PROTOCOL

Covaris

Dry Pulverization of Tissue using the cryoPREP™CP02

Contents

INTENDED USE

The cryoPREP CP02 Pulverizer is intended for use in research applications (RUO). This product is not intended for the diagnosis, prevention, or treatment of disease.

INTRODUCTION

The cryoPREP™ Extraction Systems have been designed to integrate the tissue processing workflow from point-of-collection flash freezing to biomarkers extraction with AFA™ Focused-ultrasonicators.

The cryoPREP dry pulverization increases the tissue surface area and breaks up the extra-cellular matrix thus improving extraction efficiency of biomarkers.

Combining cryoPREP dry tissue pulverization with AFA Focused-ultrasonication greatly enhances biomolecule extraction enabling increased sample yields, improved integrity, and greater complexity with reagents suited for downstream analytical methods.

A brief overview from the NCI Clinical Proteomic Tumor Analysis Consortium (CPTAC) revealed that the tissue pool of cryo-pulverized tumors prepared with cryoPREP when shared and analyzed from different facilities resulted in a very consistent result, such recommending its use in the Standard Operation Procedure of the project[1].

REVISION HISTORY

Part Number	Revision	Date	Description of change
010303	A	06/15	Release of tissue dry pulverization protocol using CP02
010303	B	07/17	CP02 Protocol update

[1] Tabb DL, Wang X, Carr SA., Reproducibility of Differential Proteomic Technologies in CPTAC Fractionated Xenografts.J Proteome Research (2016) Mar 4;15(3):691- 706 https://www.ncbi.nlm.nih.gov/pubmed/26653538

DESCRIPTION OF THE WORKFLOW

The biomarker extraction workflow is a two steps process:

1. Tissue dry cryo-pulverization with the cryoPREP CP02
2. Biomarker extraction with Covaris Adaptive Focused Acoustic (AFA) Technology.

Tissue dry Cryo-Pulverization

An overview of the workflow is explained in **Figure1**. The tissue should be loaded into the center of the tissueTUBE followed by treatment in liquid nitrogen to flash freeze the tissue. The flash frozen tissue can then be processed on the Covaris cryoPREP instrument to disrupt the extra-cellular matrix. The pulverized tissue material is then transferred into a Covaris AFA tube for AFA treatment.

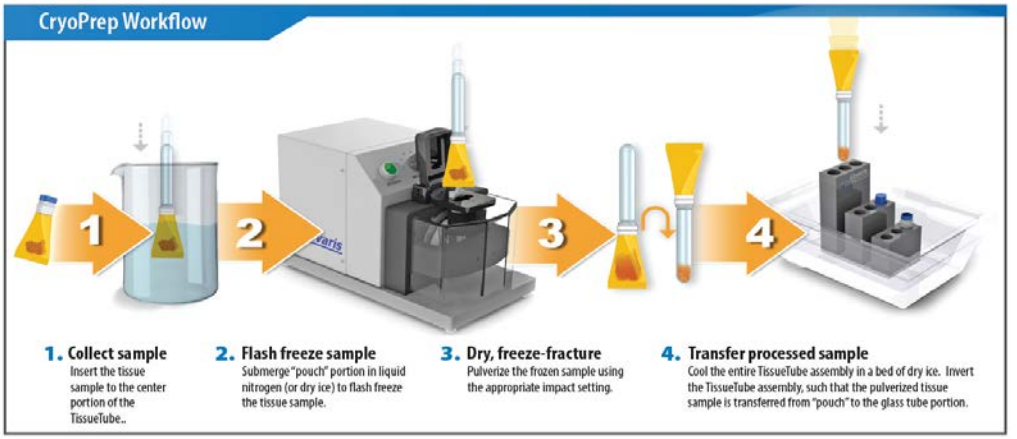
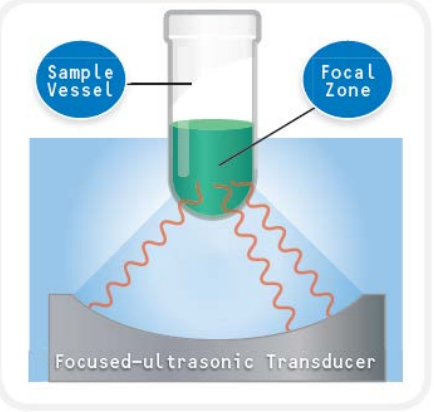


Figure 1: Overview of tissue sample preparation workflow using the Covaris cryoPREP

Biomarker extraction using AFA

Extraction of the target biomarkers is performed using patented Covaris Adaptive Focused Acoustic (AFA) technology. Different instruments are available depending of tissue mass and desired throughput. Please refer to Covaris website for details of instruments and capacities.



The AFA optimized reagents enhance protein extraction in native or denaturing buffers compatible with your downstream analytical techniques.

SAMPLE INPUT REQUIREMENTS

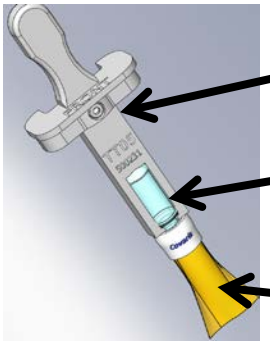
The Covaris single-use closed-tube, low temperature preparation system is compatible to work with both soft and hard tissue types. The cryoPREP CP02 can reproducibly pulverize tissue samples and plant materials. In addition to pulverizing samples, the cryoPREP CP02 aides in the transfer of the pulverized material to a homogenization vessel while at cryogenic temperatures.

MATERIALS REQUIRED

	Name	Description	Selection Guide
Tissue Dry Cryo-Pulverization	cryoPREP CP02	cryoPREP is an automated apparatus that delivers repeatable mechanical force to cryofracture flash frozen samples. This system comprises of four components: tissueTUBE, tissueTUBE holder, tissueTUBE plug and transfer tube.	PN 500001 (110V) PN 500000 (220V)
	tissueTUBE	tissueTUBE is used to cryo-pulverize tissue in the cryoPREP. Multiple options are available depending on tissue type and mass.	Table 1 below
	tissueTUBE Holder	This is a required accessory to hold tissueTUBE into the cryoPREP	Table 1 below
	Transfer Tube	Transfer tube should be connected to the tissueTUBE. Sample is transferred into the transfer tube after cryo-pulverization in the tissueTUBE. Multiple options ae available depending on the tissueTUBE and buffer volumes.	Table 2 below
	Preparation Station	This is designed for loading and stacking the transfer tubes and tissueTUBEs in an orderly manner.	tissueTUBE TT2 Cold Station (PN 500166) tissueTUBE TT1 Cold Station (PN 520157) tissueTUBE TT05 Cold Station (PN 500249)
	tissueTUBE Plug	If cryo-pulverized tissue is stored in the tissueTUBE then a plug should be used.	Table 1 below

Biomarker Extraction	truXTRAC Protein Extraction Buffers	truXTRAC protein extraction buffers are versatile, convenient and compatible with various downstream applications. The four different protein buffers are: - truXTRAC Protein Extraction Buffer N - truXTRAC Protein Extraction Buffer SuperB - truXTRAC Protein Extraction Buffer DF - truXTRAC Protein Extraction Buffer TP	Table 4 below
	Covaris AFA Focused-ultrasonicator	AFA is a highly controllable acoustic based non-contact and isothermal process that preserves native protein conformation and biological activity in the presence of appropriate buffers.	Contact us
	Holder/ Rack for Covaris AFA instrument	These are specially designed tools for holding the microTUBE transfer tube in a specific position for the AFA Focused-ultrasonicator process to happen efficiently	Tables 3a and 3b below

1. Illustration of components of tissue dry pulverization workflow



2. Selection of tissueTUBE and tissueTUBE Accessories

Use the Table 1 to choose appropriate tissueTUBE based on tissue mass.

Sample mass	tissueTUBE	tissueTUBE Plug	tissueTUBE Insertion Tool	tissueTUBE Holder
< 50 mg (*)	TT05M (PN 520139)	TT05M-P (PN 520141)	TT05 (PN 500231)	TT1 (PN 500095)
< 50 mg of hard tissue (heart, muscle, kidney) (*)	TT05M-XT (PN 520140)	TT05M-P (PN 520141)	TT05(PN 500231)	TT1 (PN 500095)
(*) Legacy TT05 (PN 520071) and TT05XT (520072) are also available for this application, please contact applicationsupport@covaris.com				
< 500 mg	TT1 (PN 520001)	TT1-P (PN 520006)	TT1 (PN 500159)	TT1 (PN 500095)
< 500 mg hard tissue (bone, seeds, tablets, plant material)	TT1-XT (PN 520007)	TT1-P (PN 520006)	TT1 (PN 500159)	TT1 (PN 500095)
500 mg - 2 g	TT2 (PN 520021)	TT2-P (PN 520023)	N/A	TT2 (PN 500096)

Table 1

3. Selection of transfer Tube

Use the Table 2 to choose appropriate transfer tube based on extraction buffer volume.

tissue TUBE	Buffer volume	Transfer Tube (PN)	tissueTUBE Adapter*	tissueTUBE Insertion Tool	tissueTUBE Holder
TT05M (PN 520139) or TT05M-XT (PN 520140)	300µl-1.0ml	milliTUBE 1ml (PN 520128)	N/A	TT05 (PN 500231)	TT1 (PN 500095)
	1.0ml-2.0ml	milliTUBE 2ml (PN 520132)	N/A	TT05 (PN 500231)	TT1 (PN 500095)
TT1 (PN 520001) or TT1-XT (PN 520007)	300µl-1.0ml	milliTUBE 1ml (PN 520128)	TT1ADPM (PN 520142)	TT1 (PN 500159)	TT1 (PN 500095)
	1.0ml-1.5ml	15*19mm TC15 (PN 520048)	TT1ADP13 (PN 520017)	TT1 (PN 500159)	TT1 (PN 500095)
	1.0ml-2.0ml	milliTUBE 2ml (PN 520132)	TT1ADPM (PN 520142)	TT1 (PN 500159)	TT1 (PN 500095)
	1.0ml - 4.0ml	13*65mm TC13 (PN 520010)	TT1ADP13 (PN 520017)	N/A	TT1 (PN 500095)
	1.0ml - 12ml	16*100mm TC 16 (PN 520011)	N/A	N/A	TT1 (PN 500095)
TT2 (520021)	5ml - 25ml	20*125mm TC 20 (PN 520012)	N/A	N/A	TT2 (PN 500096)

(*) Adapter is only required for the TT1 and TT1XT tissueTUBEs. Not required for TT05M, TT05M-XT or TT2 tissueTUBEs.

Table 2

4. Selection of Holders/Racks Required for AFA treatment

Use Tables 3a and 3b to choose the appropriate holder and rack based on the instrument.
For processing single samples follow Table 3a and for processing multiple samples follow Table 3b.

Transfer Tube Name and Part Number	M220 Holder & Insert	S-Series Holder
milliTUBE 1mL (PN 520128)	(PN 500414) & (PN 500422)	Holder milliTUBE 1 ml (PN 500371)
milliTUBE 2mL (PN 520132)	NA	Holder milliTUBE 2 ml (PN 500375)
13*65mm TC13 (PN 520010)	NA	Holder 13x65mm Tube (PN 500011)
16*100mm TC16 (PN 520011)	NA	Holder 16x100mm Tube (PN 500012)
20*125mm TC20 (PN 520012)	NA	Holder 20x125mm Tube (PN 500051)

Table 3a

Transfer Tube Name	ME220 Holder & Waveguide	E220 evolution Rack	E220 Rack	LE220 Rack
milliTUBE 1mL (PN 520128)	(PN 500520) & (PN 500534)	Rack E220e 4 Place milliTUBE 1 ml (PN 500431)	Rack 24 Place milliTUBE 1 ml (PN 500368)	Rack 24 Place milliTUBE 1 ml (PN 500368)
milliTUBE 2mL (PN 520132)	NA	NA	Rack 24 Place milliTUBE 2 ml (PN 500376)	Rack 24 Place milliTUBE 2 ml (PN 500376)
13*65mm TC13 (PN 520010)	NA	NA	NA	Rack 24 place (PN 500033)
16*100mm TC16 (PN 520011)	NA	NA	NA	Rack 12 place (PN 500031)
20*125mm TC20 (PN 520012)	NA	NA	Rack 12 Place (PN 500032)	NA

Table 3b

5. Selection of truXTRAC Protein Extraction Buffers

Use the following table to choose the appropriate extraction buffer based on the downstream application

	Native Protein Extraction				Denaturing Protein Extraction			
Protein Extraction Buffer	**Enzyme Activity**	**IP**	**ELISA**	**NATIVE PAGE**	**SDS PAGE**	**IEF**	**2D-GE**	**LC/MS**
truXTRAC Buffer N (PN 520099)	++	++	+++	++	+	-	+	++
truXTRAC Buffer SuperB (PN 520112)	-	-	-	-	+++	-	++	+++
truXTRAC Buffer DF (PN 520093)	-	-	-	-	++++	+++	+++	++++
truXTRAC Buffer TP (PN 520103)	-	-	-	-	++	++++	++++	-

(+) = Applicable (++) = Recommended (+++) = Highly Recommended (-) = Not Applicable

Table4

For more details about the truXTRAC buffer and its compositions please click on the link below.
http://covaris.com/resources/protocols/#toggle-id-6

Safety Data Sheets: http://covaris.com/resources/safety-data-sheets

STORAGE

The cryoPREP CP02 components may be stored at room temperature.
Ambient temperature range: 19°C to 25°C (66°F to 77°C)
Ambient humidity range: 30% to 70%

OTHER SUPPLIES

- Dry Ice
- Liquid Nitrogen

PROTOCOL

I. Tissue Dry Pulverization

1. **Load Sample into the tissueTUBE –** Using gloved hands, pinch the bottom 1/3rd of the tissueTUBE, and insert the sample through the top opening of the tissueTUBE as illustrated in Figure 2, using forceps or tweezers. Place the sample 1/3 of the way up the bottom of the tissueTUBE in the center away from the edges.

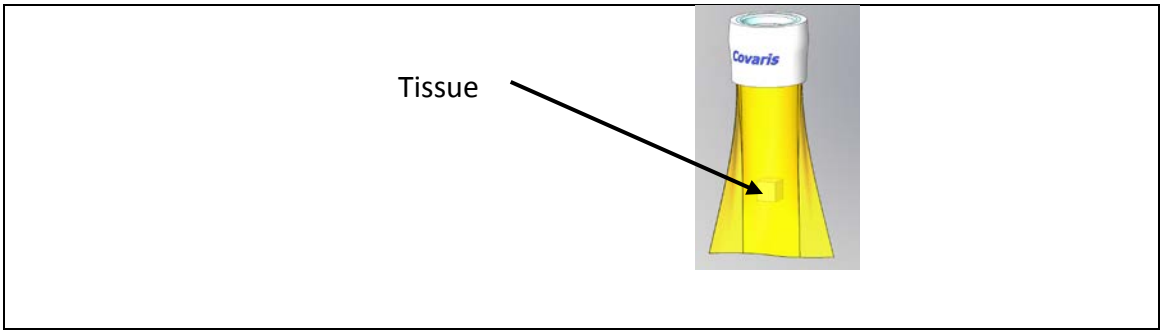


Figure 2 - tissueTUBE with frozen tissue

2. **Attach Transfer Tube –** If the tissueTUBE was sealed with a plug, replace the plug with a transfer tube. When using the TT05, TT1 or TT2 tissueTUBEs, screw in the tubes all the way, and then open one-quarter turn to allow venting during cryofracturing.

NOTE: Transfer tubes have to be loosened to prevent pressure build up.

3. **Pre-chill the tissueTUBE-Transfer Tube complex –** Label the tissueTUBE before placing it on dry ice. If using the Prep Station, the larger transfer tubes (TC 16 or TC 20) can be pre-chilled by placing them into the second position in the Prep Station shown in Figure 3.

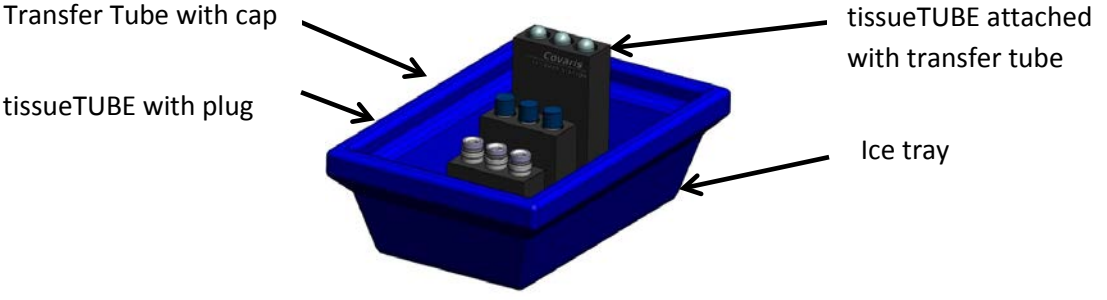


Figure 3 – Prep station set up

4. **Snap Freeze the sample –** While holding the transfer tube or the insertion tool (for TT05 and some transfer tubes with TT1 only), freeze the sample by immersing the tissueTUBE into liquid nitrogen for 30-90 seconds depending on tissue mass. When using liquid nitrogen, dip only the sample pouch and avoid dipping the cap or transfer tube. Incubation longer than 30 seconds in liquid nitrogen will sometime cause trapped air to become liquefied. Once you take the tissueTUBE out of the liquid nitrogen, please wait 3-5 seconds before inserting into the cryoPREP to allow for the liquefied air to evaporate prior to loading into the cryoPREP.

NOTE: Before processing samples, test labels for durability at cryogenic temperatures

5. **Load into cryoPREP –** Open the cryoPREP lid and quickly insert the previously frozen tissueTUBE into the cryoPREP. The pouch will slide down into the sample holder until it reaches an internal "shelf." This "shelf" ensures the sample is aligned in the impact zone of the tissueTUBE.

NOTE 1: Use the TT05 Insertion Tool (PN 500231) for the milliTUBE 1 ml and 2 ml transfer tubes when using the TT05M and TT05M-XT tissue bags.

NOTE 2: When using milliTUBE 1 ml and 2 ml with the TT1 and TT1XT bags use TT1 Insertion Tool (PN 500159) and adapters described in **Table 2**.

6. **Operate cryoPREP to deliver impact –** Close the cover, using Table 6 below, select the desired impact level (1 to 6) based on the recommendations from the cryoPREP Tissue Impact Chart below, and press green "ACTIVATE" button. The cryoPREP hammer will impact and pulverize the sample. Upon impact, most tissues will flatten. The tissueTUBE flexible pouch is designed to withstand one impact.

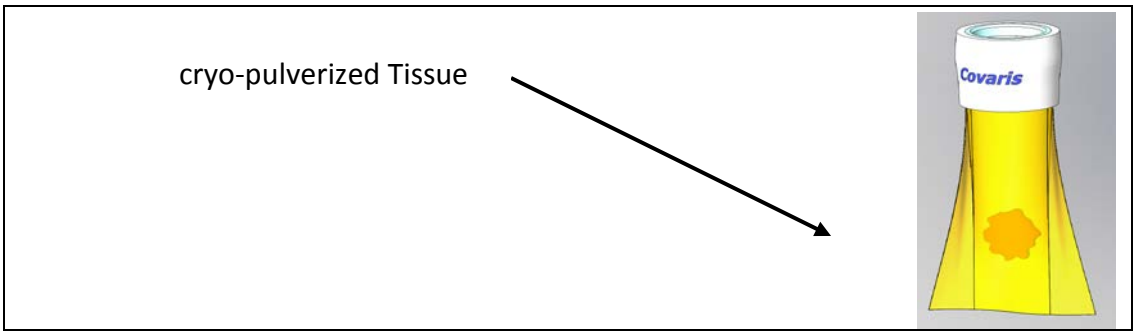


Figure 4 – Pulverized tissue

If a second impact is needed to pulverize a sample, please employ the TT1XT or TT05M-XT tissueTUBEs. After the first impact, carefully inspect the tissueTUBE for punctures. If a puncture is identified, transfer the sample to a new pouch before attempting a second impact. Re-freeze the sample before delivering the second impact. The TT1XT and TT05M-XT are designed to withstand up to three impacts under normal conditions. More than three impacts are not recommended.

NOTE: After each impact re-freeze the sample by dipping the tissueTUBE in to liquid nitrogen.

	TT05M/TT05M-XT	TT1/TT1XT		TT2	
Mass range / Tissue Type	15 - 50mg	50 - 300mg	300-500mg	500-1000mg	1000-2000mg
Brain	1	2	3	4	6
Liver	2	3	3	4	6
Kidney	3	4	4	6	6
Muscle	4	4	4	6	6
Heart	5	5	5	6	6
Ocular tissue	5	6	6	6	6
Vascular tissue	5	6	6	6	6
Skin	6	6	6	6	6
Tumor	5	5	5	6	6
Bone	6	6	6	6	6
Cartilage	6	6	6	6	6

Table 6 - cryoPREP Tissue Impact Chart

7. **Remove tissueTUBE with pulverized sample –** Raise the lid and grasp the transfer tube or handle to remove the tissueTUBE. Keep the tissueTUBE with the pulverized sample on the bottom. Using your fingers quickly snap the tissueTUBE to disperse the flattened tissue. If you notice larger than desired portions of tissue remaining intact, repeat steps 4, 5 and 6.

NOTE: tissueTUBEs may be used as a storage vessel by re-attaching the plug.

8. **Transfer pulverized sample to transfer tube –** Using your fingers, quickly snap the tissueTUBE to disperse the flattened tissue. Immediately invert the tubes so the tissueTUBE is on top and tap/flick the tissueTUBE to transfer the tissue particles into the bottom of the transfer tube. This step should be done quickly to avoid any melting and adhesion of sample to tube walls. Alternatively, the buffer can be added to the tissue bag and suspended sample transferred into the tube using a pipette with a wide bore.

9. **Store sample –** Unscrew the tissueTUBE from the Transfer tube and affix the cap to seal the transfer tube. Discard the tissueTUBE and its cap (if used) appropriately.

2. Biomarker Extraction

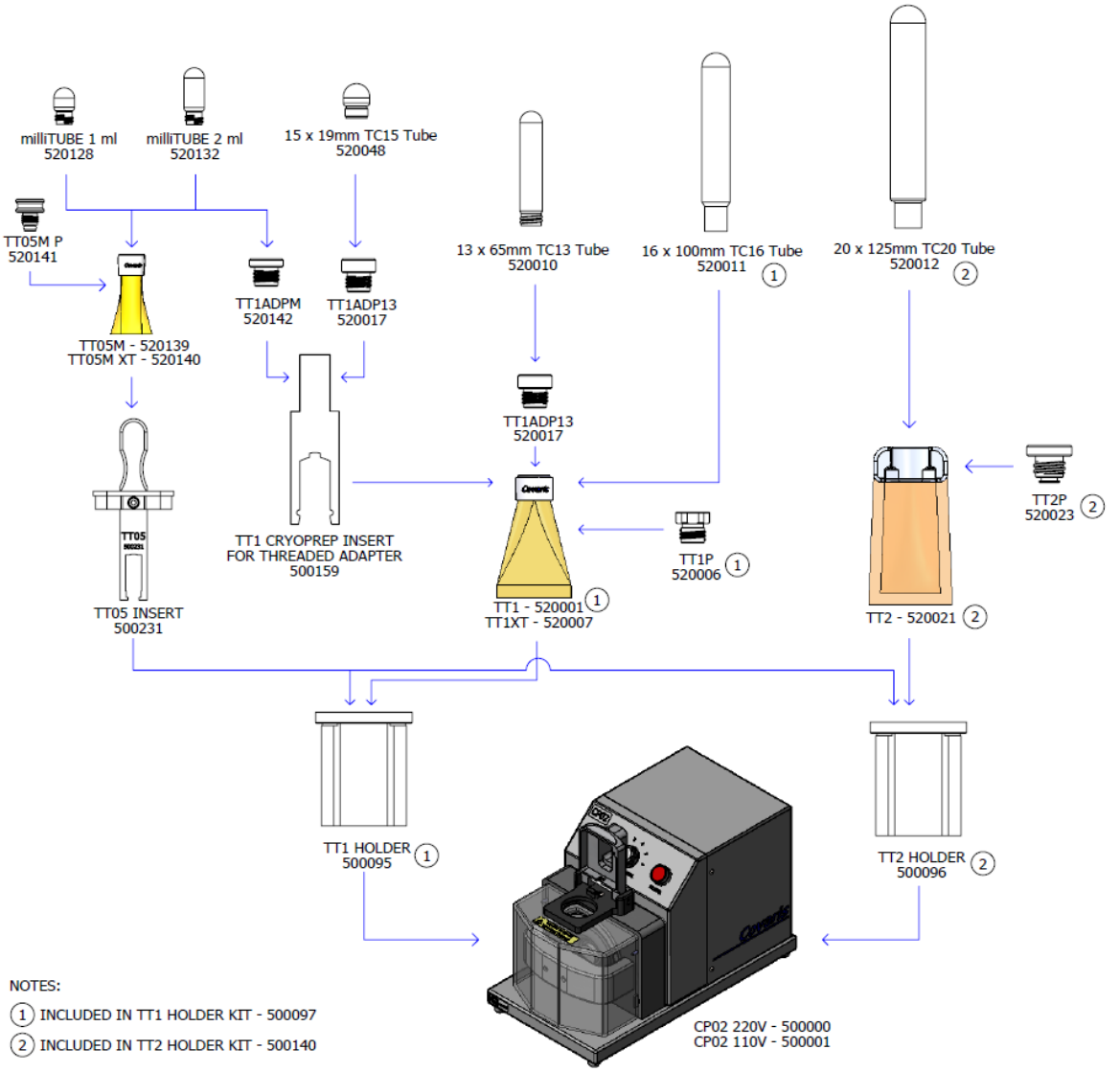
1. **Process Sample –** Add the appropriate extraction buffer to the transfer tube and place it in Covaris AFA instrument of interest.

> **NOTE:** Return pulverized samples to a cryogenic temperature until ready for AFA treatment to avoid sample degradation and adhesion to tube wall. RNA extraction requires immediate attention.

2. **Protocols and Methods –** Follow the recommended AFA processing method based on the bio- molecules of interest.
   Please click on the link below to access the protocols, method section and User Manual for any instrument of interest.
   http://covaris.com/resources/protocols/

SUPPLEMENTAL MATERIAL

Appendix A: Schematic Diagram of cryoPREP and consumables



PUBLICATIONS

1. Tabb DL,Wang X,Carr SA., Reproducibility of Differential Proteomic Technologies in CPTAC Fractionated Xenografts.J Proteome Research (2016) Mar 4;15(3):691- 706 https://www.ncbi.nlm.nih.gov/pubmed/26653538
2. Kuan-lin Huang,Shunqiany li,PhilipMertins.,Tabb DL,Wang X,Carr SA., Proteogenomic integration reveals therapeutic targets in breast cancer xenografts.Nature communications (2017) Mar 28.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5379071
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4. Laura Arrigoni, Andreas S. Richter., Standardizing chromatin research: a simple and universal Method for ChIP-seq. Nucleic Acids Research,2016,Vol.44 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4838356/pdf/gkv1495.pdf
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6. J Tammam, C Ware., Down-regulation of the Notch pathway mediated by a γ-secretase inhibitor induces anti-tumour effects in mouse models of T-cell leukaemia. Br J Pharmacol. 2009 November; 158(5): 1183-1195 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2782329/

CITATION AND REFERENCES

1. Please click on the link below to see publications relating to tissue disruption and homogenization using cryoPREP http://covaris.com/resources/publications/#toggle-id-7
2. Please click on the link below to see publications relating to analytical mass spec measurement after tissue disruption http://covaris.com/resources/publications/#toggle-id-8
3. Please click on the link below to see publications relating to Biomarker discovery from plant material http://covaris.com/resources/publications/#toggle-id-9
4. Please click on the link below to see publications relating to release of toxic compounds from tissues. http://covaris.com/resources/publications/#toggle-id-10
5. Please click on the link below to see publications relating to small molecules and metabolite release from tissues. http://covaris.com/resources/publications/#toggle-id-25