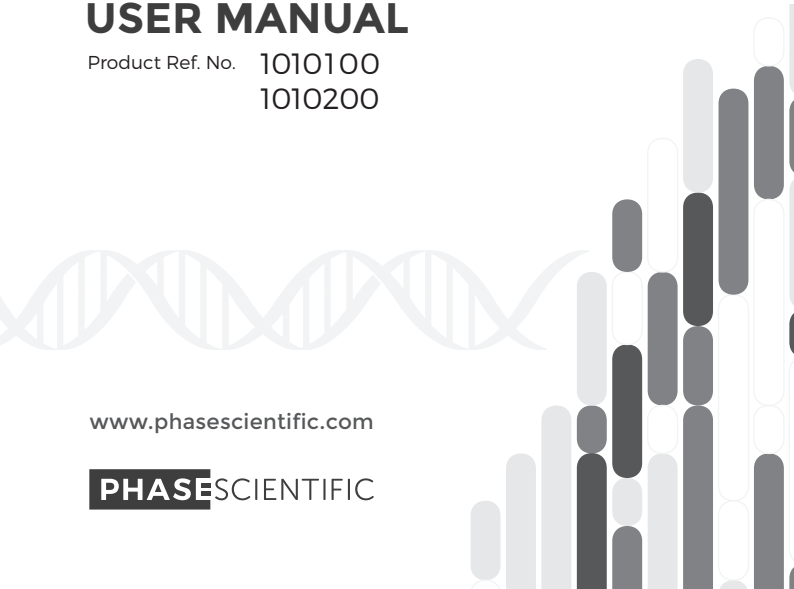
PHASiFY™ MAX

cfDNA Extraction Kit

for Plasma Samples

USER MANUAL

Product Ref. No. 1010100
1010200



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Contents

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™ MAX cfDNA extraction kit is uniquely designed to purify 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.

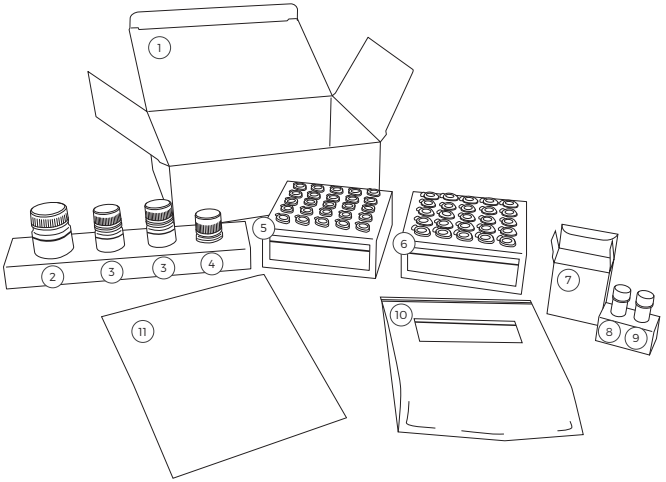
cfDNA are highly fragmented nucleic acids present in plasma, serum and other body fluids. They are largely comprised of mononucleosomal DNA fragments (~170 bp) and can also contain some dinucleosomal (~300 bp) and trinucleosomal (~500 bp) fragments. The recent discovery of cfDNA originating from tumor cells and the fetus in the plasma of cancer patients and pregnant women respectively has unlocked liquid biopsy molecular diagnostics for cancer and non-invasive prenatal testing.

The PHASIFY™ MAX cfDNA extraction kit allows for unprecedented isolation of cfDNA from plasma using a proprietary liquid phase extraction method.

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

KIT COMPOSITION (1 mL PLASMA INPUT)

Ref. No. 1010100



PHASIFY™ MAX cfDNA Extraction Kit - 1 mL

1. RT Box
2. Buffer D2
3. D1
4. Solution A2
5. Solution B
6. Solution C
7. COLD Box
8. A1
9. Solution D3
10. Microcentrifuge tubes
11. Quick Start Guide

CONTENTS OF KIT (1 mL PLASMA INPUT) PSM106b

Content	Quantity	Storage
RT Box		
Solution A2 Solution B Solution C D1 Buffer D2 2 mL microcentrifuge tube	3 mL 25 x 460 µL 25 x 250 µL 2 x 8.7 g 20 mL 25 empty tubes	15-30˚C
COLD Box		
A1 Solution D3	25 mg 70 µL	4˚C

EQUIPMENT & REAGENTS NOT INCLUDED

In addition to the PHASIFY™ MAX 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.

2. Make sure the solutions are prepared as stated
   - **a. Solution A1:**
     Add 875 µL DNase / RNase-free water into the vial of A1 (25 mg) and mix well. Store at 4˚C.
   - **b. Solution D1:**
     Add 8.7 mL Buffer D2 into ONE bottle of D1 (8.7 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

1. Add 30 µL **Solution A1**, 1 mL plasma, and 100 µL **Solution A2** into an empty 1.5 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.

3. 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 ALL contents in the tube to **Solution B**. Vortex thoroughly until the solution is homogenous.

4. Centrifuge for 1 min at 7,000 × g.

5. 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 x g.

7. Pipette 120 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.*

8. Into the 2 mL microcentrifuge tube from Step 7, add in order: 740 µL **Solution D1**, 2 µL **Solution D3**, and 860 µL 100% isopropanol. Vortex thoroughly until the solution is homogenous.

9. Incubate for 5 min.

10. Place the tube in the centrifuge with the cap hinge facing outwards in the rotor. Centrifuge for 5 min at 16,000 × g.

11. 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.

12. Centrifuge for 2 min at 16,000 × g, maintaining the same tube hinge orientation as Step 10.

13. Discard the supernatant carefully without disturbing the pellet. Add 1 mL cold 70% ethanol. Do not break up the pellet or vortex.

14. Centrifuge for 2 min at 16,000 × g, maintaining the same tube hinge orientation as Step 12.

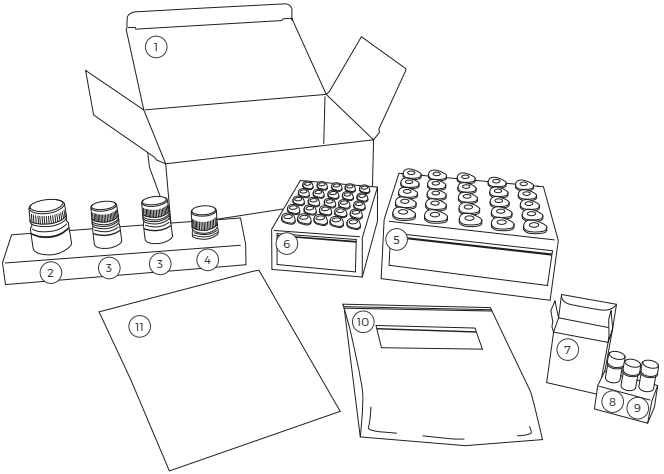
15. Discard all supernatant carefully without disturbing the pellet. Leave the tube cap open and dry the pellet for 10 min. [Over-drying can make DNA hard to re-dissolve]

16. 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. 1010200



PHASIFY™ MAX cfDNA Extraction Kit - 2 mL

1. RT Box
2. Buffer D2
3. D1
4. Solution A2
5. Solution B
6. Solution C
7. COLD Box
8. A1
9. Solution D3
10. Microcentrifuge tubes
11. Quick Start Guide

CONTENTS OF KIT (2 mL PLASMA INPUT) PSM.205

Content	Quantity	Storage
RT Box		
Solution A2 Solution B Solution C D1 Buffer D2 2 mL microcentrifuge tube	6 mL 25 x 1170 µL 25 x 270 µL 2 x 8.7 g 20 mL 25 empty tubes	15-30°C
COLD Box		
A1 Solution D3	2 x 25 mg 70 µL	4°C

EQUIPMENT & REAGENTS NOT INCLUDED

In addition to the PHASIFY™ MAX 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) Microcentrifuge capable of 16,000 x g 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.

2. 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 8.7 mL Buffer D2 into ONE bottle of D1 (8.7 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

1. 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.

2. Incubate at 37˚C for 15 min.

3. Briefly centrifuge the provided **Solution B** tubes before use (to collect any liquid at the cap). Vortex the treated plasma after incubation and transfer ALL contents in the tube to **Solution B**. Vortex thoroughly until the solution is homogenous.

4. Centrifuge for 6 min at 2,300 × g.

5. 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 x g.

7. Pipette 120 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.*

8. Into the 2 mL microcentrifuge tube from Step 7, add in order: 740 µL **Solution D1**, 2 µL **Solution D3**, and 860 µL 100% isopropanol. Vortex thoroughly until the solution is homogenous.

9. Incubate for 5 min.

10. Place the tube in the centrifuge with the cap hinge facing outwards in the rotor. Centrifuge for 5 min at 16,000 × g.

11. 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.

12. Centrifuge for 2 min at 16,000 × g, maintaining the same tube hinge orientation as Step 10.

13. Discard the supernatant carefully without disturbing the pellet. Add 1 mL cold 70% ethanol. Do not break up the pellet or vortex.

14. Centrifuge for 2 min at 16,000 × g, maintaining the same tube hinge orientation as Step 12.

15. 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]

16. 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 USE LIMITATIONS

The PHASIFY™ MAX 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 Total DNA

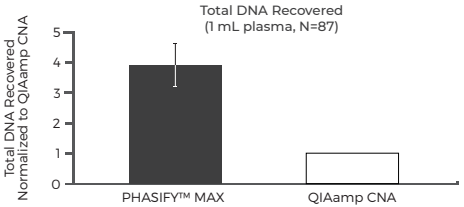


Figure 1 - The cfDNA was extracted from 89 tissue positive cancer patient plasma samples (1mL) using PHASIFY™ MAX and the QIAamp CNA kit. Total DNA recovery was measured using Quant-iT™ PicoGreen™ dsDNA Assay Kit. On average PHASIFY™ MAX demonstrated a 3.8-fold increase in total DNA recovery compared to QIAamp CNA (p-value <0.0001 Wilcoxon Signed-Rank). Note, 2 outliers were excluded in which PHASIFY™ MAX generated 209x and 800x greater recovery.

II. Improved Yield of Total Mutant Copies (ctDNA)

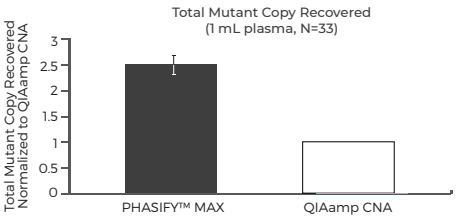


Figure 2 - Of the 89 tissue positive samples tested, either BRAF, KRAS or NRAS mutations were detected in 33 samples by both PHASIFY™ MAX and the QIAamp CNA kit. Mutation detection was performed by probe-based droplet digital PCR (ddPCR). On average, PHASIFY™ MAX demonstrated a 2.4-fold (2.7-fold median) increase in total mutant copy recovery compared to QIAamp CNA (p-value 0.0006 Wilcoxon Signed-Rank).

III. Improved Mutation Detection and Clinical Sensitivity

Tumor Type	*PHASIFY™ MAX (MAF)*	*QIAamp CNA (MAF)*
Pancreatic	0.35%	ND
Pancreatic	0.65%	ND
Pancreatic	0.41%	ND
Appendiceal	0.35%	ND
Ovarian	2.80%	ND
Ovarian	0.44%	ND
Colorectal	2.10%	ND
Colorectal	0.24%	ND
Colorectal	0.27%	ND
Colorectal	0.25%	ND
Colorectal	0.50%	ND
Colorectal	0.35%	ND
Colorectal	0.58%	ND

ND = Not Detected, MAF = Mutant allele frequency

Figure 3 - In 13 samples, mutations were detected in samples extracted with PHASIFY™ MAX, but not with QIAamp CNA.

IV. Greater Recovery of Desired cfDNA Fragments

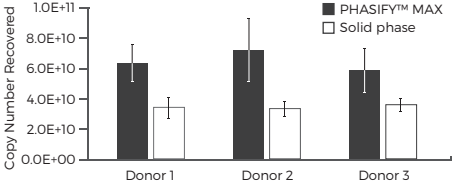


Figure 4

Figure 4 - 25 ng of a 145 bp fragment was spiked into 1 mL of human plasma from 3 different donors (n=3). cfDNA was then extracted using PHASIFY™ MAX or a solid phase extraction kit. Recovery of the 145 bp fragment was determined by probe-based ddPCR.

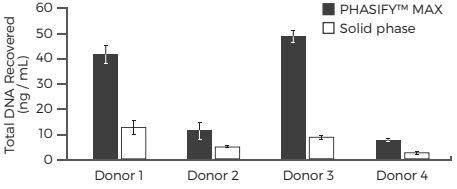


Figure 5

Figure 5 - DNA was extracted from 1 mL healthy human plasma from 4 different donors (n ≥ 3) using PHASIFY™ MAX or a solid phase extraction kit. Total DNA yields were determined by Qubit. Results were confirmed with ddPCR using probe / primers complimentary to EGFR DNA sequence (data not shown).

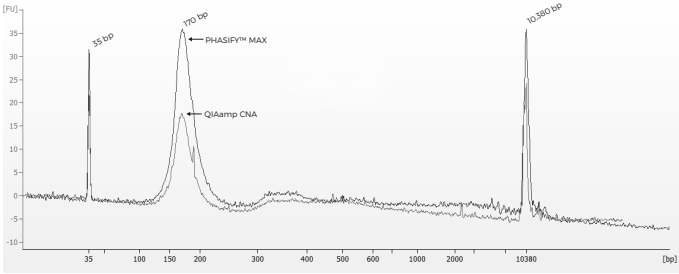


Figure 6 - DNA was extracted from 1 mL healthy human plasma extracted using PHASIFY™ MAX and the QIAamp Circulating Nucleic Acid kit. The extracted DNA were analysed on Agilent Bioanalyzer 2100, where 35bp and 10,380bp peaks correspond to internal markers.

Improved recovery of cfDNA with PHASIFY™ MAX cfDNA extraction kit has been demonstrated with spiked-in healthy human plasma and cancer patient plasma using Qubit, Droplet Digital PCR (ddPCR) and Bioanalyzer analyses.

V. Reproducible Results Between Extractions

The recovery of PHASIFY™ MAX cfDNA extraction kit is consistent and reproducible amongst different samples.

Plasma ID	Donor 1	Donor 2	Donor 3	Donor 4
Total DNA Recovered (ng)	41.13	11.93	5.25	2.69
	42.38	11.66	5.61	2.04

Figure 7 - DNA was extracted from 1 mL plasma from 4 different human donors in duplicates using PHASIFY™ MAX. Total DNA yields were determined by Qubit.

VI. Consistent cfDNA Yield and Performance at Higher Sample Volume Input

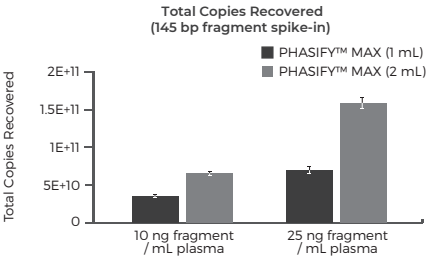


Figure 8 - 145bp fragment DNA was spiked into healthy human plasma at 10ng /mL and 25ng / mL concentrations and extracted with PHASIFY™ MAX 1mL and 2mL kits. Total 145bp DNA fragment recovery was measured with probe-based ddPCR.

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) will not significantly impact results for most applications.
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 8). 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 A1 with Solution A2 can reduce the digestion / lysis activity. • Incorrect preparation of Solution D1. Check that Solution D1 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 Solution C is less than the suggested volume.	• Insufficient bottom phase transferred from Solution B to Solution C. Transfer all top phase of Solution C to the next step, 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 µL 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 after Step 14 (e.g. impurities, gel-like pellet).	• This may occur when an excess amount of bottom phase (the wrong phase) is accidentally transferred from Solution C. Perform extra cold 70% ethanol washing step by repeating 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 resuspend.	• This may be due to over-drying. Mildly vortex the tube after adding resuspension buffer, briefly centrifuge the tube and let sit before freezing. We recommend resuspending the DNA pellet immediately after drying.

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 µL for the 1 mL plasma input kit and 900-950 µL 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 might result in reduced purity of the pellet. We recommend to avoid transfer of the wrong phase in all cases.

Can I extract more than 120 µL of red top phase of Solution C in Step 6?

We recommend transferring only 120 µL of the red top phase. Transferring more of the top phase may result in higher recovery/yield, but it increases the risk of accidentally pipetting and transferring contaminants from the bottom phase. 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 11 and 12) before the 70% ethanol wash to increase the purity of the samples. Note, additional washing steps may reduce DNA recovery/yield.

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™ MAX 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™ MAX cfDNA extraction kit, please do not hesitate to contact us by:

Email:	phasify@phasesci.com
Service hotline:	+1(657) 233-5880 (US)
	+(852) 3892-7200 (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

1. Collect blood in an EDTA collection tube.
2. Centrifuge the blood samples at 1,600 × g for 20 min at 4˚C in the swinging bucket centrifuge.
3. Carefully transfer the plasma to a new microcentrifuge tubes without disturbing the buffy coat.
4. Centrifuge the plasma samples at 16,000 × 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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Information subjected to change without notice.
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™ MAX cfDNA Extraction Kit to the following terms:

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