

SPUTUM PROCESSING WORKSHEET, SPW QUESTION BY QUESTION (QxQ), VERSION 1.0

I. GENERAL INSTRUCTIONS

The Sputum Processing Worksheet (SPW) is to be completed while processing the sputum sample collected at the clinic visit.

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

Please answer every question on this form. *NOTE: All response options in the paper form may not appear in CDART (e.g., ‘Don’t know’, ‘Declines to answer’, etc.).* Beside each item input is a small double bracket icon which looks like this: . Clicking this icon displays a field dialogue box in which the “Field Status” selection menu allows you to choose from the following options: ‘Refused’, ‘No response’, ‘Doesn’t know’, ‘Not applicable’, ‘Maximum value’, ‘Minimum value’, and ‘Missing’. **See MOP 6 – Section 3.2 for additional instructions on how to select a Field Status option.**

Whenever numerical responses are required, enter the number so that the last digit appears in the rightmost box. Enter leading zeroes where necessary to fill all boxes (e.g., enter 0.25 rather than .25).

II. INSTRUCTIONS FOR INDIVIDUAL ITEMS

Header Information: Consists of key fields which uniquely identify each subject and recorded occurrence of a form. For the “ID NUMBER”, record the 3-character, 6-digit number assigned to the specific participant. For the “Event”, record if this is happening at Baseline or another event.

- Item 0a.** Record the date the data was collected or abstracted in the MM/DD/YYYY format either by selecting the pop-up calendar in CDART or entering the date in the space provided.
- Item 0b.** Record the SOURCE 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 SOURCE data, please contact the GIC in order to receive your own individual staff code.
- Item 0c.** Record the start time (i.e., hour/minute) of the sputum processing in the HH:MM AM/PM format in the space provided.
- Item 1.** Record the weight of the entire sputum sample in grams in the space provided.
- Item 2.** Select the check boxes to answer among the four possible choices all that apply to the salivary contamination.
- Item 3.** Select the check boxes to answer among the four possible choices all that apply to the sputum sample consistency.
- Item 4.** Select the check boxes to answer among the eight possible choices all that apply to the mucus “plugs”.

Item 5. Select the check boxes to answer among the five possible choices all that apply to the color of the mucus “plugs”.

Item 6. Record any other general notes and/or comments about the sputum sample in the space provided.

Item 6a. Select only one option among the five possible choices and follow the instructions for continuing as follows:

- If **Condition 1** selected, go to Items 7-12.
- If **Condition 2** selected, go to Items 7-12.
- If **Condition 3** selected, go to Items 7-8.
- If **Condition 4** selected, go to Items 7-8 with induced sample; then Items 9-12 with spontaneous sample.
- If **Condition 5** selected, go to Item 6a1.

Item 6a1. Select only one option among the three possible choices and follow the instructions for continuing as follows:

- If **Condition A** selected, go to Items 7-12.
- If **Condition B** selected, go to Items 7-12.
- If **Condition C** selected, go to Items 7-8.

Item 7.

Item 7a. Record the weight of the weighing tray in grams in the space provided.

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

Item 7c. Record the guanidine volume in milliliters in the space provided.

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

Item 8.

Item 8a. Record the weight of the micro centrifuge tube in grams in the space provided.

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

Item 8c. Record the volume of Zymo added in microliters in the space provided.

Item 8d. Select **Save and Reload** in CDART to automatically calculate the volume per aliquot in microliters based on the values entered in Items 8b and 8c [i.e., $(8b + 8c) / 2$]. **NOTE:** If Item 8b and/or 8c is missing or not entered appropriately, Item 8d will not calculate.

NOTE: Weigh an empty micro centrifuge tube. Zero the balance. Measure ~0.500 g of whole sputum sample and record the weight (where there is < 0.500 g available, don't use less than 0.250 g). Add an equal volume (~500 µL) of Zymo Research RNA/DNA Shield to the sample and mix. Divide the sample into two equal aliquots and store at -80°C. Ship sample on dry ice.

Item 9.

Item 9a. Record the weight of the centrifuge tube in grams in the space provided.

- Item 9b.** Record the weight of the sputum sample in grams in the space provided.
- Item 9c.** Record the volume of EDTA-DPBS added to the sample in milliliters in the space provided.
- Item 9d.** Record the volume of supernatant removed in milliliters in the space provided. *NOTE: See section 10 for the process.*
- Item 9e.** Record the volume of EDTA added to stock sputolysin to make 0.2% DDT in milliliters in the space provided.
- Item 9f.** Record the volume of 0.2% DDT added to the sample in milliliters in the space provided.
- Item 9g.** Record the volume of DTT containing supernatant removed in milliliters in the space provided.
- Item 9h.** Record the resuspension volume of EDTA-DPBS in milliliters in the space provided. *NOTE: Should be 0.25 – 2 mL.*

NOTE: Weigh a 50 mL conical test tube. Zero the balance. After separating any clear runny saliva from the more viscous “main” sample, add remaining “main” sample to 50 mL conical test tube and record weight in grams. Add 8x sample weight of cold EDTA-DPBS buffer, homogenize for 15 minutes on a rotating tumbler, then spin at 790 g at 10 minutes. Remove half the volume of supernatant (leaving behind the “master” sample) and spin this at 1500 g for 10 minutes. Collect the supernatant and store at -80°C (see text below section 10). To the “master” sample, add a volume of 0.2% sputolysin (diluted with 1mM EDTA) that is equal to the same volume you previously removed from the “master” sample (i.e., half the volume of DPBS you added to initial weight of sample. For example, if initial volume of DPBS added to the sample = 8 mL, you need to add 4 mL of 0.2% sputolysin as follows: 0.8 mL DTT from stock vial (1%) + 3.2 mL of cold DPBS = 4 mL of 0.2% sputolysin). Pipet the sample up and down with P1000 pipette to break up any clumps (this is especially necessary for very viscous/thick samples), then homogenize the sample for 15 minutes on a rotating tumbler - then filter the sample through a pre-wetted 48-53 µm nylon mesh into new 50 mL tube. Spin the sample filtrate at 790 g for 10 minutes. Remove and store the entire volume of DTT containing supernatant at -80°C (see EDTA-DTT Supernatant Table in 10 below). Resuspend the cell pellet in 0.25 – 2 mL of cold EDTA-DPBS depending on thickness of pellet (“thin” pellets require less volume; “thick” pellets require more volume).

Item 10.

- Items 10a1-10a3.** Record the number of Nucleotides, Cytokines, and Cytokine Zymo Research RNA/DNA aliquots in the spaces provided, respectively.
- Items 10b1-10b3.** Record the volume per aliquot of Nucleotides, Cytokines, and Cytokine Zymo Research RNA/DNA stored in microliters in the spaces provided, respectively.

NOTE: If the supernatant volume is greater than 8.6 mL, obtain four 1000 µL aliquots for nucleotides and four 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 200 µL. **Mix one of the next 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., 200-500 μ L for nucleotides, 200 μ L for cytokines) until finished.

All supernatant samples are immediately stored in a -80°C freezer.

Items 10c1-10c2. Record the volume stored of Sputolysin Sup 01 and 02 in milliliters in the spaces provided, respectively.

Item 11.

Items 11a1-11a2. Record the number of Dead and Live cells in Quadrant 1 in the spaces provided, respectively.

Item 11a3. Select **Save and Reload** in CDART to automatically calculate the Total number of cells in Quadrant 1 based on the values entered in Items 11a1 and 11a2. *NOTE: If Item 11a1 and/or 11a2 is missing or not entered appropriately, Item 11a3 will not calculate.*

Item 11a4. Record the number of Squamous Epithelial cells in Quadrant 1 in the space provided. *NOTE: This cell count is not included in the Dead/Live cell count.*

Items 11b1-11b4. For Quadrant 2, follow the instructions as in Items 11a1-11a4 as the question pattern is identical.

Items 11c1-11c4. For Quadrant 3, follow the instructions as in Items 11a1-11a4 as the question pattern is identical.

Items 11d1-11d4. For Quadrant 4, follow the instructions as in Items 11a1-11a4 as the question pattern is identical.

Items 11e1-11e4. Select **Save and Reload** in CDART to automatically calculate the total numbers for Dead, Live, Total, and Squamous Epithelial cells based on the values entered in Items 11a1-11a4, 11b1-11b4, 11c1-11c4, and 11d1-11d4, respectively. Select the manual refresh icon (i.e., ) in CDART to automatically calculate the total number for Total cells based on the values entered in Items 11c1-11c4. *NOTE: If one or more of Items 11a1-11a4, 11b1-11b4, 11c1-11c4, and/or 11d1-11d4 is missing or not entered appropriately, Items 11e1, 11e2, 11e3, and/or 11e4 will not calculate, respectively.*

NOTE: Count live (clear) and dead (cell interior is blue) cells in each of the four corner quadrants. Include bronchial epithelial cells (BECs) but exclude RBCs. 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 four cytospin slides.

Item 11f. Select **Save and Reload** in CDART to automatically calculate the sample concentration in cells per milliliter based on the value calculated in Item 11e3 [i.e., $(11e3 / 4) \times 2 \times 10^4$]. *NOTE: If Item 11e3 is missing or not calculated appropriately, Item 11f will not calculate.*

Item 11g. Select **Save and Reload** in CDART to automatically calculate the total cell count (reference range: $\frac{1}{2}$ - 1 million) based on the values entered and/or calculated in Items 9h and 11e3 [i.e., $((11e3 / 4) \times 2 \times 10^4) \times 9h$]. *NOTE: If Items 9h and/or 11e3 is missing or not entered and/or calculated appropriately, Item 11g will not calculate.*

Item 11h. Select **Save and Reload** in CDART to automatically calculate the number of cells/weight based on the values entered and/or calculated in Items 9b and 11g [i.e., 11g / 9b]. *NOTE: If Items 9b and/or 11g is missing or not entered and/or calculated appropriately, Item 11h will not calculate.*

Item 11i. Select **Save and Reload** in CDART to automatically calculate the viability based on the values entered and/or calculated in Items 11e2 and 11e3 [i.e., (11e2 / 11e3) x 100]. *NOTE: If Items 11e2 and/or 11e3 is missing or not entered and/or calculated appropriately, Item 11i will not calculate.*

Item 11j. Record the final adjusted sample concentration in cells per milliliter based on the resuspension volume (range = 0.5 – 1.0 x 10⁶/mL) in the space provided.

Item 12.

Item 12a. Record the number of Hema 3 stained slides stored in the space provided.

Item 12b. Record the amount of cell suspension used in the Hema 3 stained slides in microliters in the space provided.

NOTE: Slides are made using 60-90 μ L of cell suspension (at 0.5 - 1X10⁶/mL). Make one slide using 60 μ L, 2nd slide using 70 μ L, 3rd slide using 80 μ L, and a 4th slide using 90 μ L. Spin six minutes at 450 rpm. NOTE: These volumes are based on the Shandon Cytospin IV model. If you are using a different cytopspin instrument, refer to the manufacturer's instructions on appropriate sample concentrations and volumes to use. Following air drying, fix all four slides in 95% ethanol – DO NOT COVER SLIP THE SLIDES. Ship all slides to the sputum reading center. Store all slides in SOURCE box at room temperature.

Item 13. Record any other additional comments in the space provided.

Item 14. Record the time (i.e., hour/minute) sputum processing ended in the HH:MM AM/PM format in the space provided.

Select **Save and Close** at the bottom of the page/screen.