing 20 µl Dilution Buffer and 0.5 µl DNARelease Additive. Make sure the sample is covered with the solution. Mix by vortexing and spin down. Incubate for 2–5 minutes at room temperature, followed by 2 minutes at 98°C. Spin down again, and use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

4.2.5 Fingernails

a) Direct protocol: Place a small nail sample (approximately 1 x 2 mm, in other words < 1 mg) directly into a 20 µl PCR reaction.

Note: When amplifying longer DNA fragments (> 1 kb), dilution protocol is recommended.

b) Dilution protocol: Place an approximately 7 mg nail sample in a tube that contains 50 µl Dilution Buffer and 1.5 µl DNARelease Additive. Make sure the sample is covered with the solution. Mix by vortexing and spin down. Incubate for 2–5 minutes at room temperature followed by 2 minutes at 98°C. Spin down again, and use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

Note: Using finely diced sample may yield better results, e.g. improved sensitivity.

4.2.6 Formalin-fixed paraffin-embedded (FFPE) tissues

a) Direct protocol: Not recommended

b) Dilution protocol:
FFPE tissue section
Place one 7–10 µm thick FFPE human tissue section into a tube containing 50 µl Dilution Buffer, 1.5 µl of DNARelease Additive and 50 µl TE, pH 8.* Crush with a pipette tip and spin down. Make sure the sample is covered with the solution. Incubate for 1 hour at 60 °C followed by 10 minutes at 98°C. After cooling and centrifuging (16 000 x *g*, 2 min), transfer the supernatant into a new tube. Use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

In some cases, if the amount of tissue debris or DNA in the supernatant is very high, the supernatant may need to be diluted. Make a 1:10 or 1:100 dilution in H_2O or TE buffer and use 0.5 µl of the dilution as a template in a 20 µl PCR reaction.

FFPE tissue on a microscope slide (unstained)
Prepare a 1:1 mixture of Dilution buffer and TE, pH 8.0. Mix by vortexing and spin down. Pipette 100 µl of the mixture on a 4–7 µm thick FFPE tissue on the microscope slide. Scrape the tissue off with a pipette tip and place the solution and tissue into a clean tube. Add 1.5 µl of DNARelease Additive.* Mix the solution with a pipette tip and spin down. Make sure the sample is covered with the solution. Incubate for 1 hour at 60°C followed by 10 minutes at 98°C. After cooling and centrifuging (16 000 x *g*, 2 min), transfer the supernatant into a new tube. Use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

In some cases, if the amount of tissue debris or DNA in the supernatant is very high, the supernatant may need to be diluted. Make a 1:10 or 1:100 dilution in H_2O or TE buffer and use 0.5 µl of the dilution as a template in a 20 µl PCR reaction.

Note: The DNA in FFPE tissue is usually fragmented, limiting the size of PCR products that can be successfully amplified. We recommend amplicon sizes less than 300 bp. In some cases extending the first incubation over night improves the PCR yield.

4.3 Liquid samples

4.3.1 Saliva

a) Direct protocol: Add 0.2–0.5 µl of saliva directly into a 20 µl PCR reaction.

b) Dilution protocol: Add 5 µl of saliva in a tube that contains 20 µl Dilution Buffer and 0.5 µl DNARelease Additive. Mix by vortexing and spin down. Incubate at 98°C for 2 minutes. Spin down again, and use 0.5 µl of the supernatant as a template in a 20 µl PCR reaction.

Note: Only fresh saliva is recommended. If the saliva sample is to be stored for a longer period before PCR, it is recommended to use commercial saliva collection tubes (such as Oragene®•DNA from Genotek).

4.3.2 Amniotic fluid

a) Direct protocol: Add 0.5–2 µl of amniotic fluid directly into a 20 µl PCR reaction.

b) Dilution protocol: Not recommended.

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* The protocol is optimized for 1–4 cm^2 tissue sections. For smaller or larger samples, adjust the volumes accordingly.

5. Notes about reaction components

5.1 Enzyme

Phusion Human Specimen DNA Polymerase is a proofreading polymerase that contains a unique processivity-enhancing domain, making this polymerase robust, rapid and accurate. The hot start technology is based on a reversibly binding Affibody® protein.[1,2]

Phusion Human Specimen DNA Polymerase possesses 5′→3′ DNA polymerase activity and 3′→5′ exonuclease activity.

When cloning fragments amplified with Phusion Human Specimen 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 DyNAzyme™ II DNA Polymerase (F-501), for example. However, before adding the overhangs it is very important to remove all the DNA polymerase by purifying the PCR product carefully. Any remaining Phusion Human Specimen DNA Polymerase will degrade the A overhangs, creating blunt ends again. A detailed protocol for TA cloning of PCR fragments amplified with any of the Phusion DNA Polymerases can be found on Finnzymes' website (www.finnzymes.com).

5.2 Phusion® Human Specimen PCR Buffer

The 2x Phusion Human Specimen PCR Buffer has been optimized for Direct PCR from human samples. It contains the dNTPs and provides 1.5 mM $MgCl_2$ concentration in the final reaction.

5.3 Dilution Buffer

The Dilution Buffer has been optimized to release DNA from a wide variety of human samples when supplemented with DNARelease Additive (see Section 5.4).

5.4. DNARelease™ Additive

DNARelease Additive is used in the dilution protocol to improve the release of DNA from human samples.

5.5 Primers

The recommendation for the 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 on Finnzymes' website (www.finnzymes.com) to determine the Tm values of primers and optimal annealing temperature.

6. Notes about cycling conditions

6.1 Initial denaturation

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

6.2 Denaturation

Keep the denaturation time as short as possible. Usually 1 second 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.

6.3 Primer annealing

Note that the optimal annealing temperature for Phusion Human Specimen DNA Polymerase may differ significantly from that of *Taq*-based polymerases. Always use the Tm calculator and instructions on Finnzymes' website (www.finnzymes.com) 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 an annealing temperature equal to the Tm of the lower Tm primer. If necessary, 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).

6.4 Extension

The extension is performed at 72°C. The recommended extension time is 15 seconds for amplicons ≤ 1 kb, and 15 s/kb for amplicons >1 kb.

7. Control reactions

7.1 Direct PCR control reactions using the control primer mix

We recommend performing Direct PCR control reactions with both direct and dilution protocols using the control primers supplied with this kit. As a template, use the same tissue material as in the actual experiment.

The control primer mix is a mix of degenerate primers in H_2O that amplify a 237 bp fragment of mammalian genomic DNA. The amplified region is a highly conserved non-coding region upstream of the SOX21 gene.[3] Each primer concentration is 25 µM.

Primer #1 (24-mer)
5′- AGCCCTTGGGGASTTGAATTGCTG -3′
Melting point: 73.5°C

Primer #2 (27-mer)
5′- GCACTCCAGAGGACAGCRGTGTCAATA -3′
Melting point: 72.2°C (R=A), 75.3°C (R=G)

Table 3. Pipetting instructions for control reactions.

Component	20 µl reaction	50 µl reaction
H_2O	add to 20 µl	add to 50 µl
2x Phusion® Human Specimen PCR Buffer	10 µl	25 µl
Universal control primer mix	0.4 µl	1 µl
Phusion® Human Specimen DNA Polymerase	0.4 µl	1 µl
Sample (see Section 4) Direct protocol: Dilution protocol:	Amount depends on the sample 0.5 µl	Amount depends on the sample 1.25 µl

Table 4. Cycling instructions for control reactions.

Cycle step	Temp.	Time	Cycles
Lysis of cells	98°C	5 min	1
Denaturation Annealing/Extension	98°C 72°C	1 s 15 s	35–40
Final extension	72°C 4°C	1 min hold	1

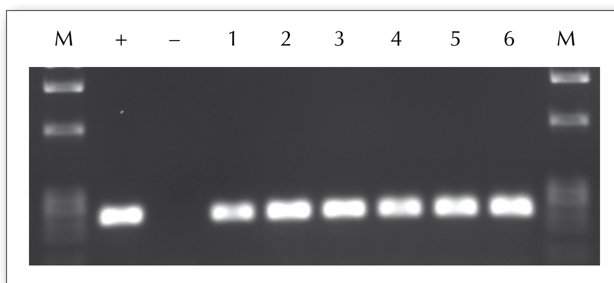

Figure 1. Amplification of 237 bp DNA fragments from various human samples using the Universal control primer mix included in the kit. Samples were placed directly into 20 µl PCR reactions: buccal swab (lane 1), hair (lane 2), tooth (lane 3), fingernail (lane 4), saliva (lane 5) and amniotic fluid (lane 6). + denotes the positive control reaction with purified DNA and – denotes the no-template control.

7.2 Positive and negative controls

Positive control: When optimizing the reactions, it is recommended to perform a positive control with purified DNA using your own primers to ensure that the PCR conditions are optimal. If the positive control with purified DNA fails, the PCR conditions should be optimized before continuing further.

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

8. Troubleshooting

No product at all or low yield
General: If the positive control reaction with purified DNA using your own primers does not yield a product: • Make sure the pipetting and cycling protocols were performed as recommended. • Check primer design. • Optimize annealing temperature (run a temperature gradient). • Titrate template amount. • Optimize denaturation time. • Increase extension time. **Direct protocol:** If the actual samples yield no product, but the positive control reaction with purified DNA using your own primers and Direct PCR control reaction are working: • Increase the PCR reaction volume to 50 µl. • With liquid samples: Titrate the template amount (e.g. saliva 0.2–0.5 µl in 20 µl reaction) • With solid samples: Use less sample material as a template (e.g. 0.35 mm punch) or try finely crushed sample. • Use the dilution protocol. **Dilution protocol:** If the actual samples yield no product, but the positive control reaction with purified DNA using your own primers and Direct PCR control reaction are working: • Dilute the supernatant 1:10 or 1:100 with H_2O or TE buffer, and use 0.5 µl as a template in PCR. You can also try higher volume of undiluted supernatant as a template (up to 2 µl in a 20 µl PCR reaction). • Try incubating the dilution reaction at an elevated temperature (up to 65°C) instead of room temperature. • Make sure to perform the 98°C incubation (see Section 4.1). • With FFPE samples: Try longer incubation time at 60°C (e.g. over night). Make sure to perform the second incubation at 98°C. • Titrate the sample size/volume in the dilution reaction. • Try finely crushed sample material in the dilution reaction.
Non-specific products - High molecular weight smears or low molecular weight discrete bands
• Increase the annealing temperature or perform temperature gradient. • Reduce the total number of cycles. • Decrease primer concentration. • Shorten extension time. • Design new primers.

9. References

1. Nord K. *et al.* (1997) *Nature Biotechnol.* 15: 772–777.
2. Wikman M. *et al.* (2004) *Protein Eng. Des. Sel.* 17: 455–562.
3. Woolfe A. *et al.* (2005) *PLoS Biology* 3: 116–130.

Shipping and storage

Phusion Human Specimen Direct PCR Kit is shipped on gel ice. Upon arrival, store the components at –20°C. The Dilution Buffer can also be stored at +4°C once it is thawed. The kit is stable for one year from the packaging date when stored and handled properly.

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FINNZYMES
Part of Thermo Fisher Scientific

FINNZYMES OY
Keilaranta 16 A, 02150 Espoo, Finland
Tel. +358 9 2472 3010
Fax +358 9 2472 3200
www.finnzymes.com