Immune Tolerance Network

# ITN091AI VIBRANT: Urine Processing for Single Cell Analysis and Proteomics Standard Operating Procedure

**Instructions:** Study personnel must be trained and authorized by the site Principal Investigator to perform urine processing. Personnel are required to read and understand the following **ITN091AI VIBRANT Urine Processing for Single Cell Analysis and Proteomics Standard Operating Procedure**. The following procedures MUST be followed for all specimens.

## 1. PURPOSE

To describe the procedure for processing and freezing urine cells for single cell analysis and urine supernatant for proteomics.

## 2. REQUIRED SUPPLIES

### *Items Supplied by ITN:*

- CryoStor CS10 (BioLife Solutions, #210102)
- TheraPEAK X-VIVO-10 without phenol red and gentamicin (Lonza, #BEBP02-055Q)
- cOmplete Protease inhibitor tablets (Roche, #05892970001)
- Sterile 2 mL cryovials
- Sterile Conical Tubes (50 mL)
- Sterile Conical Tubes (15 mL)
- Mr. Frosty Container (filled with isopropanol and pre-chilled at 4°C for at least 4 hours)

### *Items Supplied by Clinical Sites:*

- Gloves, goggles, lab coat
- Alcohol-proof black marker for labeling tubes
- 70% Ethanol
- Sterile Urine Collection Cup
- Sterile disposable transfer pipettes
- Sterile Serological Pipettes (1 mL, 10 mL, and 25 mL)

- Ice bucket with wet ice
- Isopropanol

### *Equipment Supplied by Clinical Sites:*

The following laboratory equipment is required for urine processing.

- Serological Pipettors
- BSL 1-2 Safety Cabinet
- -70 to -80°C Mechanical Freezer (preferably on back-up power and monitored for maintenance of temperature)
- 4°C Refrigerator
- Flammable Safety Cabinet for Isopropanol Storage (preferred)
- Swinging-bucket centrifuge capable of accommodating 50 mL tubes (4°C preferred)
- Microcentrifuge capable of accommodating 2 mL cryovials (4°C preferred)
- Vortex (preferred but not required)

## 3. PROCEDURES

### *General Precautions*

- Informed consent must be obtained from all participants before any research procedures, including specimen collection, are performed.
- Always follow appropriate precautions for handling of human specimens, including appropriate use of personal protective equipment.
- Perform all processing in a BSL 1-2 Safety Cabinet.
- Always use sterile technique.
- Always prepare materials and workspace by wiping with 70% ethanol.
- Before working with isopropanol, individuals should be trained in its proper handling and storage. For reference: http://www.labmanager.com/lab-health-and-safety/2008/12/working-with-isopropyl-alcohol?fw1pk=2#.VsuyffkrLIU.

### *Storage of Reagents*

- Store all reagents at 4°C, unless otherwise noted.
- X-VIVO-10 and CryoStor CS10 are cGMP-grade solutions. Use sterile technique at all times. This

includes minimizing exposure of solution to environmental contaminants.

- Be sure the Mr. Frosty container is filled with isopropanol and pre-chilled at 4°C for at least four hours before use. Note that the isopropanol in the Mr. Frosty should be replaced after every five uses, per manufacturer's recommendation.

- Isopropanol: The compound should be stored in a tightly closed container in a cool, dry, well-ventilated area away from incompatible substances. It should be kept away from heat, sparks, flames, and other sources of ignition, as well as strong oxidizers, acetaldehyde, chlorine, ethylene oxide, acids, and isocyanates. Isopropyl alcohol is highly flammable and can easily ignite. Vapors may form explosive mixtures with air, traveling to a source of ignition and flash back. Use of water spray to fight fires may be inefficient. A flammable safety cabinet is the best storage option.

### *Preparation for Processing Procedure*

- There are three urine processing procedures designated as Procedure A, Procedure B, and Procedure C. The appropriate procedure differs by visit and is specified in the table below. The table also specifies the number of tubes required per visit (included in the visit-specific collection kit) as well as the volume of urine supernatant required for each processing type.

| **Week** | | **0** | **4** | **8** | **10** | **11** | **12** | **14** | **18** | **22** | **26** | **30** | **34** | **38** | **48** | **60** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Visit** | **-1** | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **U** | **DSC** |
| Volume "neat" urine super (mL) | | 10 | | | 20 | | 10 | | 10 | | 10 | | 10 | 10 | 10 | 10 | | 10 |
| Volume "protease" urine super (mL) | | 10 | | | 10 | | | | 10 | | 10 | | | 10 | 10 | 10 | | 10 |
| Urine cell pellet | | x | | | x | | | | x | | x | | | x | x | x | | x |
| **Follow Urine Processing Procedure** | | **A** | | | **B** | | **C** | | **A** | | **A** | | **C** | **A** | **A** | **A** | | **A** |

- For urine processing procedure A:
  - For initial urine processing:
    - 4 x 50 mL conical tubes (two labeled "centrifugation" and two labeled "supernatant")
  - For urine supernatant:
    - 1 x 15 mL conical tube labeled "supernatant + protease inhibitor"
    - 10 x 2 mL cryovials labeled "neat"
    - 10 x 2 mL cryovials labeled "protease"
  - For cell pellet:
    - 2 x 2 mL cryovials labeled "pellet"

- For urine processing procedure B:
  - For initial urine processing:
    - 4 x 50 mL conical tubes (two labeled "centrifugation" and two labeled "supernatant")
  - For urine supernatant:
    - 1 x 15 mL conical tube labeled "supernatant + protease inhibitor"
    - 20 x 2 mL cryovials labeled "neat"
    - 10 x 2 mL cryovials labeled "protease"
  - For cell pellet:
    - 2 x 2 mL cryovials labeled "pellet"

- For urine processing procedure C:
  - For initial urine processing:
    - 4 x 50 mL conical tubes (two labeled "centrifugation" and two labeled "supernatant")

- For urine supernatant:
  - 10 x 2 mL cryovials labeled “neat”

### *Collection of Urine Specimen*

1. Urine will be collected prior to renal biopsy at applicable visits.

2. Obtain midstream urine in a pre-labeled sterile urine collection cup (must not be first morning void). A minimum of 15 mL of urine is required. A maximum of 90 mL of urine may be used.

3. Place on ice until processed. Processing should occur immediately. All processing procedures will be done on ice.

## *Urine Processing Procedure A (For Visit 0, 7, 9, 12, 13, 14, DSC)*

### *Initial Urine Processing Procedure*

a. Swirl the urine in the collection cup to suspend particles that have dropped to the bottom.

b. By pouring, split the total urine volume (15-90 mL) equally into two sterile 50 mL conical tubes labeled “centrifugation”.

c. Spin the two conical tubes at 200g for 10 minutes in swinging-bucket centrifuge (4°C, if available).

d. Using a sterile 25 mL serological pipet, carefully transfer urine supernatants into 1 (for volumes ≤ 40 mL) or 2 (for volumes > 40 mL) clean sterile 50 mL conical tube(s) labeled “supernatant”, being careful not to disturb the pellets.

e. Keep both the supernatant and cell pellets on ice for further processing.

### *Urine Supernatant Processing Procedure*

1. Urine supernatant will be processed two ways: **10 mL** without protease inhibitor (“neat”) and **10 mL** with protease inhibitor (“protease”). If there is < 20 mL of urine supernatant, fill the “neat” urine supernatant cryovials first and use any remaining sample for the protease inhibitor-treated cryovials.

**For the “neat” urine supernatant without protease inhibitor:**

2. Using supernatant from step 5 above (Initial processing procedure), with a sterile 1 mL serological pipet, transfer 1 mL of the urine supernatant into each of 10 pre-labeled sterile 2 mL “neat” cryovials.

3. Freeze the labeled cryovials at -70 to -80°C until shipment.

**For the protease inhibitor-treated urine supernatant:**

4. Using a sterile 10 mL serological pipet, transfer 11 mL of urine supernatant (from Step 5 above) from the 50 mL conical to a sterile 15 mL conical tube labeled “supernatant + protease inhibitor”. Keep on ice.

5. Push one protease inhibitor tablet from the foil packaging directly into this 15 mL conical tube.

6. Vortex the tube or shake vigorously until the tablet has dissolved.

7. Using a sterile 1 mL serological pipet, transfer 1 mL of the protease inhibitor-treated urine supernatant into each of 10 pre-labeled sterile 2 mL "protease" cryovials.

8. Freeze the labeled cryovials at -70 to -80°C until shipment.

### *Urine Cell Pellet Cryopreservation Procedure*

1. Using a sterile 1 mL serological pipet, re-suspend each urine pellet from Step 5 of the Initial Processing Procedure in 0.5 mL ice-cold X-VIVO-10 by gently pipetting up and down five times, making sure to wash down any sediment on the sides of the tube. Transfer each re-suspended cell pellet to a pre-labeled "pellet" 2 mL cryovial (two total).

2. Using a sterile 1 mL serological pipet, add an additional 0.5 mL of ice-cold X-VIVO-10 to each 50 mL centrifuge tube to recover residual cells. Be sure to rinse down the sides of the tube to recover all cells and transfer to the same 2 mL "pellet" cryovials as in step 1 above.

3. Discard the 50 mL conical tubes.

4. Centrifuge the cryovials in a micro-centrifuge at 200g for 5 minutes (at 4°C, if available).

5. Using a disposable transfer pipette, remove and discard as much supernatant as possible without disturbing the cell pellet.

6. Using a sterile 1 mL serological pipet, add 1 mL ice-cold X-VIVO-10 to each cell pellet, re-suspend by gently pipetting up and down five times.

7. Centrifuge the cryovials in a micro-centrifuge at 200g for 5 minutes (at 4°C, if available).

8. Using a disposable transfer pipette, remove and discard as much supernatant as possible without disturbing the cell pellet.

9. Wipe down the outside of the CryoStor CS10 cryopreservation media container with 70% ethanol or isopropanol before opening.

10. Using a sterile 1 mL serological pipet, add 0.25 mL ice-cold CryoStor CS10 to each cell pellet and re-suspend by gently pipetting up and down five times.

11. Place the cryovials in a Mr. Frosty container (filled with isopropanol and pre-chilled at 4°C for at least four hours) and place the Mr. Frosty directly into a -70 to -80°C freezer.

12. After 15 minutes, slap the Mr. Frosty container to facilitate nucleation, and return the Mr. Frosty to the -70 to -80°C freezer for a minimum of 24 hours and maximum of 7 days.

13. Transfer the cryovials from the Mr. Frosty to a freezer box and store in the -70 to -80°C freezer until shipment.

## *Urine Processing Procedure B (For Visit 3 only)*

### *Initial Urine Processing Procedure*

1. Swirl the urine in the collection cup to suspend particles that have dropped to the bottom.

2. By pouring, split the total urine volume (15-90 mL) equally into two sterile 50 mL conical tubes labeled "centrifugation".

3. Spin the two conical tubes at 200g for 10 minutes in swinging-bucket centrifuge (4°C, if available).

4. Using a sterile 25 mL serological pipet, carefully transfer urine supernatants into 1 (for volumes ≤ 40 mL) or 2 (for volumes > 40 mL) clean sterile 50 mL conical tube(s) labeled "supernatant", being careful not to disturb the pellets.

5. Keep both the supernatant and cell pellets on ice for further processing.

### *Urine Supernatant Processing Procedure*

1. Urine supernatant will be processed two ways: **20 mL** without protease inhibitor ("neat") and **10 mL with protease inhibitor** ("protease"). If there is not enough urine supernatant, fill cryovials in this order:
    a. First priority = 10 cryovials with 1 mL "neat" supernatant
    b. Second priority = 10 cryovials with 1 mL protease inhibitor-treated supernatant
    c. Third priority = 10 additional cryovials with 1 mL "neat" supernatant

**For the "neat" urine supernatant without protease inhibitor:**

2. Using supernatant from Step 5 above (Initial processing procedure), with a sterile 1 mL serological pipet, transfer 1 mL of the urine supernatant into each of **10** pre-labeled "neat" sterile 2 mL cryovials.

3. Using a sterile 10 mL serological pipet, transfer 11 mL of urine supernatant from Step 5 above from the 50 mL conical to a sterile 15 mL conical tube labeled "supernatant + protease inhibitor". Keep on ice until Step 6 below for the protease inhibitor-treated urine supernatant.

4. Using the remaining supernatant from Step 5 above in the 50 mL conical tube, with a sterile 1 mL serological pipet, transfer 1 mL of the urine supernatant into each of **10** pre-labeled "neat" sterile 2 mL cryovials.

5. Freeze the labeled cryovials at -70 to -80°C until shipment.

**For the protease inhibitor-treated urine supernatant:**

6. Using the 15 mL conical tube containing 11 mL of urine supernatant from step 3 above, push one

protease inhibitor tablet from the foil packaging directly into this tube.

7. Vortex the tube or shake vigorously until the tablet has dissolved.

8. Using a sterile 1 mL serological pipet, transfer 1 mL of the protease inhibitor-treated urine supernatant into each of 10 pre-labeled sterile 2 mL "protease" cryovials.

9. Freeze the labeled cryovials at -70 to -80°C until shipment.

### *Urine Cell Pellet Cryopreservation Procedure*

1. Using a sterile 1 mL serological pipet, re-suspend each urine pellet from Step 5 of the Initial Processing Procedure in 0.5 mL ice-cold X-VIVO-10 by gently pipetting up and down five times, making sure to wash down any sediment on the sides of the tube. Transfer each re-suspended cell pellet to a pre-labeled "pellet" 2 mL cryovial (two total).

2. Using a sterile 1 mL serological pipet, add an additional 0.5 mL of ice-cold X-VIVO-10 to each 50 mL centrifuge tube to recover residual cells. Be sure to rinse down the sides of the tube to recover all cells and transfer to the same 2 mL "pellet" cryovials as in step 1 above.

3. Discard the 50 mL conical tubes.

4. Centrifuge the cryovials in a micro-centrifuge at 200g for 5 minutes (at 4°C, if available).

5. Using a disposable transfer pipette, remove and discard as much supernatant as possible without disturbing the cell pellet.

6. Using a sterile 1 mL serological pipet, add 1 mL ice-cold X-VIVO-10 to each cell pellet, re-suspend by gently pipetting up and down five times.

7. Centrifuge the cryovials in a micro-centrifuge at 200g for 5 minutes (at 4°C, if available).

8. Using a disposable transfer pipette, remove and discard as much supernatant as possible without disturbing the cell pellet.

9. Wipe down the outside of the CryoStor CS10 cryopreservation media container with 70% ethanol or isopropanol before opening.

10. Using a sterile 1 mL serological pipet, add 0.25 mL ice-cold CryoStor CS10 to each cell pellet and re-suspend by gently pipetting up and down five times.

11. Place the cryovials in a Mr. Frosty container (filled with isopropanol and pre-chilled at 4°C for at least four hours) and place the Mr. Frosty directly into a -70 to -80°C freezer.

12. After 15 minutes, slap the Mr. Frosty container to facilitate nucleation, and return the Mr. Frosty to the -70 to -80°C freezer for a minimum of 24 hours and maximum of 7 days.

13. Transfer the cryovials from the Mr. Frosty to a freezer box and store in the -70 to -80°C freezer until

shipment.

## *Urine Processing Procedure C (For Visits 5 and 11)*

### *Initial Urine Processing Procedure*

1. Swirl the urine in the collection cup to suspend particles that have dropped to the bottom.

2. By pouring, split the total urine volume (15-90 mL) equally into two sterile 50 mL conical tubes labeled "centrifugation".

3. Spin the two conical tubes at 200g for 10 minutes in swinging-bucket centrifuge (4°C, if available).

4. Using a sterile 25 mL serological pipet, carefully transfer urine supernatants into 1 (for volumes ≤ 40 mL) or 2 (for volumes > 40 mL) clean sterile 50 mL conical tube(s) labeled "supernatant", being careful not to disturb the pellets.

5. Keep the supernatant on ice for further processing. **Cell pellets can be discarded.**

### *Urine Supernatant Processing Procedure*

1. Urine supernatant will be processed **one way**: **10 mL** without protease inhibitor ("neat").

**For the "neat" urine supernatant without protease inhibitor:**

2. Using supernatant from step 5 above (Initial processing procedure), with a sterile 1 mL serological pipet, transfer 1 mL of the urine supernatant into each of **10** pre-labeled sterile 2 mL "neat" cryovials.

3. Freeze the labeled cryovials at -70 to -80°C until shipment.