TruePrime™ Whole Cell-Free DNA Amplification Kit

For apoptotic cell-free DNA analysis

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Patent /license

TruePrime™ apoptotic cell-free DNA amplification kit includes *TthPrimPol*. Patent for *TthPrimPol* is pending. Owner: SYGNIS AG or one of its subsidiaries.

INTENDED USE

FOR RESEARCH USE ONLY

TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis is intended for molecular biology use and in vitro use only. This product is not intended for diagnosis, prevention or treatment of a disease in human beings or animals.

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KIT CONTENTS

DESCRIPTION	CAP COLOR	5 REACTIONS	20 REACTIONS
End-repair enzyme mix	Red	1 x 18 µl	4 x 18 µl
End-repair reaction buffer	Red	1 x 42 µl	4 x 42 µl
Ligation mix	Blue	1 x 180 µl	4 x 180 µl
Enhancer	White	1 x 6 µl	4 x 6 µl
Adaptor	Amber	1 x 14 µl	4 x 14 µl
Reaction buffer	Yellow	1 x 30 µl	4 x 30 µl
dNTPs	Green	1 x 30 µl	4 x 30 µl
Nuclease-free water	Translucent	1 x 1000 µl	4 x 1000 µl
Enzyme 1	Purple	1 x 30 µl	4 x 30 µl
Enzyme 2	Orange	1 x 5 µl	4 x 5 µl

Enzyme 1: *TthPrimPol*; Enzyme 2: Phi29 DNA polymerase.
Adaptor sequence: 5' TAACATTTGTTGGCCACTCAGGCCAACAAATGTTAT 3'

SHIPPING AND STORAGE

TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis is shipped in dry ice. Upon receipt, the kit should be stored immediately at -20ºC in a non-frost-free (constant-temperature) freezer. If stored correctly, the product can be kept for at least six months after shipping without displaying any reduction in performance. For longer periods, store the kit at -80ºC. Avoid repeated freeze-thaw cycles if possible.

HANDLING

This kit is sensitive to small amounts of DNA. Wear gloves at all times and prepare the reaction in a laminar flow hood or similar device to avoid contaminations. Use molecular biology grade clean reagents, sterile reaction tubes and DNA-free pipette tips. Thaw End-repair enzyme mix, Ligation mix, Enzyme 1, Enzyme 2 and dNTPs on ice. All other components can be thawed at room temperature.

Any human body fluids should always be considered as potentially infectious, and appropriate care should be taken when handling these fluids.

All chemicals should be considered as potentially hazardous. This material may contain substances or activities that are harmful to human health. It should not be ingested, inhaled, or brought into contact with skin, and handled with appropriate care in accordance with the principles of good laboratory practice. In case of contact with skin wash immediately with water. For more specific information, please consult the Material Safety Data Sheets (MSDS) available on-line at www.expedeon.com.

QUALITY CONTROL

Each batch of TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis is tested against predetermined specifications to ensure consistent product quality. Enzymes used in the kit have been tested separately to ensure adherence to specifications.

REAGENTS AND EQUIPMENT TO BE SUPPLIED BY THE USER

- Sterile vials, pipettes and pipette tips. Use low-retention plasticware if possible.
- Sterile, ideally DNA-free certified 0.2 ml PCR tubes
- Microcentrifuge
- Cold block
- Thermocycler
- Vortexer
- Magnetic stand
- Ethanol
- AMPure® XP Beads

Optional: QIAamp Circulating Nucleic Acid Kit (Qiagen, 55114) for cell-free DNA purification. Quant-iT™ PicoGreen® dsDNA quantification reagent (Invitrogen, P7581), Qubit™ dsDNA HS or BR Assay Kit (ThermoFisher, Q32851 or Q32850) or similar reagents for amplified DNA quantification.

INTRODUCTION

The TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis combines the novel TruePrime™ DNA amplification technology, with key novel steps of cell-free DNA pretreatment, composed of an end-repair + dA tailing reaction and ligation (Figure 1) of hairpin-adaptors (Figure 2), which enables the efficient amplification of apoptotic cell-free DNA by TruePrime™ following the rolling circle DNA amplification method.

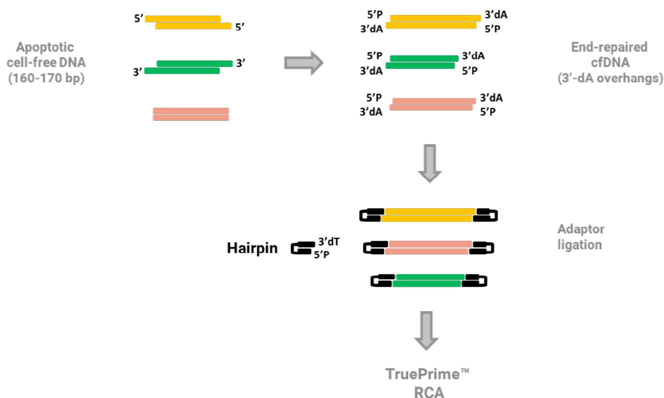

Figure 1. Cell-free DNA pretreatment to eliminate protruding ends, restore 5' phosphates and 3' hydroxyl groups, and add 3' dA overhangs.

Figure 2. DNA sequence and structure of the hairpin-adaptor used in the TruePrime™ apoptotic cell-free DNA amplification kit.

TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis uses a novel multiple displacement amplification method based on the combination of the recently discovered DNA primase *TthPrimPol* and the highly processive and high-fidelity Phi29 DNA polymerase to amplify genomic DNA starting from cell-free DNA obtained from plasma, serum, urine, CSF or any other bodily fluid. *TthPrimPol* generates primers on the hairpin-adaptors that are extended by Phi29 DNA pol. The strong strand displacement capacity of Phi29 DNA polymerase allows *TthPrimPol* to synthesize new primers on the new hairpins generated, resulting in exponential isothermal DNA amplification (Figure 3).

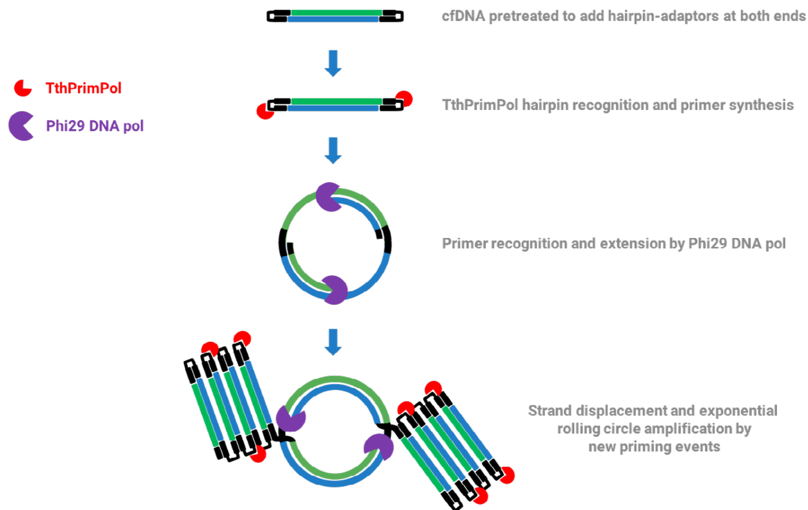

Figure 3. Amplification mechanism of the novel TruePrime™ kit for apoptotic cell-free DNA

PROTOCOL

TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis uses a novel and reliable method to achieve accurate DNA amplification from cell-free DNA. Dedicated buffers and enzymes deliver microgram quantities of DNA (see "Expected amplification yields" section). Please note that the apparent yield may change depending on the quantification chosen. Store amplified DNA at 4ºC for short-term storage or -20ºC for long-term storage.

1. **End-repair + dA tailing reaction:** 30′ 20ºC and 30′ 65ºC
    - Sample volume: 50 µl
    - Reaction volume: 60 µl
2. **Adaptor ligation:** 15′ 20ºC
    - Sample volume: 60 µl
    - Reaction volume: 93.5 µl
3. **Cleanup of adaptor-ligated DNA:** 30′
    - Elution volume: 15 µl
4. **TruePrime™ amplification:** 3h 30ºC and 10′ 65ºC
    - Sample volume: 15 µl
    - Reaction volume: 50 µl

DETAILED PROTOCOL

Starting Material: up to 150 ng of purified cell-free DNA. If the cfDNA volume is less than 50 µl, add nuclease-free water to a final volume of 50 µl.

1. End-repair + dA tailing reaction

1.1. Add the following components to a sterile nuclease-free PCR tube:

- End-repair enzyme mix 3 µl
- End-repair reaction buffer 7 µl
- Cell-free DNA 50 µl
- Total volume 60 µl

1.2. Set a 100 µl or 200 µl pipette to 50 µl and pipette the final volume up and down at least 10 times to mix up.

Note: mixing well is important. Bubbles will not interfere with performance.

1.3. Place in a thermocycler, with the heated lid set to ≥ 75ºC, and run the following program:

- 30 minutes @ 20ºC
- 30 minutes @ 65ºC
- Hold at 4ºC

Note: samples can be stored at -20ºC at this point, but a slight loss (~20%) in yield may be observed, so it is recommended to continue with the adaptor ligation step.

2. Adaptor ligation

2.1. Add the following components directly to the end repair mix:

- End repair mix (Step 1.3) 60 µl
- Ligation mix 30 µl
- Enhancer 1 µl
- Adaptor 2.5 µl
- Total volume 93.5 µl

Note: The ligation mix and enhancer can be mixed previously and this is stable for at least 8 hours at 4ºC, taking care of not adding the Adaptor to this premix.

2.2. Set a 100 µl or 200 µl pipette to 80 µl and pipette the final volume up and down at least 10 times to mix completely.

Caution: The ligation mix is very viscous. Be careful to properly mix the ligation reaction, otherwise the ligation efficiency could be reduced. Bubbles will not interfere with performance.

2.3. Incubate at 20ºC for 15 minutes in a thermocycler with the heated lid off.

Note: Samples can be stored overnight at -20ºC.

3. Cleanup of adaptor-ligated DNA

3.1. AMPure XP Beads must be allowed to warm to room temperature for at least 30 minutes before use. Vortex AMPure XP Beads to resuspend them.

3.2. Add 84 µl (0.9X) AMPure XP Beads to the Adaptor-ligated DNA. Mix well by pipetting up and down at least 10 times or vortexing for 3-5 seconds. If centrifuging samples after mixing, stop the centrifugation before the beads could settle out.

3.3. Incubate samples for at least 5 minutes at room temperature.

3.4. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant.

3.5. When the solution is clear (~5 minutes), carefully remove and discard the supernatant, taking care of not disturbing the beads that contain the DNA.

Caution: Do not discard the beads.

3.6. Add 200 µl of 80% freshly prepared ethanol to the tube in the magnetic stand. Incubate at room temperature for 30 seconds, and then remove and discard the supernatant, taking care of not disturbing the beads that contain the DNA.

3.7. Repeat the previous step for a total of two washes. All visible liquid after the second wash must be removed. If necessary, briefly spin the tube, place it back on the magnetic stand and remove ethanol traces with a p10 pipette.

3.8. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown, and start to crack they are too dry.

3.9. Remove the tube from the magnetic stand and elute the DNA from the beads by adding 17 µl of nuclease-free water.

3.10. Mix well, by pipetting up and down at least 10 times, or vortexing for 3-5 seconds. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

3.11. Place the tube on the magnetic stand. When the solution is clear (~5 minutes), transfer 15 µl to a new PCR tube.

Note: Samples can be stored at -20ºC.

4. **TruePrime™ amplification:** Purified samples (from step 3.11) are amplified.

4.1. Preparation of amplification mix: Prepare the amplification mix adding the components in the order listed in Table 1.

COMPONENT	VOLUME/REACTION
Nuclease-free water	19.3 µl
Reaction buffer	5 µl
dNTPs	5 µl
Enzyme 1	5 µl
Enzyme 2	0.7 µl

Table 1: Preparation of amplification mix

Note: Scale up accordingly (10% excess recommended) when performing several reactions at the same time. Mix the amplification mix by vortexing and store on ice until use.

4.2. Amplification reaction:

4.2.1. Add 35 µl of the amplification mix (Table 1) to the 15-µl DNA samples.

4.2.2. Mix by pipetting and incubate at 30ºC for 3 hours. Inactivate the reactions at 65ºC for 10 minutes. Cool down to 4ºC.

Note: Incubation time can be increased to 6 hours if higher amplification yields are required.

4.2.3. Quantify the samples to determine the amplification yield. Picogreen® or Qubit™ dsDNA Assay Kit from ThermoFisher can be used for this purpose. Please follow the manufacturer's recommendations.

Note: Store the amplified DNA at 4ºC for short-term storage or -20ºC for long-term storage.

PICOGREEN® QUANTIFICATION OF TRUEPRIME™ AMPLIFIED DNA

This protocol is designed for quantification of double stranded TruePrime™ amplified DNA using PicoGreen® reagent.

1. Make a 1:150 dilution of PicoGreen® stock solution in 1x TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA). Each quantification reaction requires 20 µl. Example: for 30 measurements add 4 µl of PicoGreen® to 596 µl 1x TE. Protect the solution from light at all times to avoid photodegradation of the PicoGreen® reagent.

2. Prepare a standard curve using genomic DNA. Prepare a 16 µg/ml stock solution of gDNA in 1x TE buffer.

3. Prepare 200 µl of 1.6, 0.8, 0.4, 0.2 and 0.1 µg/ml of genomic DNA using 1x TE.

4. Transfer 20 µl of each DNA standard in duplicate into a 96-well plate labelled A.

5. Dilute each amplified DNA sample 1:100 (2 µl sample + 198 µl 1x TE) in a 96-well plate labelled B.

6. Place 2 µl of the 1:100 DNA sample dilution into the 96-well-plate labelled A and add 18 µl of 1x TE (dilution 1:1000). Residual 1:100 dilution (plate labelled B) might be stored at -20°C for further analysis.

7. Add 20 µl of PicoGreen® dilution to each sample (amplified DNAs and DNA standards) in the 96-well-plate labelled A. Gently shake the plate to mix the samples and reagent.

8. Measure fluorescence in a microplate reader (excitation wavelength ~480nm, emission wavelength ~520nm).

9. Calculate the concentration of the amplification product: Generate a standard curve of fluorescence versus concentration of DNA standards. Determine the concentration of TruePrime™ amplified products from the equation of the line derived from the standard curve. Dilution factor during the assay must be taken into consideration when calculating total yields.

QUBIT™ QUANTIFICATION OF TRUEPRIME™ AMPLIFIED DNA

We recommend the Qubit™ procedure, as it produces more reliable results in our hands. Please follow the manufacturer's recommendations.

EXPECTED AMPLIFICATION YIELDS

Below you will find the DNA amplification yields obtained from different cell-free DNA inputs purified from human plasma and subjected to the TruePrime™ Whole Cell-Free DNA Amplification Kit - For apoptotic cell-free DNA analysis workflow. DNA yield was quantified using PicoGreen® reagent. Apparent yields will on average be about 25% lower with the Qubit™ method, since there is some consensus that the PicoGreen® method overestimates the DNA concentration.

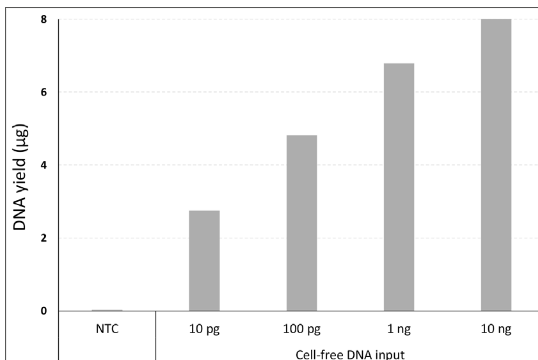

Figure 4. Expected amplification DNA yields depending on the cell-free DNA amount initially processed (NTC = no template control).

These yields might vary depending on:

- Type of blood collection tube
- Blood storage temperature
- Time between sample collection and plasma/serum preparation
- Time between blood centrifugation and plasma/serum transfer
- Cell-free DNA purification kit

We recommend the following materials and conditions to get pure apoptotic cell-free DNA preps and minimize the contaminations with genomic DNA from nucleated cells present in the blood (e.g. leukocytes), that could interfere the amplification process of apoptotic cell-free DNA.:

- Blood collection tube: specialized tubes like Cell-free DNA BCT® (Streck) or PAXgene® Blood ccfDNA Tube (PreAnalytix, 768115)
- Time between extraction and plasma preparation: less than 2 hours
- Blood storage temperature: 4°C
- Double spin procedure:
  - » First centrifugation: RT, 1600 rcf, 10 minutes
  - » Second centrifugation: RT, 16000 rcf, 10 minutes
- Plasma collection: 3.5 ml per 10 ml of blood (first centrifugation) and 3 ml per 3.5 ml of plasma (second centrifugation)
- Cell-free DNA purification: Qiagen QIAamp® Circulating Nucleic Acid Kit (Cat.No. 55114)
- Cell-free DNA quantification: Qubit™ quantification

Some variance between samples is expected depending on the source and status of each sample.

Processing of samples for subsequent analysis

For performing subsequent analysis on TruePrime™ amplification products we highly recommend to purify the samples beforehand as TruePrime™ components can have inhibitory effects. We recommend the Qiagen Qiaquick® PCR purification kit (Cat No./ID: 28104), the Qiagen QIAamp® DNA Mini Kit (Cat No./ID: 51304), or ethanol precipitation. All have worked in our hands, with slight advantages for the columns.

Processing of samples for subsequent NGS

The size distribution of the amplification fragments will be approximately between 1 and 20 kb, with a peak around 9 kb. The DNA can be handled in general like non-amplified high molecular weight genomic DNA. Fragmentation of amplified DNA will work well for example with the Covaris sonication fragmentation using parameters suited for extracted genomic DNA. Library construction can be performed with all commonly available construction kits.

Coverage

The completeness of DNA amplification from cell-free DNA may vary, leading to different degrees of coverage breadth in samples processed in parallel. We recommend to either perform low depth NGS, check coverage, and select samples for deeper sequencing based on the results.

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TROUBLESHOOTING GUIDE

PROBLEM	SOLUTION
Reduced yield or no amplification product	**Contamination of template DNA** Use sterile laboratory equipment and barrier pipette tips. Work in a laminar-flow hood. Use molecular biology grade nuclease-free water to prepare all samples. **Inactive Enzymes** End-repair enzyme mix, Ligation mix, Enzyme 1 and Enzyme 2 should be properly stored at -20°C. The freezer must be a non-frost-free (constant-temperature) freezer.
Poor performance in downstream applications	**Presence of non-specific amplification product** Use sterile laboratory equipment and barrier pipette tips. Work in a laminar-flow hood. Use molecular biology grade nuclease-free water to prepare all samples.

ORDERING INFORMATION

TruePrime™ Whole Cell-Free DNA Amplification Kit -
For apoptotic cell-free DNA analysis

SIZE	CAT. NO.
5 reactions	340005
20 reactions	340020

CUSTOMER SERVICE

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CLICK-TO-ORDER

TECHNICAL SUPPORT

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