



INSTRUCTIONS FOR SPUTUM PROCESSING WORKSHEET FORM SPW, VERSION 6.0, QUESTION BY QUESTION (QxQ)

I. GENERAL INSTRUCTIONS

The Sputum Processing Worksheet Form is completed while processing the sputum sample collected at Clinic Visit 5.

Note: The completion of this form is necessary to run the following reports: “Visit 5 Shipping Manifest-Box 2” and “Visit 5 Shipping Contents Report”. Ensure that this form has been entered before any of the listed reports are run.

Header Information: The header information consists of key fields which uniquely identify each recorded instance of a form. For the Event field, record if this is happening at Visit 5 or another event.

0a. Form Date: Record the date form was completed. Select the date from the pop-up calendar in the data management system (DMS) or type the date in the space provided. Dates should be entered in the mm/dd/yyyy format.

0b. Staff Code: Record the SPIROMICS staff code of the person who collected or abstracted the data. This code is assigned to each person at each site by the GIC. If you do not have a staff code and are collecting SPIROMICS data, please contact the GIC in order to receive your own individual staff code.

0c. Date of Collection: Record the date the data was collected or abstracted. Select the date from the pop-up calendar in the data management system (DMS) or type the date in the space provided. Dates should be entered in the mm/dd/yyyy format.

0d. Processing Start Time: Record the sputum processing start time in hours:minutes in 24 clock time.

II. DETAILED INSTRUCTIONS FOR EACH ITEM

Item 1. **Weight of Entire Sample** Record the weight of entire sample in grams in the space provided.

Color and Description of Sample

Item 2. **Salivary Contamination** Select all the options among the four possible choices that apply.

- Select Minimal if the salivary contamination is minimal.
- Select Mild if the salivary contamination is mild.
- Select Moderate if the salivary contamination is moderate.
- Select Excessive if the salivary contamination is excessive.

Item 3. **Consistency** Select all the options among the three possible choices that apply.

- Select Watery if the sample consistency is watery.
- Select Muroid (involving, resembling, or of the nature of mucus) if the sample consistency is muroid.
- Select Purulent (consisting of, containing, or discharging pus) if the sample consistency is purulent.

Item 4. **Mucus “plugs” (material that is colored and/or more opaque than clear surrounding saliva)**

Select all the options among the eight possible choices that apply.

- Select Numerous if the mucus “plugs” are numerous.
- Select Moderate number if the mucus “plugs” are moderate in number.
- Select Sparse if the mucus “plugs” are sparse in number.
- Select Large if the mucus “plugs” are large in number.
- Select Small if the mucus “plugs” are small in number.
- Select Dense/flocculent if the mucus “plugs” are dense/flocculent in number.
- Select Diffuse opacity if the mucus “plugs” are diffuse opacity.
- Select None if there are no mucus “plugs.”

Item 5. **Color of mucus “plugs”** Select all the options among the five possible choices that apply.

- Select Clear if the mucus “plugs” appear clear.
- Select White if the mucus “plugs” appear white in color.
- Select Yellow/tan if the mucus “plugs” appear yellow/tan in color.
- Select Brown if the mucus “plugs” appear brown in color.
- Select Green if the mucus “plugs” appear green in color.

General Notes/Comments

Item 6a. **Sputum processing condition** Select one of the options among the five possible choices that apply and follow the instructions for continuing.

- Select Condition 1: Induced initial sample Wt. is > 1g if the initial sample Wt. is > 1g. [Perform Steps 7-14]
- Select Condition 2: Induced initial sample Wt. is 0.5g-1g if the initial sample Wt. is 0.5g-1g. [Perform Step 7, then 10 -14]
- Select Condition 3: Induced initial sample Wt. is <0.5g if the initial sample Wt. is <0.5g. [Perform Steps 10-14]
- Select Condition 4: Induced initial sample Wt. is <0.5g and a spontaneous sputum sample was acquired prior to induction if the initial sample Wt. is <0.5g and a spontaneous sputum sample was acquired prior to induction. [Perform Steps 7-8 with induced sample; then 10-14 with spontaneous sample.]
- Select Condition 5: No induced sputum sample was produced; but a spontaneous sample was acquired if no induced sputum sample was produced; but a spontaneous sample was acquired.

Item 6a1. **Sputum processing for Condition 5** Select one of the options among the three possible choices that apply and follow the instructions for continuing.

- Select Condition A: Spontaneous sample Wt. is > 1g if the spontaneous sample Wt. is > 1g. [Perform Steps 7-14]
- Select Condition B: Spontaneous sample Wt. is 0.5g-1g if the spontaneous sample Wt. is 0.5g-1g. [Perform Step 7, then 10 -14]
- Select Condition C: Spontaneous sample Wt. is <0.5g if the spontaneous sample Wt. is <0.5g. [Perform Steps 10-14]

Items 7a-c. **Processing Whole Sample using the Mucin Method** Record the value of each item in the space provided.

Item 7a. Record the weight of the weighing tray in grams. This field is not required.

Item 7b. Record the weight of the whole sputum in grams.

Item 7c. Record the weight of the guanidine volume in milliliters.

Note: Sample size should be from 0.100-0.250g. Add 0.5ml of guanidine reduction buffer (6M) to the sample and store at 2-8 degrees C in the refrigerator.

Items 8a-b. **Processing Microbiology Sample** Record the value each item in the space provided.

Item 8a. Record the weight of the micro centrifuge tube in grams. This field is not required.

Item 8b. Record the weight of the whole sputum in grams.

Item 8c. Record the volume of Zymo added in μl .

Item 8d. Record the volume per aliquot $(8b+8c)/2$.

Note: Weigh an empty micro centrifuge tube. Zero the balance. Measure $\sim 0.200\text{g}$ of whole sputum sample **and record the weight**. Add an equal volume ($\sim 200\mu\text{l}$) of Zymo Research RNA/DNA Shield to the sample and mix. Divide the sample into 2 equal aliquots and store at -80°C . Ship sample on dry ice.

Items 9a-b. **Processing %Solids/Osmotic Pressure Sample** Record the value of each item in the space provided.

Item 9a. Record the weight of the micro centrifuge tube in grams. This field is not required.

Item 9b. Record the weight of the whole sputum in grams.

Note: Weigh an empty micro centrifuge tube. Zero the balance. Use a 200ul air displacement pipette and draw up whatever can be achieved. Transfer entire volume ($50\text{-}200\mu\text{l}$) of sample to micro centrifuge tube. Record the weight in grams of the sample and store in -80°C . Sample can be transferred to -20°C prior to delivery.

Items 10a1-g1. **Processing Total Remaining Whole Sample** for Cytokines, Nucleotides, Cell Counts, Cytospins and RNA Record the value of each item in the space provided.

Item 10a1. Record the weight of the centrifuge tube in grams. This field is not required.

Item 10b1. Record the weight of the whole sputum in grams.

Item 10c1. Record the volume of EDTA-DPBS added to sample in milliliters.

Item 10d1. Record the volume of supernatant removed in milliliters (see section 11 below for process).

Item 10e1. Record the volume of EDTA added to stock sputolysin to make 0.2% DDT in milliliters.

Item 10f1. Record the volume of 0.2% DDT added to the sample in milliliters.

Item 10g1. Record the volume of DTT containing supernatant removed in milliliters.

Item 10h1. Record the volume of EDTA-DPBS in milliliters (0.25-2 ml).

Note: Weigh a 50ml centrifuge tube. Zero the balance. Add remaining sample to centrifuge tube and record weight in grams. Add 8x sample weight of cold EDTA-DPBS buffer, homogenize for 15 min on a rotating tumbler, then spin at 790g at 10 min. Remove half the volume of supernatant (leaving behind the "master sample") and spin this at 1500g for 10 min. Collect the supernatant of this sample and store at -80 degrees C (* see below). To the "master" sample, add a volume of 0.2% sputolysin that is equal to the same volume you just removed from the master sample (note: stock sputolysin is at 1% - dilute this to 0.2% with 1mM EDTA as in following example: if you require 3ml of 0.2% sputolysin, add 0.6ml of stock sputolysin (1%) to 2.4ml of 1mM EDTA). Homogenize the sample for 15 min. on a rotating tumbler then filter the sample through a 48-53 μm nylon mesh into new 50ml tube. Spin the sample filtrate at 790Xg for 10 minutes. Remove and store the entire volume of DTT containing supernatant at -80 degrees C. Resuspend the cell pellet in cold EDTA-DPBS (0.25 – 2ml depending on thickness of pellet).

Items 11a1-f1. **EDTA-DPBS Supernatants for Nucleotides and Cytokines** Record the value of each item in the space provided.

Item 11a1. Record the total number of Nucleotides aliquots obtained.

Item 11b1. Record the volume per aliquot Nucleotides stored in microliters.

Item 11c1. Record the total number Cytokines aliquots obtained.

Item 11d1. Record the volume per aliquot of Cytokines stored in microliters.

Item 11e1. Record the number of aliquots of Cytokine mixed with Zymo Research RNA/DNA shield.

Item 11f1. Record the volume per aliquot of Cytokine mixed with Zymo Research RNA/DNA stored in microliters shield.

Note: If the supernatant volume is greater than 8.6 ml, obtain 4 1000 µl aliquots for nucleotides, 4 1000 µl aliquots for cytokines. Of the remaining sample, take 600 µl and mix it 1:1 with Zymo Research RNA/DNA shield. **Apply the label called “SPU_DPBS_Zymo” to the aliquot containing the Zymo Research RNA/DNA shield. If the sample volume is less than 8.6 ml** start by getting one nucleotide sample between 200-500 µl and one cytokine sample at 200µl. **Mix one of the cytokine aliquots 1:1 with Zymo Research RNA/DNA shield and apply the label called “SPU_DPBS_Zymo” to this aliquot.** If there is sample leftover after that, then continue alternating between nucleotide and cytokine aliquots (i.e., 200-500µl for nucleotides, 200µl for cytokines) until finished. All supernatant samples are immediately stored in a -80 degrees C freezer.

Item 11g1. **EDTA-DTT Supernatants** Record the volume stored of Sputolysin Sup 01 in milliliters.

Item 11g2. **EDTA-DTT Supernatants** Record the volume stored of Sputolysin Sup 02 in milliliters.

Items 12a1-12J. **Cell Counts** Record the value each cell count in the space provided.

Item 12a1. Record the number of dead cells in Quadrant 1.

Item 12a2. Record the number of live cells in Quadrant 1.

Item 12a3. The number of total cells in Quadrant 1 will be calculated and autofill once you have entered the data for Q12a1 and 12a2. Save and reload or Save and click form and refresh .

Item 12a4. Record the number of Squamous Epithelial cells in Quadrant 1. This count is not included in the live/dead cell count.

Item 12b1. Record the number of dead cells in Quadrant 2.

Item 12b2. Record the number of live cells in Quadrant 2.

Item 12b3. The number of total cells in Quadrant 2 will be calculated and autofill once you have entered the data for Q12b1 and 12b2. Save and reload or Save and click form and refresh .

Item 12b4. Record the number of Squamous Epithelial cells in Quadrant 2. This count is not included in the live/dead cell count.

Item 12c1. Record the number of dead cells in Quadrant 3.

Item 12c2. Record the number of live cells in Quadrant 3.

Item 12c3. The number of total cells in Quadrant 3 will be calculated and autofill once you have entered the data for Q12c1 and 12c2. Save and reload or Save and click form and refresh .

Item 12c4. Record the number of Squamous Epithelial cells in Quadrant 3. This count is not included in the live/dead cell count.

Item 12d1. Record the number of dead cells in Quadrant 4.

Item 12d2. Record the number of live cells in Quadrant 4.

Item 12d3. The number of total cells in Quadrant 4 will be calculated and autofill once you have entered the data for Q12d1 and 12d2. Save and reload or Save and click form and refresh .

Item 12d4. Record the number of Squamous Epithelial cells in Quadrant 4. This count is not included in the live/dead cell count.

Item 12e1. The total number of dead cells will be calculated and autofill once you have entered the data for Q12a1, 12b1, 12c1, and 12d1. Save and reload or Save and click form and refresh .

Item 12e2. The total number of live cells will be calculated and autofill once you have entered the data for Q12a2, 12b2, 12c2, and 12d2. Save and reload or Save and click form and refresh .

Item 12e3. The number of total cells will be calculated and autofill once you have entered the all data for cell count chart. Save and reload or Save and click form and refresh .

Note: Count live (clear) and dead (cell interior is blue) cells in each of the 4 corner quadrants. Count BEC's, but exclude RBC's and squamous epithelial cells.

Item 12e4. The total number of squamous epithelial cells will be calculated and autofill once you have entered the data for Q12e1, 12e2, 12e3 and 12e4. Save and reload or Save and click form and refresh .

Note: *Count live (clear) and dead (cell interior is blue) cells in each of the 4 corner quadrants. Include bronchial epithelial cells (BEC's), but exclude RBC's. Count squamous epithelial cells but do not include them in the total live/dead cell count. Perform total cell count and cell viability. Adjust the cell concentration of the sample to $0.5 - 1 \times 10^6$ cells/ml and make 4 cytospin slides.

Item 12f. The value of the Initial Sample Concentration (cells/ml) = $([12e3 / 4] \times 2 \times 10^4)$ will be calculated and autofill once you have entered all the data for the dead/live cell and there is a calculated value in 12e3. Save and reload or Save and click form and refresh .

Item 12g. The value of the Total Cell Count ($([12e3 / 4] \times 2 \times 10^4) \times 10h1$) will be calculated and autofill once you have entered the data for resuspension volume of EDTA-DPBS Q10h1 and all the data for the dead/live cells and there is a calculated value in 12e3(ref range: 1/2 million – 1 million). Save and reload or Save and click form and refresh .

Item 12h. The value of the number of cells/weight = $(12g) / \text{weight of selected sample (10b1)}$ will be calculated and autofill from the recorded value of the weight of sputum 10b1 and the value for 12g. Save and reload or Save and click form and refresh .

Item 12i. The value Viability = $(12e2 \text{ live cells} / 12e3 \text{ total cells}) \times 0.1$ will be calculated and autofill once you have entered all the data for live and dead cells. Save and reload or Save and click form and refresh .

Item 12j. Record the Final Adjusted Sample Concentration (cells/ml) based on resuspension volume (range = $0.5 - 1.0 \times 10^6/\text{mL}$).

Item 13. Cytospins

Note: Slides are made using 60 μL -90 μL of cell suspension (at $0.5 - 1 \times 10^6/\text{mL}$). Make 1 slide using 60 μL , 2nd slide using 70 μL , 3rd slide using 80 μL and a 4th slide using 90 μL . Spin 6 min. at 450rpm. Note: these volumes are based on the Shandon Cytospin IV model. If you are using a different cytospin instrument, refer to the manufacturer's instructions on appropriate sample concentrations and volumes to use. Following air drying, fix all 4 slides in 95% - DO NOT COVER SLIP THE SLIDES. Ship all slides to the slide reading center. Store all slides in SPIROMICS box at room temperature.

Item 13a. Record the number of slides stored of Hema 3 stained slides in the space provided.

Item 13b. Record the amount of amount of cell suspension used (ml) in the Hema 3 stained slides in the space provided.

Item 14. **Zymo Research RNA/DNA cell pellet** Select only one option among the two possible choices that apply.

- Select No if cells for RNA were not collected.
- Select Yes if cells for RNA not collected.

Item 14a. The value for Zymo Research RNA/DNA cell pellet will be calculated and autofill from the recorded value of the total cell count Q12g and the sample concentration 12f and when the value from 13b is entered. Save and reload or Save and click form and refresh .

Note: The sample is spun down at 790g for 10 minutes. Discard the supernatant and resuspend the pellet in 0.5ml of DPBS. Add 0.5 ml of Zymo Research RNA/DNA shield to the sample. Store in -80°C. The number of cells left in the Zymo Research RNA/DNA pellet will be equal to the TCC volume (12g) minus the total number of cells used to make slides in 13b (0.0 – 0.3 ml X 12f).

To determine quantity of DPBS to use in resuspension:

If the cell pellet is really small bring up in less fluid; close to .5 ml

If the cell pellet is big and robust use a higher quantity of fluid; 2ml

It is best to add a little of the solution at a time then to add a lot that will cause the sample to be watery. If you have a watery sample it is too diluted and you will have to start the process over again, reconcentrate, and spin down.

Item 15 **Comments** Record any additional comments in the space provided. Item 16 Processing Ended Time: Record the sputum processing end time in hours:minutes in 24 clock time.

Save and close the form.