4BB™ TruePrime®

apoptotic cell-free DNA amplification kit

HANDBOOK

4basebio

INDEX

ORDERING INFORMATION

PRODUCT	SIZE	CAT. NO.
4BB™ TruePrime® apoptotic cell-freee DNA amplification kit	5 reactions	340005
4BB™ TruePrime® apoptotic cell-freee DNA amplification kit	20 reactions	340020

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

SHIPPING AND STORAGE

4BB™ TruePrime® apoptotic cell-free DNA amplification kit is shipped in dry ice. Upon receipt, the kit should be stored immediately at -20°C 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 -80°C. 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 contamination. Use molecular biology grade clean reagents, sterile reaction tubes and DNA-free pipette tips. Thaw the 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 by following with the principles of good laboratory practice. In case of contact with skin, immediately wash with water. For more specific information, please consult the Material Safety Data Sheets (MSDS) available online at www.4basebio.com.

QUALITY CONTROL

Each batch of 4BB™ TruePrime® apoptotic cell-free DNA amplification kit 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 4BB™ TruePrime® apoptotic cell-free DNA amplification kit 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.

The kit combines the novel 4BB™ 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 4BB™ TruePrime® by utilizing the rolling circle DNA amplification method.

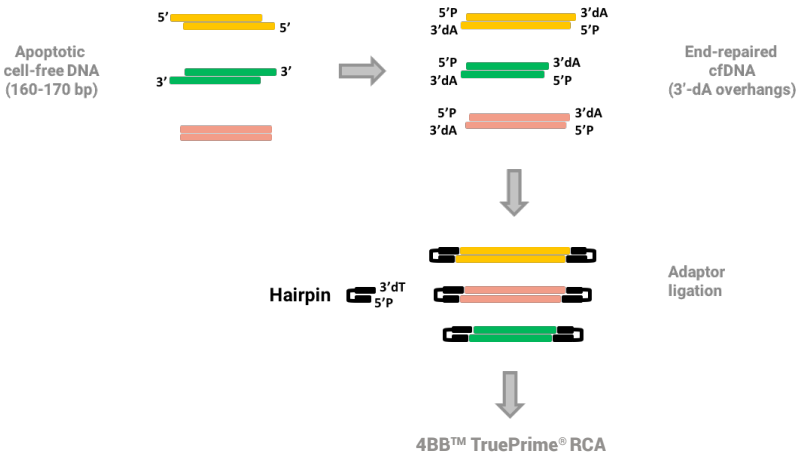

Figure 1. Cell-free DNA pretreatment to eliminate protruding ends, restore 5′ phosphates and 3′ hydroxyl groups, and add 3′ dA overhangs.

Figure 2. DNA sequence and structure of the hairpin-adaptor used in the 4BB™ TruePrime® apoptotic cell-free DNA amplification kit.

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).

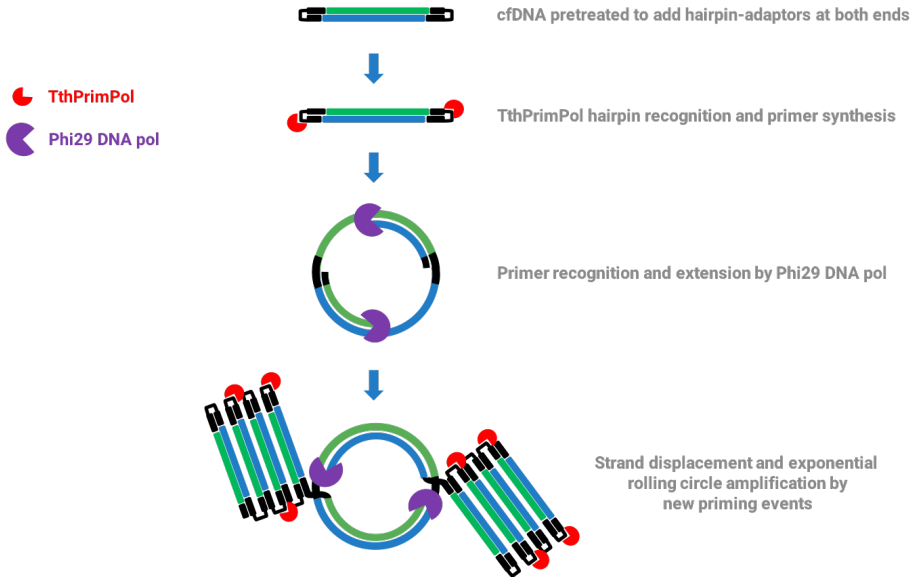

Figure 3. Amplification mechanism of the novel 4BB™ TruePrime® kit for apoptotic cell-free DNA

PROTOCOL

4BB™ TruePrime® apoptotic cell-free DNA amplification kit 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 minutes at 20ºC and 30 minutes at 65ºC
   - Sample volume: 50 µl
   - Reaction volume: 60 µl
2. **Adaptor ligation:** 15 minutes at 20ºC
   - Sample volume: 60 µl
   - Reaction volume: 93.5 µl
3. **Cleanup of adaptor-ligated DNA:** 30 minutes at room temperature

   Elution volume: 15 µl
4. **TruePrime® amplification:** 3 hours at 30ºC and 10 minutes at 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 at 20ºC
- 30 minutes at 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 the 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. 4BB™ 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 4BB™ TRUEPRIME® AMPLIFIED DNA

This protocol is designed for quantification of double stranded 4BB™ 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 ~480 nm, emission wavelength ~520 nm).
9. Calculate the concentration of the amplification product: Generate a standard curve of fluorescence versus concentration of DNA standards. Determine the concentration of 4BB™ 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 4BB™ 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 4BB™ TruePrime® apoptotic cell-free DNA amplification 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.

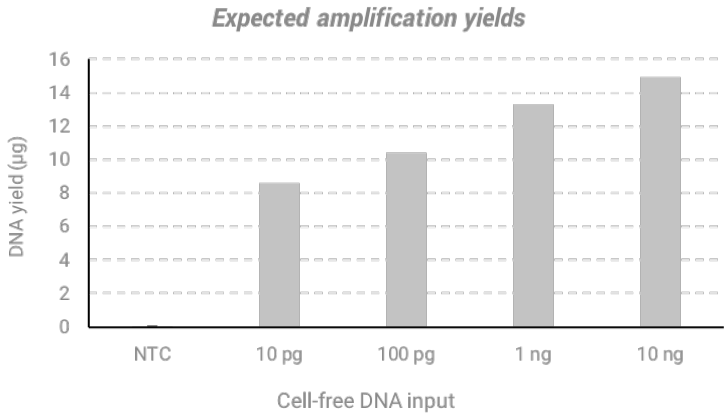

Figure 4. Expected amplification DNA yields depending on the cell-free DNA amount initially processed (NTC = no template control).

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 4BB™ TruePrime® amplification products we highly recommend purifying the samples beforehand, as 4BB™ TruePrime® components can have inhibitory effects. For the purification 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.

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.

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