



## **truPREP® Protein FFPE Kit (Manual 16)**

Isolation  
(PN 520359)

Isolation, Purification  
(PN 520363)

Isolation, Purification (PN 520363)  
& Digestion (PN 520364)

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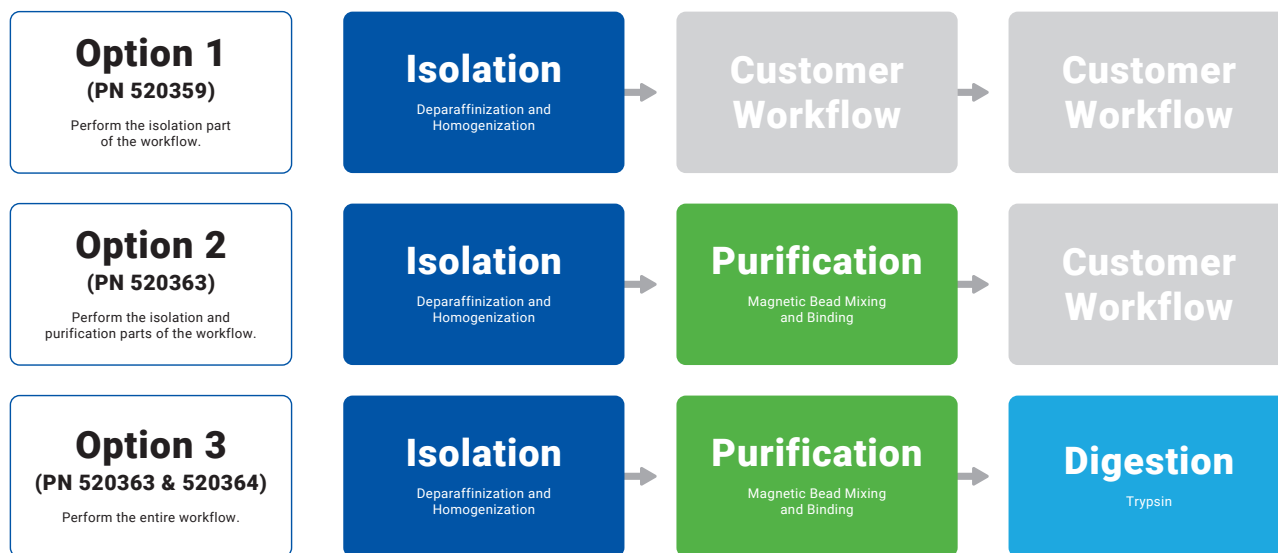
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## Introduction

Achieve reliable results with the Covaris truPREP Protein FFPE Kit, which offers three modular options for fast and reproducible protein sample preparation from Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples. This innovative solution allows for the flexibility to utilize a complete Covaris workflow or integrate with your established purification and/or digestion workflow.



**Figure 1.** Summary of truPREP Protein Kit workflow options.

- **Option 1 (Isolation) PN 520359 | truPREP Protein FFPE Kit - Isolation (Manual) (16)** includes a proprietary non-toxic deparaffinization method used in conjunction with Covaris' Adaptive Focused Acoustics® (AFA®) technology on the R230 Focused-ultrasonicator to effectively homogenize tissues and extract proteins. Streamline your workflow with easy loading of your 10 µm FFPE scroll into individually capped tubes for easy processing. Upon completion of this section customers can continue with their own established purification workflows.
- **Option 2 (Isolation & Purification) PN 520363 | truPREP Protein FFPE Kit – Isolation, Purification (Manual) (16)** integrates the deparaffinization and extraction option with an optimized Protein Aggregation Capture (PAC) workflow for efficient protein purification. Customers at this point can continue with their established digestion workflow.
- **Option 3 (Isolation, Purification, & Digestion) PN 520363 & 520364 | truPREP Protein FFPE Kit – Isolation, Purification (Manual) (16) & truPREP Protein FFPE Kit – Digestion (Manual) (16)** includes the Isolation and Purification option along with Promega Trypsin Gold for an overnight digestion, resulting in a purified peptide hydrolysate for further downstream analysis.

The Covaris protocol detailed below encompasses the complete workflow: Isolation, Purification, and Digestion. Each respective section begins with steps for preparing the necessary reagents and accessories. At the conclusion of each of the first two sections, you may choose to continue with the Covaris protocol or apply your own preferred method, depending on which option you have chosen.

## Kit Contents

This kit contains reagents and consumables for 16 samples, which can be processed in batch size of 8 or 16 samples. In addition, method files and plate definitions for the R230 instrumentation are provided.

## Instrumentation and Accessories

The following Covaris instruments and accessories are required to use this kit. These accessories are not included and must be purchased separately. They are available as a package under the PN 500827 truPREP Protein FFPE Hardware Accessory Kit (Manual).

Plate definitions and method files can be downloaded from the Covaris [website](#). Further instructions for use are outlined in the instrument set up section below.

| Product Name                                                                             | Part # | Instrument or Accessory                       | Product Image                                                                       |
|------------------------------------------------------------------------------------------|--------|-----------------------------------------------|-------------------------------------------------------------------------------------|
| R230 Focused-ultrasonicator                                                              | 500620 | Instrument (not included in kit)              |  |
| truPREP Protein FFPE Hardware Accessory Kit (Manual)                                     | 500827 | Accessory Kit<br>(to be purchased separately) |                                                                                     |
| This accessory kit includes the following components.                                    |        |                                               |                                                                                     |
| prepTUBE 16 Shuttle & Shuttle Crossbars (Manual) *Referred to in protocol as "Shuttle"   | 500830 | Accessory (included in PN 500827)             |                                                                                     |
| prepTUBE 0.75 mL R230 Sample Holder Adapter *Referred to in protocol as "Adapter"        | 500831 | Accessory (included in PN 500827)             |                                                                                     |
| prepTUBE 0.75 mL 16 Home Rack (Manual) *Referred to in protocol as "Home Rack"           | 500829 | Accessory (included in PN 500827)             |                                                                                     |
| prepTUBE 0.75 mL Heat Block *Referred to in protocol as "TPX Heat Block"                 | 500828 | Accessory (included in PN 500827)             |                                                                                     |
| *This heat block was designed to be compatible with the QInstrument heater PN 2016-0100. |        |                                               |                                                                                     |

Please refer to the **Appendix A** for additional instructions and diagrams related to the hardware accessories.

## Reagents and Consumables Included

| Consumable/Reagent                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Option 1 (PN 520359)<br>Isolation | Option 2 (PN 520363)<br>Isolation, Purification | Option 3 (PN 520363 & 520364)<br>Isolation, Purification, & Digestion |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|-------------------------------------------------|-----------------------------------------------------------------------|
| prepTUBE TPX with Bead*<br>qty. 18                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | ✓                                 | ✓                                               | ✓                                                                     |
| FFPE Protein Rehydration Buffer<br>1 mL   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | ✓                                 | ✓                                               | ✓                                                                     |
| Deparaffinization Solution<br>8 mL   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | ✓                                 | ✓                                               | ✓                                                                     |
| Tissue Lysis Buffer<br>2 mL   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | ✓                                 | ✓                                               | ✓                                                                     |
| Collection Tube<br>qty. 16   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                   | ✓                                               | ✓                                                                     |
| Reducing Agent**<br>5 mg   -20 °C                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                   | ✓                                               | ✓                                                                     |
| Alkylation Reagent<br>qty. 2   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                   | ✓                                               | ✓                                                                     |
| Magnetic Bead Mixture<br>100 µL   2–8 °C                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                   | ✓                                               | ✓                                                                     |
| WB5 Diluent<br>2.4 mL   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                   | ✓                                               | ✓                                                                     |
| Ethanol -Wash Buffer Diluent<br>1.6 mL   Room Temp.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                   | ✓                                               | ✓                                                                     |
| Promega Trypsin Gold***<br>1 Vial   -20 °C                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                   |                                                 | ✓                                                                     |
| <p>*Two additional prepTUBE TPX with bead have been included to be utilized during the heat block calibration step. These are not to be run with the assay after the calibration.</p> <p>**Item can be stored at room temperature (RT) prior to reconstitution.</p> <p>***Item is shipped at ambient temperatures but should be stored at -20 °C upon receipt of the product. Once reconstituted, the item should be used at once or if only processing 8 samples at a time the remaining material can be stored reconstituted at -20 °C.</p> |                                   |                                                 |                                                                       |

**Figure 2.** Reagents and Consumables included in each respective kit.

## Items Supplied by End User

| Reagents                                        | Option 1 (PN 520359)<br>Isolation | Option 2 (PN 520363)<br>Isolation, Purification | Option 3 (PN 520363 & 520364)<br>Isolation, Purification, & Digestion |
|-------------------------------------------------|-----------------------------------|-------------------------------------------------|-----------------------------------------------------------------------|
| 100% Ethanol<br>Sigma E7023*                    |                                   | ✓                                               | ✓                                                                     |
| 100% Isopropanol<br>Sigma I9516*                |                                   | ✓                                               | ✓                                                                     |
| 100% Acetonitrile<br>Sigma 34851-1L*            |                                   | ✓                                               | ✓                                                                     |
| Nuclease-free Water<br>Invitrogen AM9932*       |                                   | ✓                                               | ✓                                                                     |
| 50 mM Ammonium Bicarbonate<br>Sigma A6141-500G* |                                   |                                                 | ✓                                                                     |
| 50 mM Acetic Acid<br>Sigma 320099-500ML*        |                                   |                                                 | ✓                                                                     |
| *Recommended product.                           |                                   |                                                 |                                                                       |

**Figure 3.** Reagents provided by end user for each respective option.

| Hardware                                                                                                      | Option 1 (PN 520359)<br>Isolation | Option 2 (PN 520363)<br>Isolation, Purification | Option 3 (PN 520363 & 520364)<br>Isolation, Purification, & Digestion |
|---------------------------------------------------------------------------------------------------------------|-----------------------------------|-------------------------------------------------|-----------------------------------------------------------------------|
| Dry Block Heater<br>(QInstruments PN 2016-0100)                                                               | ✓                                 | ✓                                               | ✓                                                                     |
| 8-Channel Decapper (optional)                                                                                 | ✓                                 | ✓                                               | ✓                                                                     |
| Microtome (Leica PN<br>14051856372 or equivalent)                                                             | ✓                                 | ✓                                               | ✓                                                                     |
| Vortex Mixer                                                                                                  |                                   | ✓                                               | ✓                                                                     |
| Thermoshaker (Eppendorf PN<br>5382000023 or equivalent)                                                       |                                   | ✓                                               | ✓                                                                     |
| Magnetic Separator Compatible<br>with 1.5 mL Microcentrifuge<br>Tubes (Invitrogen PN 12321D<br>or equivalent) |                                   | ✓                                               | ✓                                                                     |
| Heating Block Compatible with<br>1.5 mL Microcentrifuge Tubes<br>(Eppendorf PN 2231001035 or<br>equivalent)   |                                   |                                                 | ✓                                                                     |

**Figure 4.** Hardware required by customer for each respective option.

A fixed-angle benchtop centrifuge with appropriate tube adapters may be used in place of a swinging bucket plate centrifuge. Please contact Covaris Application Support ([applicationsupport@covaris.com](mailto:applicationsupport@covaris.com)) for additional recommendations.

Workflows

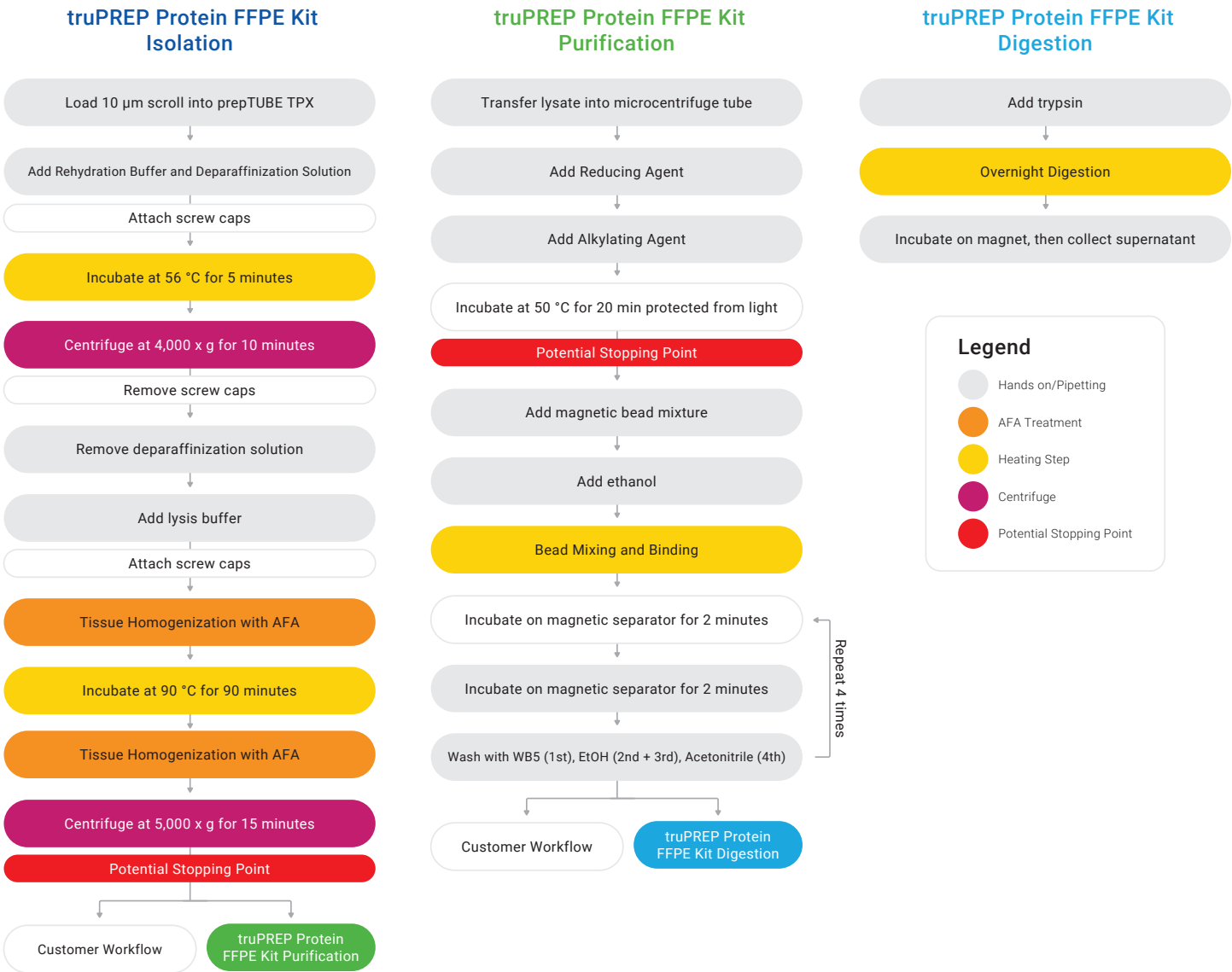


Figure 5. truPREP Protein FFPE Kit workflow.

## Instrument Setup

### R230 Focused-ultrasonicator

Refer to the R230 Instrument Manual ([https://www.covaris.com/wp/wp-content/uploads/pn\\_010528.pdf](https://www.covaris.com/wp/wp-content/uploads/pn_010528.pdf)) and R230 User Guide ([https://www.covaris.com/wp/wp-content/uploads/pn\\_010480.pdf](https://www.covaris.com/wp/wp-content/uploads/pn_010480.pdf)) for complete setup.

1. Turn on R230 Focused-ultrasonicator prior to starting reagent preparation step.
2. Install prepTUBE 0.75 mL R230 Sample Holder Adapter (PN 500381) into the R230 by pressing down until the rack clicks into place. This rack will remain installed throughout the entire workflow.

**NOTE:** See **Appendix A** for further instructions.

## SonoLab Setup

1. Current versions of SonoLab can be found at <https://www.covaris.com/r-series/>. To check which version of SonoLab is currently installed on your R230 search under the about tab in upper left-hand corner in the Covaris icon.
2. Check for SonoLab software updates and use the latest available version. Consumables may require a SonoLab update to run protocol. Current versions of SonoLab can be found at <https://www.covaris.com/r-series/>.
3. Navigate to <https://www.covaris.com/truprep-protein/> to select the appropriate plate method and plate definition files. Installation instructions can be found on the link provided above. After download follow the instructions for the following files and install it on the R230 Focused-ultrasonicator prior to processing samples:
  - **Plate Definition:** "R230\_500831 Rack\_190925 Tube\_26WL\_17.50S"
  - **Method File:** truPREP PN 520359 Tissue Homogenization ("Tissue Homogenization" in this document)

| Peak Incident Power (PIP) (Watts) | Duty Factor (%) | Cycles per Burst | Bath Temperature (°C) | Treatment Time (seconds) |
|-----------------------------------|-----------------|------------------|-----------------------|--------------------------|
| 350                               | 32              | 1,000            | 12                    | 300                      |

**Figure 6.** Detailed method file for truPREP PN 520359 Tissue Homogenization.

4. Follow steps on SonoLab for installation of the downloaded methods and plate definitions.
5. Once installed, select the "Tissue Homogenization" method from the drop-down menu to allow for the water bath temperature to equilibrate and reach set point.



# Section 1: Isolation

## Important Notes on FFPE Samples

The yield of protein from FFPE tissue blocks is highly variable. Factors such as fixation time, ratio of tissue to paraffin, type of tissue (highly connective or granular), age, and storage conditions of the FFPE block can lead to variability in yields. This is especially evident in older FFPE sample blocks or those stored at elevated temperatures.

The deparaffinization and extraction process is aided by a special accessory, the prepTUBE 16 Shuttle & Shuttle Crossbars (Manual) and prepTUBE 0.75 mL 16 Home Rack (Manual), which enables the processing of 8 or 16 samples per run without need to remove individual tubes during pipetting.

## FFPE Sample Input Requirements and Guidelines

**Curls/Scrolls Input Requirements:** Total thickness of 10 µm, non-trimmed FFPE section. This protocol has been optimized for a sample input of one 10 µm FFPE tissue section per tube.

### 1.1 Reagent Preparation

For the Isolation section of the truPREP Protein FFPE Kit there are no reagent preparation steps needed. All of the reagents provided are in their final working format.

### 1.2 prepTUBE 0.75 mL Heat Block Preparation

1. Provided with the truPREP Protein FFPE Kit – Isolation and Isolation, Purification there are 2 additional prepTUBE TPX with bead to be utilized for equilibrating the prepTUBE 0.75 mL Heat Block.
2. Preheat the dry block heater\* equipped with prepTUBE 0.75 mL Heat Block to 56 °C and measure temperature in a prepTUBE TPX filled with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 56 °C ± 2 °C within the vessel.
3. Preheat the dry block heater\* equipped with the prepTUBE 0.75 mL Heat Block to 90 °C and measure temperature in a prepTUBE TPX with bead filled with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 90 °C ± 2 °C within the vessel.

**NOTE:** \*QInstrument heater (PN 2016-0100) Provided by customer. The dry block heater may require > 5 °C offset to reach the correct temperature in the vessel.

### 1.3 Deparaffinization

Before beginning this step, please refer to **Appendix A** for guidance of the accessories noted in this section.

## 1.4 FFPE Sample Input Requirements and Guidelines

**Curls/Scrolls Input Requirements:** Total thickness of 10 µm, non-trimmed FFPE section

1. Remove cap of prepTUBE TPX with bead.
2. Load one 10 µm FFPE scroll per prepTUBE TPX with bead.
3. Add 50 µL Rehydration Buffer to the prepTUBE TPX with bead.
4. Add 450 µL Deparaffinization Solution and cap the prepTUBE TPX with bead.



**WARNING:** DO NOT VORTEX THE TUBE OR MIX THE CONTENTS.

5. Insert the tubes into the **Shuttle** sitting on the **Home Rack**.

**NOTE:** This configuration (**Shuttle** without Stabilizer bar +Tubes) will be called "**Assembly**" hereafter.

6. Transfer the **Assembly** to the preheated (56 °C) dry block heater equipped with the prepTUBE 0.75 mL **Heat Block**.
7. Incubate for 5 minutes at 56 °C.



**WARNING:** Use caution when removing the rack from the TPX **Heat Block**.

8. Remove the **Assembly** and place back into the **Home Rack**.
9. Transfer the **Assembly** with **Home Rack** into a benchtop centrifuge equipped with a swinging bucket rotor and counterbalance accordingly.
10. Centrifuge for 10 minutes at 4,000 x g.
11. Remove the **Assembly** + **Home Rack** from the centrifuge.
12. Remove cap from prepTUBE TPX with bead, then carefully take off the upper layer of FFPE Protein Deparaffination Tube.

**NOTE:** Two phases will be visible after centrifugation. The aqueous phase at the bottom of the tube contains the tissue. The FFPE Protein Deparaffinization Solution will be on top. When removing the FFPE Protein Deparaffinization Solution, ensure that the aqueous phase at the bottom of the tube and the interphase are not disturbed. It is recommended to use a single pipettor for this step. Approximately 400 µL should be removed, leaving the Rehydration Buffer and up to 50 µL of FFPE Protein Deparaffinization Solution behind. For extra caution if desired the FFPE Protein deparaffinization solution can be removed in smaller 100 µL increments up to 400 µL total volume removal.

13. Discard the supernatant.

## 1.5 Tissue Homogenization and Decrosslinking

1. Add 100 µL Tissue Lysis Buffer to each sample and cap the prepTUBE TPX with bead.

**NOTE:** Covaris Lysis Buffer is detergent-based, hence the need to purify samples to ensure LC-MS compatibility.

2. Attach the Stabilizer Clamps to the **Assembly** to hold the tubes in place during AFA.

**NOTE:** See **Appendix A** for illustrated instructions for attaching and removing Stabilizer clamps.

3. Move the **Assembly** with Stabilizer Clamps out of the **Home Rack** and into the Focused-ultrasonicator on top of the prepTUBE 0.75 mL R230 Sample Holder Adapter which has already been assembled.
4. In the SonoLab software once the **Assembly** is in place in the R230 execute the "Tissue Homogenization" method.
5. Remove the **Assembly** with stabilizer clamps from the R230 Focused-ultrasonicator and insert it into the preheated (90 °C) dry block heater equipped with the prepTUBE 0.75 mL **Heat Block**.
6. Incubate for 90 minutes at 90 °C

**NOTE:** The **Shuttle** will be extremely hot at this point. Please use caution when removing from **Heat Block**.

7. Move the **Assembly** with Stabilizer Clamps out of the dry block heater and back into the Focused-ultrasonicator on top of the R230 Rack Insert.
8. Process the samples using the "Tissue Homogenization" program for the second time.
9. Transfer the **Assembly** and Stabilizer Clamps into the **Home Rack**, and then remove Stabilizer Clamps.

**NOTE:** See appendix for illustrated instructions for attaching and removing Stabilizer clamps.

10. Transfer the **Assembly** with **Home Rack** into a benchtop centrifuge equipped with a swinging bucket rotor and counterbalance accordingly.
11. Centrifuge for 15 minutes at 5,000 x g.
12. Remove the **Assembly** with **Home Rack** from the centrifuge. Take care not to shake or bump the samples.
13. **Optional:** Quantify concentration of extracted proteins.



**STOPPING POINT:** Storing samples at this point – At this point Sample can be stored at - 20 °C for up to 5 days. If samples are frozen upon thawing repeat Steps 11 & 12 before continuing to your next process.

## Notes

1. If using BCA for quantification, samples must be diluted 1:5 in nuclease free water to ensure buffer compatibility with the assay. For increased accuracy, it is recommended to dilute standards with a solution consisting of 1 part Rehydration Buffer, 2 parts Tissue Lysis Buffer, and 12 parts nuclease-free water.
2. Be careful not to transfer any of the upper white layer into your BCA plate, as this can artificially increase absorbance measurements. Position the pipette tip at the bottom of the tube to avoid aspirating the white haze that may remain on the top.
3. At this stage in the workflow, if you have purchased the PN-520363 truPREP Protein FFPE Kit – Isolation, Purification (Manual) (16) please proceed with the second step of the workflow (Purification). If customers have only purchased the Isolation - (PN 520359) truPREP Protein FFPE Kit- Isolation (Manual) (16) Kit at this point in the workflow customers may proceed with their own desired purification and digestion methods.

## Section 2: Purification

### 2.1 Reagent Preparation

#### Reducing Agent

Reconstitute the Reducing Agent by adding 139  $\mu$ L of nuclease-free water to the lyophilized powder and pipette/vortex thoroughly. Freeze any unused Reducing Agent at -20 °C.

#### Alkylation Reagent

Reconstitute one vial of Alkylation Reagent by adding 100  $\mu$ L nuclease-free water to the lyophilized powder and pipette/vortex thoroughly. One vial will be sufficient for processing 8 samples. Discard any excess reconstituted Alkylation Reagent.

#### Magnetic Bead Mixture

1. Place provided magnetic bead mixture on a magnetic rack to separate bead mixture from storage buffer.
2. Remove storage buffer and then add 500  $\mu$ L of nuclease-free water and pipette thoroughly.
3. Place mixture on magnetic rack to isolate the beads from the nuclease-free water
4. Remove supernatant and repeat this wash step 2 additional times.
5. Reconstitute beads in 100  $\mu$ L of nuclease-free water. Store at 4 °C for up to 4 weeks.

#### WB5 Diluent

1. Prepare wash buffer directly before use.
2. Add 1.6 mL of 100% Isopropanol to the WB5 Diluent and vortex to mix.

#### Wash Buffer Diluent Preparation Ethanol Wash Buffer Diluent

1. Prepare wash buffer directly before use.
2. Add 6.4 mL of 100% Ethanol to the Ethanol Wash Buffer Diluent and vortex to mix.

### 2.2 Heat Block Preparation

**NOTE:** This *Heat Block* is not the same as the prepTUBE TPX *Heat Block*. It is recommended to use a Heating Block Compatible with 1.5 mL Microcentrifuge Tubes (Eppendorf PN 2231001035 or equivalent) for the Purification and Digestion portion of this protocol.

1. Preheat the dry block heater equipped with a 1.5 mL microcentrifuge tube to 50 °C and measure temperature in a 1.5 mL microcentrifuge tube filled with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 50 °C  $\pm$  2 °C within the vessel.

**NOTE:** The dry block heater may require > 5 °C offset to reach the correct temperature in the vessel .

### 2.3 Reduction / Alkylation

1. Thaw and/or prepare Reducing Agent and Alkylation Reagent according to the instructions in "Reagent Preparation".

**NOTE:** One vial of Alkylation Reagent is sufficient for processing 8 samples.

2. Transfer 65  $\mu$ L FFPE tissue lysate to a 1.5 mL microcentrifuge tube.

3. Add 3  $\mu$ L Reducing Agent to each sample.
4. Add 7.5  $\mu$ L Alkylation Reagent to each sample.
5. Briefly vortex tubes to mix, and centrifuge if necessary to collect droplets.
6. Transfer the tubes into a preheated (50 °C) dry block heater equipped with a heating block compatible with 1.5 mL microcentrifuge tubes.
7. Incubate for 20 minutes at 50 °C protected from light.
8. Remove tubes from the dry block heater.



**STOPPING POINT:** This is the final stopping point in the protocol. Samples can be stored at this point at -20 °C for up to up 5 days.

## 2.4 Purification

1. Ensure the magnetic bead mixture, WB5 Diluent, and Ethanol Wash Buffer Diluent have been prepared as outlined in "Reagent Preparation".
2. Vortex magnetic bead mixture immediately before proceeding to the next step.
3. Add 2.5  $\mu$ L magnetic beads to all samples and pipette mix or briefly vortex.
4. Add 85  $\mu$ L 100% ethanol to all samples.
5. Transfer the tubes into a preheated (24 °C) thermoshaker equipped with a heating block compatible with 1.5 mL microcentrifuge tubes.
6. Incubate at 24 °C for 10 minutes with shaking at 1,250 rpm.
7. Transfer tubes from the thermoshaker to a magnetic separator compatible with 1.5 mL microcentrifuge tubes.
8. Wait for at least 2 minutes until the beads have settled onto the magnet.
9. Carefully open the tubes while on the magnet; remove and discard the supernatant. Avoid disturbing the bead pellet.
10. Add 200  $\mu$ L WB5 to the magnetic bead pellet and resuspend the beads by vortexing.
11. Place the microcentrifuge tubes onto the magnetic separator and wait for at least 2 minutes until the beads have settled on the magnet.
12. Carefully open the tubes while on the magnet; remove and discard the supernatant. Avoid disturbing the bead pellet.
13. Add 200  $\mu$ L Ethanol Wash Buffer to the magnetic bead pellet and resuspend the beads by vortexing.
14. Place the microcentrifuge tubes onto the magnetic separator and wait for at least 2 minutes until the beads have settled on the magnet.
15. Carefully open the tubes while on the magnet; remove and discard the supernatant. Avoid disturbing the bead pellet.
16. Repeat steps 13–15.
17. Add 180  $\mu$ L 100% acetonitrile to the magnetic bead pellet and resuspend the beads by vortexing.
18. Place the microcentrifuge tubes onto the magnetic separator and wait for at least 2 minutes until the beads have settled on the magnet.
19. Carefully open the tubes while on the magnet; remove and discard the supernatant. Avoid disturbing the bead pellet.
20. Leave the tubes on the magnet with the lids open and dry the beads for 2–3 minutes.
21. Remove tubes from the magnetic separator.
22. Proceed directly into the digestion step.

**NOTE:** At this stage in the workflow your proteins are still bound to the beads. If you have purchased the PN- 520364 truPREP Protein FFPE Kit –Digestion (Manual) (16) please proceed with the final step of the workflow (Digestion). If customers have only purchased the PN-520363 truPREP Protein FFPE Kit – Isolation, Purification (Manual) (16) at this point in the workflow customers may proceed with their desired-on bead digestion methods.

## Section 3: Digestion

### 3.1 Reagent Preparation

#### Promega Trypsin Gold

Add 100 µL of 50 mM acetic acid directly onto 100 µg of lyophilized Promega Trypsin Gold vial. Pipette mix gently and incubate for 5 minutes at room temperature. Once reconstituted there is enough Trypsin Gold for processing all 16 samples. Aliquot into fresh tubes and freeze unused enzyme at -80 °C if only processing 8 samples at a time.

Thaw Trypsin Gold at room temperature, then store on ice until use. Trypsin Gold may be refrozen but should not exceed 5 freeze/thaw cycles. If more than 5 freeze/thaw cycles are expected, aliquot the reconstituted enzyme into multiple tubes.

### 3.2 Heat Block Preparation

**NOTE:** This **Heat Block** is not the same as the prepTUBE TPX **Heat Block**. It is recommended to use a Heating Block Compatible with 1.5 mL Microcentrifuge Tubes Eppendorf PN 2231001035 or equivalent) for the Purification and Digestion portion of this protocol.

1. Preheat the dry block heater equipped with a 1.5 mL microcentrifuge tube to 37 °C and measure temperature in a 1.5 mL microcentrifuge tube filled with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 37 °C ± 2 °C within the vessel.

**NOTE:** The dry block heater may require > 5 °C offset to reach the correct temperature in the vessel.

### 3.3 Overnight Digestion

1. Thaw and/or prepare Promega Trypsin Gold according to the instructions in "Reagent Preparation".

**NOTE:** One vial of Promega Trypsin Gold is sufficient for processing 16 samples.

2. Prepare Promega Trypsin Gold digestion mixture according to the table below and vortex to mix.

| Reagent                      | Volume for One Sample | Volume for 8 Samples* |
|------------------------------|-----------------------|-----------------------|
| Promega Trypsin Gold         | 5 µL                  | 44 µL                 |
| Ammonium Bicarbonate (50 mM) | 45 µL                 | 396 µL                |

\*Calculations include 10% excess in final volume.

4. Add 50 µL of Promega Trypsin Gold digestion mixture to the bead pellet.
5. Transfer the tubes into a preheated (37 °C) thermoshaker equipped with a heating block compatible with 1.5 mL microcentrifuge tubes.
6. Incubate at 37 °C for 18 hours with shaking at 1,000 rpm.

**NOTE:** Shorter digestion times will result in lower total peptide yields and an increased incidence of missed cleavages. However, reducing digestion to a minimum of 4 hours may result in an increased number of protein identifications.

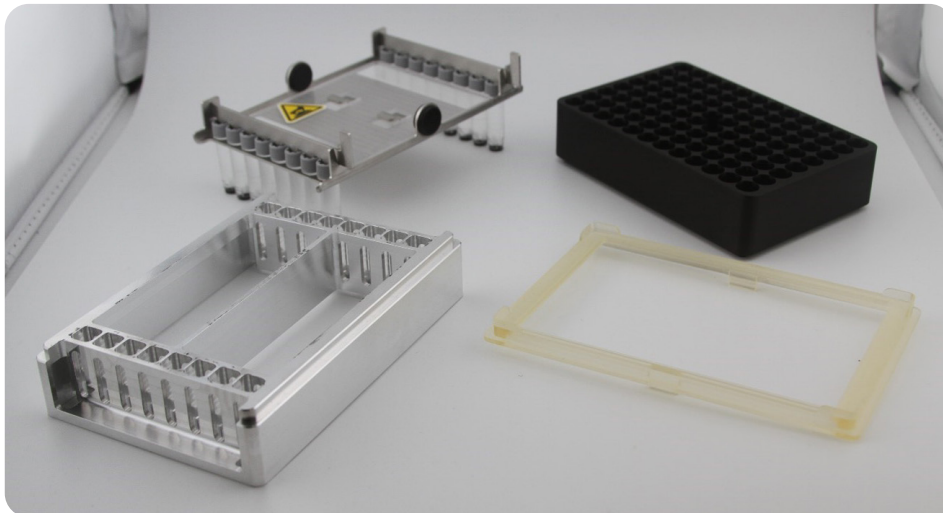
7. Transfer tubes from the thermoshaker to a magnetic separator compatible with 1.5 mL microcentrifuge tubes.

8. Wait for at least 2 minutes until the beads have settled onto the magnet.
9. Carefully open the tubes while on the magnet and transfer the supernatant to a LoBind tube or PCR tube and store appropriately to ensure sample stability.

**NOTE:** Peptide samples are ready for most downstream applications, and do not require additional purification or desalting treatments prior to analysis.

## Appendix A

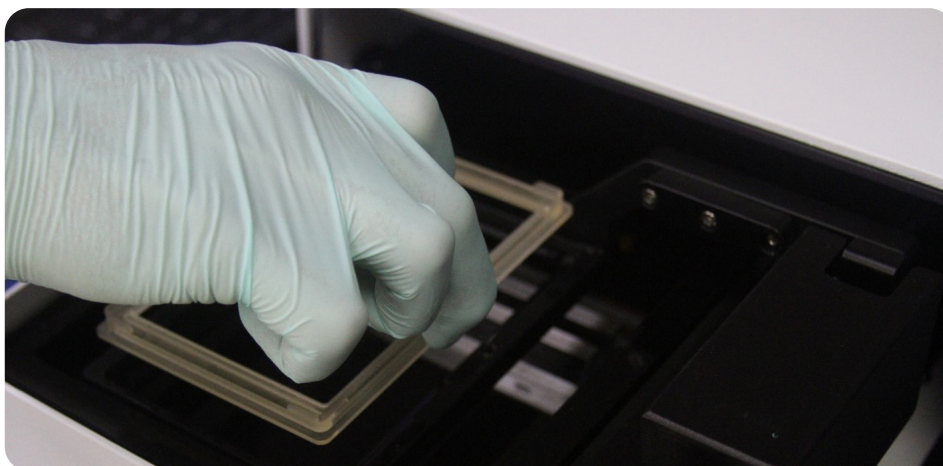
This section is to outline in greater detail the usage and setup of the truPREP Protein FFPE Hardware Accessory Kit (Manual) PN 500827. This hardware accessory kit is sold separately and is required for processing the Isolation section of the truPREP Protein FFPE kit. the adapter in place.



**Appendix Figure 1.** truPREP Protein FFPE Hardware Accessory Kit (Manual) PN 500827.

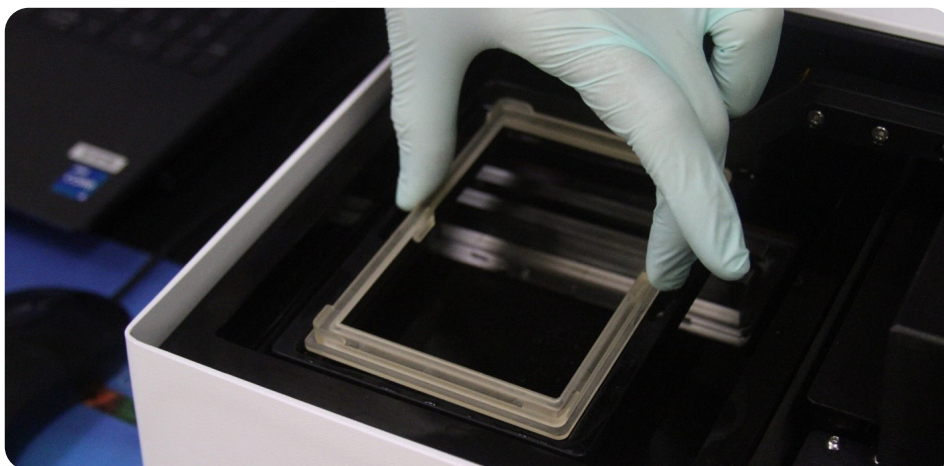
### prepTUBE 0.75 mL R230 Sample Holder Adapter (PN 500831)

- The preTUBE 0.75 mL R230 Sample Holder Adapter has Clip Features that allow it to snap into the Covaris R230.
- Once installed in the R230 this Sample Holder Adapter will remain in place for all AFA steps in the protocol.
- Clip features should face down into the water bath and the Adapter should be gently pushed into the sample arm of the instrument until the clips engage with the sample arm holding the adapter in place.
- The preTUBE 0.75 mL R230 Sample Holder (PN 500831) has Shuttle Alignment features to guide the prepTUBE 16 Shuttle & Shuttle Crossbars (Manual) (PN 500831) into position for AFA when placed onto the Rack. These features are located on the top of the Rack in all four corners.
- When inserting the sample holder into the Sample arm Shuttle Alignment features should face up away from water bath.



**Appendix Figure 2.** Insert The preTUBE 0.75 mL R230 Sample Holder Adapter into the R230.

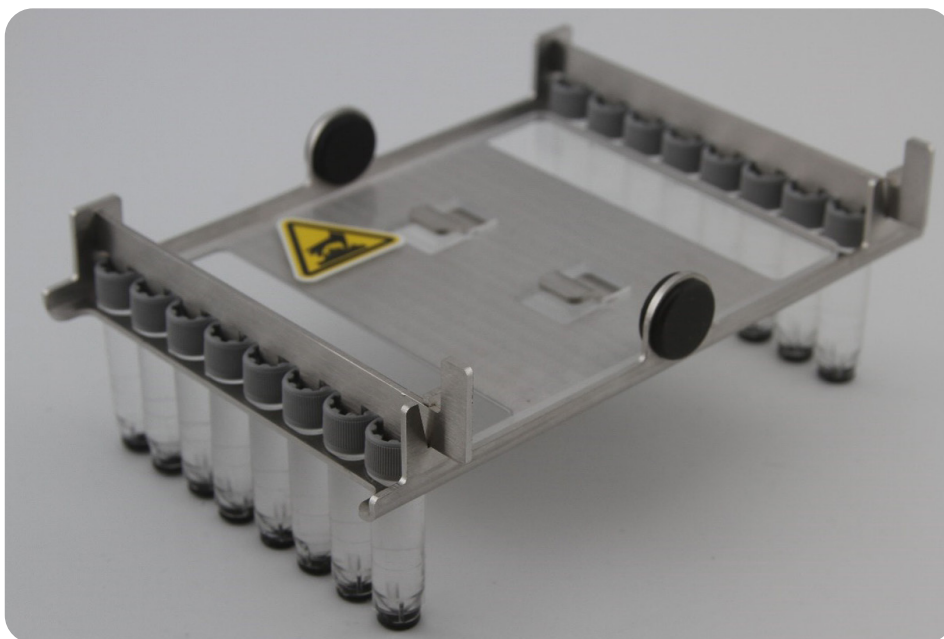




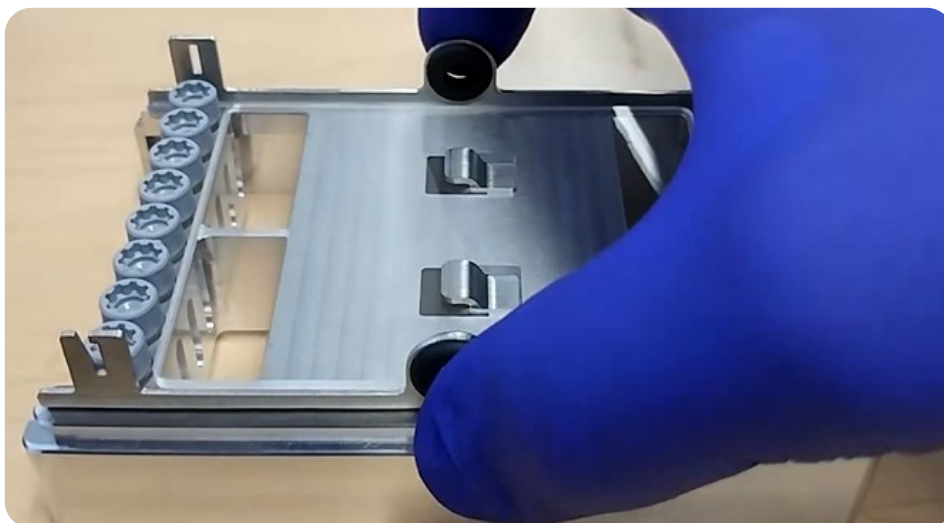
**Appendix Figure 3.** Ensuring sample holder alignment features are faced up away from the water bath.

#### **prepTUBE 16 Shuttle & Shuttle Crossbars (Manual) (PN 500830)**

- The prepTUBE 16 Shuttle & Shuttle Crossbars are utilized in this protocol as a means to house the prepTUBEs throughout the Isolation section of the protocol.
- The Shuttle crossbars should be used along with the prepTUBE 16 Shuttle when processing samples.
- Shuttle Crossbars used during Homogenization steps when on AFA Instrument.
- Shuttle Crossbars aligns tubes for transport and smooth insertions into the heat blocks.
- Shuttle crossbars should be removed before centrifugation and are not necessary during the heat block incubation steps.



**Appendix Figure 4.** prepTUBE 16 Shuttle & Shuttle Crossbars (Tubes included with kit not hardware accessory kit).



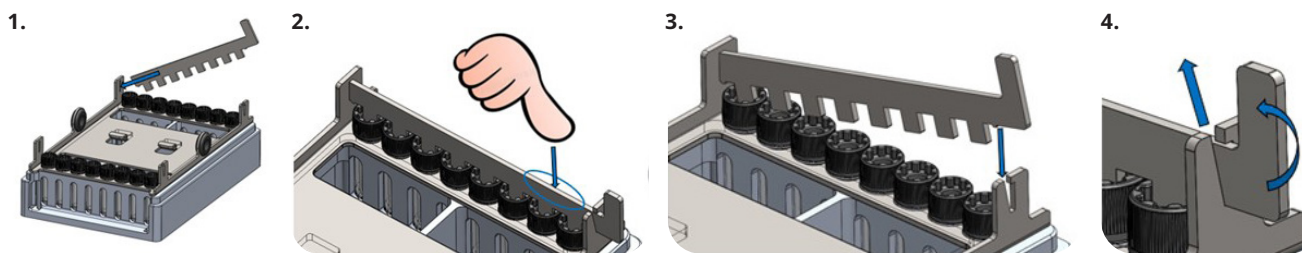
**Appendix Figure 5.** Gripper donuts for ease of use grabbing and moving the shuttle.



**Appendix Figure 6.** Gripper donuts for ease of use grabbing and moving the shuttle.



**Appendix Figure 7.** Attaching Shuttle Crossbar.



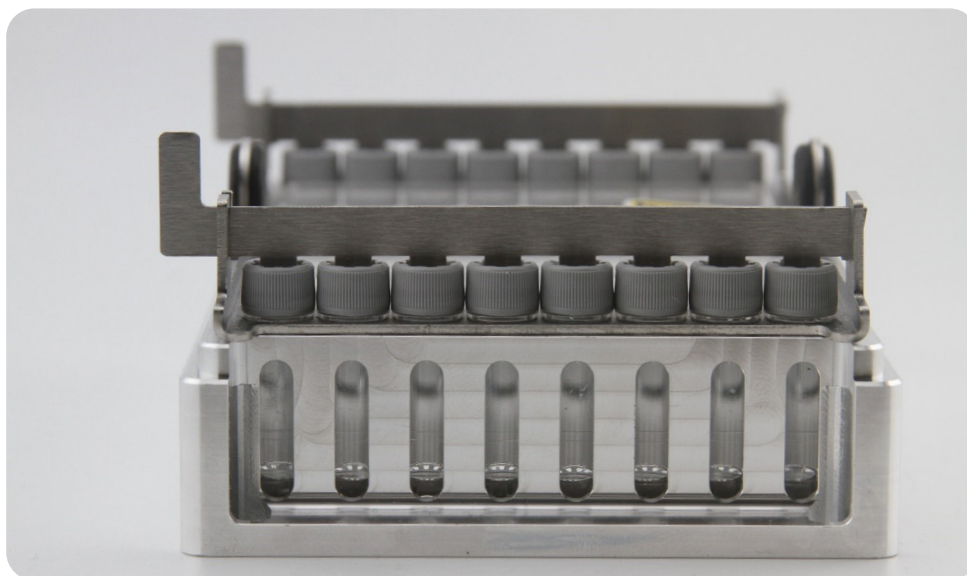
**Appendix Figure 8.** How to attach Shuttle Crossbars.

### **prepTUBE 0.75mL 16 Home rack (Manual) (PN 500829)**

The deparaffinization and extraction process is aided by a special accessory, the prepTUBE 16 Shuttle & Shuttle Crossbars (Manual) and prepTUBE 0.75 mL 16 Home Rack (Manual), which enables the processing of a minimum of 8 or 16 samples per run without need to remove individual tubes during pipetting. The open cavities on the side of the home rack allow for ease of use by removing the deparaffinization solution. This home rack allows a stable workstation in which to stabilize and organize your tube samples throughout the processing steps.



**Appendix Figure 9.** prepTUBE 0.75mL 16 Home rack (Manual) PN 500829.



**Appendix Figure 10.** prepTUBE 0.75mL 16 Home rack (Manual) PN 500829 with prepTUBE 16 Shuttle & Shuttle Crossbars (Manual) PN- 500830 and prepTUBEs.

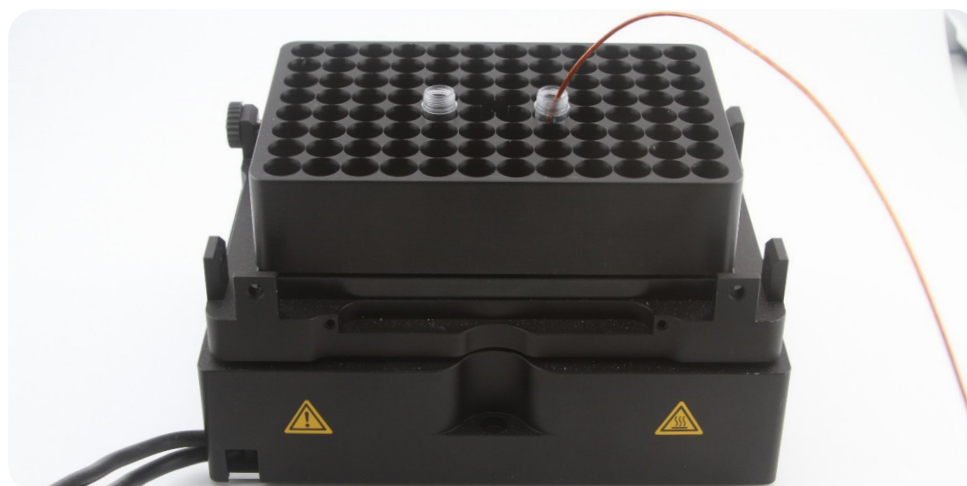


### prepTUBE 0.75mL Heat Block (PN 500828)

1. Provided with the truPREP Protein FFPE Kit – Isolation there are 2 additional prepTUBE TPX with bead to be utilized for equilibrating the prepTUBE 0.75 mL Heat Block.
2. Preheat the dry block heater equipped with prepTUBE 0.75 mL Heat Block to 56 °C and measure temperature in a prepTUBE TPX filled with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 56 °C  $\pm$  2 °C within the vessel.
3. Preheat the dry block heater equipped with the prepTUBE 0.75 mL Heat Block to 90 °C and measure temperature in a prepTUBE TPX with bead filled with water. Allow the temperature to equilibrate and adjust the heater until the final temperature is 90 °C  $\pm$  2 °C within the vessel.



**Appendix Figure 11.** prepTUBE 0.75 mL Heat Block PN: 500828.



**Appendix Figure 12.** prepTUBE 0.75 mL Heat Block PN: 500828 on QInstrument heater PN 2016-0100 (not included) calibrating heat block.

## Support and Technical Assistance

Tech Support: Ongoing assistance with the operation or application of the equipment and/or troubleshooting is provided via:

- **Telephone:**
  - US & APAC: +1 781.932.3959, during the hours of 8:30 a.m. to 5:00 p.m. (EST), Monday through Friday
  - EU: +44 (0) 845 872 0100, during the hours of 9:00 a.m. to 5:00 p.m. (GMT), Monday through Friday
- **E-mail:**
  - US Customer Service: [customerservice@covaris.com](mailto:customerservice@covaris.com)
  - EU/UK Customer Service: [emeacustomerservice@covaris.com](mailto:emeacustomerservice@covaris.com)
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  - Service and Instrumentation: [techsupport@covaris.com](mailto:techsupport@covaris.com)
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