PHASIFY ENRICH

cfDNA Extraction Kit

for Plasma Samples

USER MANUAL

Product Ref. No. 1020100 1020200

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PHASIFY™ TECHNOLOGY

The PHASIFY™ technology is a ground-breaking nucleic acid purification technique based on a proprietary liquid-liquid extraction mechanism. The novel method enables significantly improved DNA recovery and avoids common drawbacks of solid phase extraction techniques such as poor small-fragment recovery. Increased circulating cell-free DNA (cfDNA) recovery combined with condensed output sample has the potential to redefine sample quality for life science research and molecular diagnostic applications.

PRODUCT INTRODUCTION

The PHASIFY™ ENRICH cfDNA extraction kit is uniquely designed to purify and concentrate cfDNA from plasma and has demonstrated unprecedented recovery against solid phase extraction kits. The revolutionary liquid phase extraction technology results in several-fold improvement in DNA recovery compared against solid phase extraction kits.

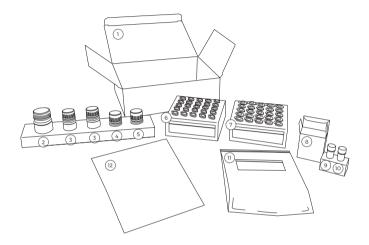
The core innovation of our novel nucleic acid extraction method is its ability to fractionate the extracted nucleic acids by size. To achieve selective size enrichment, the purified DNA is mixed with a proprietary solution to separate target DNA fragments. After size selection, the remaining contaminants will be removed and the subsequent fragments will be resuspended in a small volume. The extremely low volume for resuspension allows for an unprecedented high concentration of final DNA content to be used in downstream analysis.

The PHASIFY™ ENRICH cfDNA extraction kit allows for unprecedented isolation of cfDNA (below 500bp) from plasma and effectively remove large DNA fragment and genomic DNA sized above 1000bp using a proprietary liquid phase extraction method. By removing large-sized DNA, one can improve sensitivity and efficiency of cfDNA analysis, resulting in more reliable result.

Sample Input	Number of Reactions	
1 mL plasma	25	
2 mL plasma	2 mL plasma 25	

KIT COMPOSITION (1 mL PLASMA INPUT)

Ref. No. 1020100



PHASIFY™ ENRICH cfDNA Extraction Kit - 1 mL

- (1) RT Box
- (2) Buffer D2
- (3) D1
- (4) Solution A2

- (5) Solution P
- Solution B

 - (7) Solution C
- COLD Box

- (9) A1
- (10) Solution D3
- Microcentrifuge tubes
- (12) Quick Start Guide

CONTENTS OF KIT (1 mL PLASMA INPUT) PSSIO76

Content	Quantity	Storage
RT Box		
Solution A2	3 mL	
Solution B	25 x 460 μL	
Solution C	25 x 260 μL	
Solution P	2.2 mL	15-30°C
DI	2 x 8.8 g	
Buffer D2	18 mL	
2 mL microcentrifuge tube	25 empty tubes	
COLD Box		
Al	25 mg	/°C
Solution D3	250 μL	4°C

FOUIPMENT & REAGENTS NOT INCLUDED

In addition to the PHASIFY™ ENRICH cfDNA extraction kit - 1 mL, the following items are required:

Equipment & Materials

Water / dry bath set to 37°C

1.5 mL microcentrifuge tubes (DNase / RNase-free)

Microcentrifuge capable of 16,000 x g

Vortex-mixer

Pipettes (adjustable)

Sterile pipette tips (filtered tips are recommended)

Reagents

40% (v/v) isopropanol (molecular grade)

100% isopropanol (molecular grade)

70% (v/v) ethanol stored in -20°C (molecular grade)

DNase / RNase-free water

Resuspension buffer

PREPARATION (1 mL PLASMA INPUT)

Things to do before starting

- 1. Preheat a water / dry bath to 37°C for incubation at Step 1.
- Make sure the solutions are prepared as stated
 - a. Solution A1:

Add 875 μ L DNase / RNase-free water into the vial of A1 (25 mg) and mix well. Store at 4°C

b. Solution D1:

Add 7.6 mL Buffer D2 into ONE bottle of D1 (8.8 g) and mix well. This reaction is mildly exothermic, warming of the bottle is expected. Let bottle cool down to room temperature before use. One bottle of solution is sufficient for 14 reactions. Prepare as needed.

PROTOCOL (1 mL PLASMA INPUT)

Note: Unless specified otherwise, perform the experiment at room temperature

- Add 30 µL Solution A1, 1 mL plasma, and 100 µL Solution A2 into an empty
 5 mL microcentrifuge tube (tube not provided). Do not pre-mix Solution A1 and Solution A2. Add each component separately into plasma. Vortex thoroughly until the solution is homogenous and briefly centrifuge.
- 2 Incubate at 37°C for 15 min.
- Briefly centrifuge the provided Solution B tubes before use (to collect any liquid at the cap). Vortex and briefly centrifuge the treated plasma after incubation. Transfer <u>ALL</u> contents in the tube to Solution B. Vortex thoroughly until the solution is homogenous.
- 4. Centrifuge for 1 min at 7,000 x g.
- Briefly centrifuge the provided Solution C tubes before use (to collect any liquid at the cap). Pipette ALL the bottom phase of Solution B to Solution C.

Helpful hint: Although the system is robust, it is recommended to avoid transferring any red top phase and protein aggregate from Solution B into Solution C during Step 5.

- 6. Vortex thoroughly until the solution is homogenous. Centrifuge for 1 min at $7.000 \times g$.
- Pipette 130 µL of the red top phase of Solution C and transfer to an empty 2 mL microcentrifuge tube (provided).

Helpful hint: To facilitate top phase extraction, tilt the tube at an angle, stabilize the micropipette tip on the tube rim, and extract the top phase by collecting along the tube wall. Although the system is robust, it is recommended to avoid transferring any bottom phase from Solution C during Step 7.

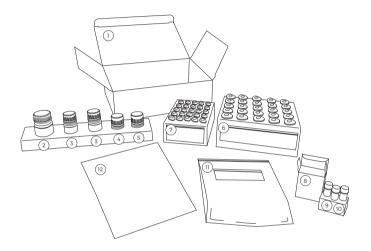
- Add 73 uL Solution P and 7 uL Solution D3.
- 9. Vortex thoroughly until homogenous then incubate for 5 min.
- Place the tube in the centrifuge with the cap hinge facing outwards in the rotor. Centrifuge for 10 min at 16,000 x g.
- Transfer ALL supernatant to an empty 2 mL microcentrifuge tube (provided) without touching the pellet.
- Into the 2 mL microcentrifuge tube from Step 7, add in order: 660 μL Solution
 D1, 2 μL Solution D3, and 860 μL 100% isopropanol. Vortex thoroughly until the solution is homogenous.
- 13. Incubate for 5 min.
- 14. Place the tube in the centrifuge with the cap hinge facing outwards in the rotor. Centrifuge for 5 min at 16.000 × a.
- Locate the position of the pellet at the bottom of the tube. Discard
 the supernatant carefully without disturbing the pellet. Add 1 mL 40%
 isopropanol. Do not break up the pellet or vortex.
- Centrifuge for 2 min at 16,000 x g, maintaining the same tube hinge orientation as Step 10.
- Discard the supernatant carefully without disturbing the pellet. Add 1 mL cold 70% ethanol. Do not break up the pellet or vortex.
- Centrifuge for 2 min at 16,000 x g, maintaining the same tube hinge orientation as Step 12.

- Discard all supernatant carefully without disturbing the pellet. Leave the tube cap open and dry the pellet for 10 min. [Over-drying can make DNA hard to re-dissolve]
- 20. Resuspend the pellet in 5 100 μL of a buffer of choice.

The purified DNA is ready for immediate use or long-term storage at -20°C or below.

KIT COMPOSITION (2mL PLASMA INPUT)

Ref No 1020200



PHASIFY™ ENRICH cfDNA Extraction Kit - 2 mL

- (1) RT Box
- Buffer D2
- (3) D1
- (4) Solution A2

- (5) Solution P
- Solution B

 - (7) Solution C
- COLD Box

- (9) A1
- (10) Solution D3
- (ii) Microcentrifuge tubes
 - (12) Quick Start Guide

CONTENTS OF KIT (2 mL PLASMA INPUT) PSS 205

Content	Quantity	Storage	
RT Box			
Solution A2	6 mL		
Solution B	25 x 1170 μL		
Solution C	25 x 280 μL	15-30°C	
Solution P	2.1 mL	15-30 C	
DI	2 x 8.8 g		
Buffer D2	18 mL		
2 mL microcentrifuge tube	25 empty tubes		
COLD Box			
A1	2 x 25 mg	4°C	
Solution D3	370 μL	40	

FQUIPMENT & REAGENTS NOT INCLUDED

In addition to the PHASIFYTM ENRICH cfDNA extraction kit - 2 mL, the following items are required:

Equipment & Materials

Water / dry bath set to 37°C

15 mL centrifuge tubes (DNase / RNase-free)

1.5 mL microcentrifuge tubes (DNase / RNase-free)

Microcentrifuge capable of 16,000 x q

Centrifuge capable of 2,300 x g

Vortex-mixer

Pipettes (adjustable)

Sterile pipette tips (filtered tips are recommended)

Reagents

40% (v/v) isopropanol (molecular grade)

100% isopropanol (molecular grade)

70% (v/v) ethanol stored in -20°C (molecular grade)

DNase / RNase-free water

Resuspension buffer

PREPARATION (2 mL PLASMA INPUT)

Things to do before starting

- 1. Preheat a water / dry bath to 37°C for incubation at Step 1.
- Make sure the solutions are prepared as stated
 - a. Solution A1:

Add 875 µL DNase / RNase-free water into ONE vial of A1 (25 mg) and mix well. Store at 4°C. One vial of solution is sufficient for 14 reactions.

Prepare as needed

b. Solution D1:

Add 7.6 mL Buffer D2 into ONE bottle of D1 (8.8 g) and mix well. This reaction is mildly exothermic, warming of the bottle is expected. Let bottle cool down to room temperature before use. One bottle of solution is sufficient for 14 reactions. Prepare as needed.

PROTOCOL (2 mL PLASMA INPUT)

Note: Unless specified otherwise, perform the experiment at room temperature

- Add 60 µL Solution A1, 2 mL plasma, and 200 µL Solution A2 into an empty 15 mL centrifuge tube (tube not provided). Do not pre-mix Solution A1 and Solution A2. Add each component separately into plasma. Vortex thoroughly until the solution is homogenous.
- Incubate at 37°C for 15 min.
- Briefly centrifuge the provided Solution B tubes before use (to collect any liquid at the cap). Vortex the treated plasma after incubation and transfer <u>ALL</u> contents in the tube to Solution B. Vortex thoroughly until the solution is homogenous.
- 4. Centrifuge for 6 min at 2,300 x g.
- Briefly centrifuge the provided Solution C tubes before use (to collect any liquid at the cap). Pipette <u>ALL</u> the bottom phase of Solution B to Solution C.

Helpful hint: Although the system is robust, it is recommended to avoid transferring any red top phase and protein aggregate from Solution B into Solution C during Step 5.

- 6. Vortex thoroughly until the solution is homogenous. Centrifuge for 1 min at $7.000 \, x \, g$.
- Pipette 140 µL of the red top phase of Solution C and transfer to an empty 2 mL microcentrifuge tube (provided).

Helpful hint: To facilitate top phase extraction, tilt the tube at an angle, stabilize the micropipette tip on the tube rim, and extract the top phase by collecting along the tube wall. Although the system is robust, it is recommended to avoid transferring any bottom phase from Solution Column Step 7.

- Add 68uL Solution P and 12 uL Solution D3.
- 9. Vortex thoroughly until homogenous then incubate for 5 min.
- Place the tube in the centrifuge with the cap hinge facing outwards in the rotor. Centrifuge for 10 min at 16,000 x g.
- Transfer ALL supernatant to an empty 2 mL microcentrifuge tube (provided) without touching the pellet.
- Into the 2 mL microcentrifuge tube from Step 7, add in order: 660 µL Solution
 p1, 2 µL Solution
 p3, and 860 µL 100% isopropanol. Vortex thoroughly until the solution is homogenous.
- 13. Incubate for 5 min.
- Place the tube in the centrifuge with the cap hinge facing outwards in the rotor. Centrifuge for 5 min at 16,000 x g.
- 15. Locate the position of the pellet at the bottom of the tube. Discard the supernatant carefully without disturbing the pellet. Add 1 mL 40% isopropanol. Do not break up the pellet or vortex.
- Centrifuge for 2 min at 16,000 x g, maintaining the same tube hinge orientation as Step 10.
- Discard the supernatant carefully without disturbing the pellet. Add 1 mL cold 70% ethanol. Do not break up the pellet or vortex.
- Centrifuge for 2 min at 16,000 x g, maintaining the same tube hinge orientation as Step 12.

- Discard all supernatant carefully without disturbing the pellet. Leave the tube cap open and dry the pellet for 10 min. [Over-drying can make DNA hard to re-dissolve]
- 20. Resuspend the pellet in 5 100 μL of a buffer of choice.

The purified DNA is ready for immediate use or long-term storage at -20°C or below.

STORAGE CONDITIONS

The RT Box should be stored at room temperature, between 15-30°C, and away from direct sunlight. The COLD Box (which contains A1 and Solution D3) should be stored at 4°C.

All components are stable for up to 1 year under the stated storage conditions.

D1 (regardless of powder or reconstituted solution form) should be stored protected from light and moisture. Reconstitute D1 only prior to use. The unfinished Solution D1 may be kept for 6 months at 15-30°C, but do not use the solution once it turns yellow.

PRODUCT USF LIMITATIONS

The PHASIFY™ ENRICH cfDNA extraction kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease. It is intended for use with plasma prepared from human whole blood samples and not for any use outside of the product claims.

PERFORMANCE MEASUREMENT

In a head-to-head study at MD Anderson Cancer Center, PHASIFY™ demonstrated threefold greater cfDNA yield against traditional solid phase extraction (Qiagen QIAamp Circulating Nucleic Acid kit), resulting in increased mutation detection in cancer patient plasma samples.

I. Improved yield of circulating tumor DNA (ctDNA)

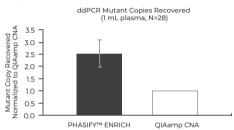


Figure 1 - The cfDNA was extracted from 28 tissue-positive cancer patient plasma samples (1 mL) using PHASIFY[™] ENRICH and the QlAamp CNA kit. On average PHASIFY[™] ENRICH demonstrated a 2.5-fold increase in mutant copy recovery compared to Olaamp CNA (P-Value <0.01 Wilcoxon Signed-Rank).

II. Enrichment of cfDNA fragments and removal of contaminating genomic DNA

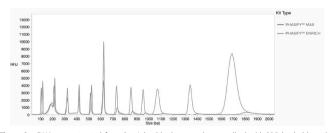


Figure 2 - DNA was extracted from 2 mt. healthy human plasma spiked with 100 bp ladder using PHASIFY™ MAX and PHASIFY™ ENRICH. Fragment analyzer trace demonstrates significant removal of fragments above 500 bp while maintaining recovery of target cfDNA.

TROUBLESHOOTING

Observation	Comments and Recommended Actions
I accidentally pipetted the wrong phase in Step 5 or Step 7.	 We have found that transferring small amounts (<10% by volume) may slightly affect enrichment performace or affect large fragment removal.
I accidentally dropped the Solution B / Solution C tube after centrifugation.	 If the Solution B / Solution C tubes are disturbed after centrifugation, the top and bottom phases may mix with each other. If the tubes are dropped or otherwise disturbed, repeat the centrifugation step and the two phases will separate again.
My DNA recovery / yield is lower than I expected.	DNA recovery and yield are influenced by numerous factors. Below are potential reasons and recommended actions: The plasma sample contains low levels of target DNA. Increase the amount of plasma input, process with the appropriate number of reactions, and combine the final resuspension. There may be insufficient mixing of the solutions at the vortexing steps (Steps 1, 3, 6, and 9). Vortex again vigorously and thoroughly until homogenous. The mixture should appear cloudy and turbid. Inefficient plasma sample digestion / lysis at Step 1 by incorrect order of solutions addition. Directly mixing Solution Al with Solution A2 can reduce the digestion / lysis activity. Incorrect preparation of Solution DI. Check that Solution DI was prepared correctly. Incorrect preparation may lead to inefficient DNA precipitation. Incomplete resuspension of pellet. Fully resuspend pellet by mild vortex or pipette up and down.
No phase forming after centrifuging Solution B tube.	Insufficient / excess plasma input. For optimal performance, use the plasma input volume as indicated on the kit. Excess reagent added in previous steps. Double check that the required amount of each reagent was added.
Abnormal bottom phase volume after Step 4.	This may be caused by improper mixing of treated plasma with Solution B components. Vortex again vigorously and thoroughly until homogenous, the mixture should appear cloudy and turbid. Centrifuge the tube again after vortex.
No phase forming after centrifuging Solution C tube.	This may be caused by an insufficient bottom phase transfer from Solution B to Solution C. Double check that the entire bottom phase solution from Solution B was transferred to Solution C tube.

Observation Comments and Recommended Actions The top phase volume of Insufficient bottom phase transferred from Solution B to Solution C is less than the Solution C. Transfer all top phase of Solution C to the next step. suggested volume. top up to the required amount with water (e.g. if the Solution C. top phase transfer volume was 100 µL, then add an additional 20 uL of water). Improper mixing of Solution B bottom phase with Solution C components. Vortex again vigorously and thoroughly until homogenous, the mixture should appear turbid. Centrifuge the tube again after vortex. The pellet looks abnormal This may occur when an excess amount of bottom phase (the wrong phase) is accidentally transferred from Solution after Step 14 (e.g. impurities. C. Perform extra cold 70% ethanol washing step by repeating gel-like pellet). steps 13 and 14 before proceeding to step 15 to remove additional 'impurities'. Note: increasing the number of washing steps may lower the DNA recovery. Pellet is difficult to This may be due to over-drying. Mildly vortex the tube after resuspend. adding resuspension buffer, briefly centrifuge the tube and let sit before freezing. We recommend resuspending the DNA pellet immediately after drying. Pellet is disturbed at Step 11. If disturbed at Step 11, the extracted supernatant may have 15. 17 or 19. possible contamination with large-sized DNA or gDNA. If disturbed at Step 15, 17 or 19, the DNA vield may be reduced. Centrifuge again with the same condition stated for that particular step. Large-sized DNA is not gDNA removal may be affected by different factors. effectively removed. The plasma sample contain too many gDNA. The cleaniness of the supernatant extraction at Step 11, 15, 17 or 19. Incorrect amount of solution P added

FREQUENTLY ASKED QUESTIONS (FAQs)

Can I input less plasma than the volume indicated on the kit?

If you have less plasma input than the volume indicated on the kit, we recommend bringing the sample volume up to the indicated volume by adding 1x PBS (pH 7.4) prior to step 1. However, we do not recommend using less than 80% v/v of plasma.

Can I input more plasma than the volume indicated on the kit?

A higher volume input might disrupt phase forming. We recommend using the plasma input volume as indicated on the kit.

How much bottom phase in Solution B should I expect to see after completing Step 4?

Under controlled conditions, the bottom phase will be approximately 800-860 μ L for the 1 mL plasma input kit and 900-950 μ L for the 2 mL plasma input kit. Regardless of the volume, we recommend extracting all the bottom phase during Step 5.

How much of the wrong phase can I accidentally transfer in Steps 5 and 7?

This will depend on your downstream application of the extracted DNA. It is possible that any transfer of the wrong phase can impact your results. We have found that transferring small amounts (<10% by volume) will not significantly impact results for most applications, however getting extra bottom phase in step 7 would affect performance of size selection. We recommend to avoid transfer of the wrong phase in all cases.

Can I extract more than 130 μ L / 140 μ L of red top phase of Solution C in Step 6 in the 1 mL / 2 mL kit respectively?

We recommend transferring only the stated volume of the red top phase. Transferring more of the top phase may affect performance of size selection, resulting in poor removal of large fragments. We recommend extracting the specified volume of the top phase.

What can I do if I want to increase the purity of the sample?

You can add another 40% isopropanol wash (i.e. repeat steps 15 and 16) before the 70% ethanol wash to increase the purity of the samples. Note, additional washing steps may reduce DNA recovery/yield.

What is the expected size(s) of DNA fragments obtained?

PHASIFYTM ENRICH kit has a gradual removal of DNA sized from 500bp to 1000bp, and complete removal of size above 1000bp. It collects mainly cfDNA monomer, dimer and trimer fragments.

What can this kit remove after extraction?

PHASIFYTM ENRICH kit can completely remove DNA sized larger than 1000bp, likely the genomic DNA released into plasma. This kit can also remove other common contaminants such as proteins.

Can this kit increase cfDNA vield compared to PHASIFY™ MAX?

PHASIFY™ ENRICH kit is tested to perform as comparable as PHASIFY™ MAX regarding cfDNA yield. PHASIFY™ MAX is aimed to collect as much DNA, while PHASIFY™ ENRICH is aimed to remove large-sized DNA in plasma samples.

SAFETY INFORMATION

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.phasescientific.com, where you can find, view, and print the SDS for the PHASIFY™ ENRICH cfDNA Extraction Kit.

If liquid containing the extraction kit buffers is spilled, clean with suitable laboratory detergent and water. If the spilled liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) bleach.

Do not add bleach directly to waste containing ethanol or isopropanol, as the reaction can result in the release of chloroform

Ensure that the waste is stored, transferred, transported, and disposed of according to applicable local, state/provincial, and/or national regulations.

TECHNICAL ASSISTANCE

If you have any queries regarding PHASIFY™ ENRICH cfDNA extraction kit, please do not hesitate to contact us by:

Email: phasify@phasesci.com
Service hotline: +1(657) 233-5880 (US)

+(852) 9135 2570 (Hong Kong)

The PHASE Technical Service Team will help to solve your concerns with our best effort.

PRODUCT WARRANTY & SATISFACTION GUARANTEE

We warrant that our goods will meet its specifications stated in this manual. This warranty lasts from the time we deliver the product until either the product expiry or "use by" date. If we do not specify the expiry date, the warranty will last for 12 months from the date we deliver the product.

The product may be used solely in accordance with the protocols provided with the product and this manual and for use with components contained in the kit only. This kit and its components are licensed for one-time use and may not be reused, refurbished or resold.

Our warranty shall not be effective if we determine, in our sole discretion, that you have altered or misused the goods or have failed to use or store them in accordance with instructions furnished by us. Our sole and exclusive liability and your exclusive remedy with respect to goods proved to our satisfaction (applying analytical methods reasonably selected by us) to be defective or nonconforming shall be the replacement of such goods free of charge.

Appendix I: Plasma Preparation and Storage

Equipment & Materials

Blood collection tubes

1.5 mL or 2.0 mL microcentrifuge tubes (DNase / RNase-free)

15 mL or 50 mL conical tubes (optional)

Swinging bucket centrifuge, set to 4°C

High speed microcentrifuge, set to 4°C

Protocol

- Collect blood in an EDTA collection tube
- 2. Centrifuge the blood samples at 1,600 \times g for 20 min at 4°C in the swinging bucket centrifuge.
- Carefully transfer the plasma to a new microcentrifuge tubes without disturbing the buffy coat.
- Centrifuge the plasma samples at 16,000 x g for 5 min at 4°C to remove residual blood cells and debris.
- 5. Extract the clean plasma samples and keep at -80°C for long-term storage.

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TECHNICAL ASSISTANCE

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For updated product information, see www.phasescientific.com

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Limited License Agreement

Use of this product indicate the agreement of any purchaser or user of the PHASIFY™ ENRICH cfDNA Extraction Kit to the following terms:

- The PHASIFY™ ENRICH cfDNA Extraction Kit may be used solely in accordance with the protocols provided with the
 product and this manual and for use with components contained in the kit only. PHASE Scientific grants no license under any of its intellectual property to use or incorporate the components of this kit with any components in included in this kit except as described in the PHASIFY™ ENRICH cfDNA Extraction Kit User Manual and additional protocols available at www.phasescientific.com.
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