



Introduction

Besides viability, pollen number is another interesting parameter in pollen analysis. Examples include:

- Assessing of pollen shed using pollen traps
- Quantification of pollen grain number per flower or anther
- Quantification of pollen grain number on a stigma after manual pollination

This *Quick Guide* describes the *Counting* feature of the Ampha Z32 for obtaining accurate pollen counts and concentrations. The *Counting* feature is used to quantify all particles in a defined volume of sample with minimal sample loss.

Remarks:

- The *Counting* is compatible with F, D, E and G chips. For E and G chip *Counting* applications, Amphasys recommends using a *Counting* buffer which reduces sedimentation and increases pollen recovery.
- Amphasys provides application specific *Counting* templates. Have a look at the [template selection](#) on our website.
- The *Counting* application is not compatible with autosampler operation and autocalibration.

Please get in touch with Amphasys support in case the template for your application is not yet available on the website, or to discuss your experimental setup (e.g. recommended buffers and additional tips for sample preparation).

The Measurement Process

1. **Purging:** After starting a *Counting* measurement, the Ampha Z32 fluidic system is purged with air. Therefore, the sample must not be attached. After purging, a notification pops up requesting the sample.
2. **Loading and Measuring:** Upon confirming the request, the measurement will start. During the first few seconds no particles will be detected, as the sample is loaded to the chip. After reaching the chip, particles will be detected.
3. **Measurement Termination:** The measurement will continue until the entire sample is consumed and has been measured. The measurement stops automatically after a period of 10 seconds without particle detection.
4. **Results:** The *Accepted* number is the total number of particles in the sample. The *Concentration* is calculated using the *Accepted* number, the microliter stop condition and the dilution factor.

Template Creation and Modification

A) Species-specific template from Amphasys

- Download the Counting templates from the Amphasys website in case your species is part of the collection. If your species is not yet available, get in touch with Amphasys support to get a customized template.
- Unzip the template collection and copy the template for your species to your desktop.
- Create a copy of the template and rename the copy. This will be your workspace.
- **Open the workspace and open the worklist. In case you intend to prepare samples with non-default sample volumes adjust the microliter stop condition. If you adjusted the settings of this workspace, you can save them for later use by creating a template from it (in the AmphaSoft menu click on *Workspace > Save as Template...*).**

B) Activating Counting in your own template

- Create a copy of your validated measurement template and rename the copy. This will be the workspace used to configure the Counting.
- Open the workspace. Open the worklist and make sure the columns *Stop Cond Cells*, *Stop Cond ul*, *Stop Cond Min*, *Stop Cond Sec* and *Counting* are displayed. If columns are missing, click on *Settings (+/-)* at the bottom of the worklist and select the column names from the list in the popup. Click *OK*. er 1 (one) into each field of the *Counting* column. This will switch on the *Counting* mode.
- Make sure all *cells*, *min* and *sec* stop conditions are set to zero.
- Configure an appropriate microliter stop condition (*Stop Cond ul*) in all measurements. The microliter stop condition will be used to calculate the cell concentration. Thus, if an accurate cell concentration is required, it must match the sample volume. Typical stop conditions for F and D chips are 500 µl and for E and G chips 2000 µl. If you prepare larger or smaller samples, please adjust the microliter stop condition accordingly.
- Save the workspace with the new settings by clicking the AmphaSoft menu *Workspace > Save as template*.
- Create a copy of the template and rename the copy. This will be your workspace for Counting.



Sample Preparation

Tips: Pollen samples from pollen traps, single anthers or from stigmas can have low pollen grain numbers. In case data analysis is challenging with low numbers, you can perform a reference measurement. In order to do that, start with a sample with high pollen number, e.g. by pooling multiple anthers, flowers or stigmas. Use this reference sample to apply axis limits and gatings to the other measurements, and to identify debris. It is recommended to perform an instrument rinsing after the reference measurement to prevent sample carryover.

For E and G chip pollen counting applications, Amphasys provides counting buffers which reduce sedimentation and increase pollen recovery. Please get in touch with Amphasys support to discuss your application.

A) Pollen from traps

- Homogenize the content of the pollen trap by shaking it well.
- Put a filter on a 5 ml tube and transfer a defined amount of sample from the pollen trap through the filter. The transferred volume should match the configured microliter stop condition, e.g. 2000 μ l.
- Make sure you recover the complete sample from the filter, e.g. by adding more buffer through the filter. If you dilute the sample with more buffer, make sure you calculate the undiluted concentration in the end, or use the dilution factor field (e.g. add a dilution factor of 2 if you diluted 1 ml pollen trap sample with 1 ml fresh buffer).

Tip: Instrument settings may need to be adjusted depending on the fluid used in the pollen trap. Please discuss your application proposal with Amphasys support.

B) Pollen from anthers or flowers

- Add anthers or flowers into an Eppendorf tube and extract the pollen using the instructions in the *Sample Preparation Quick Guide*.
Important: The total buffer volume used for pollen extraction should match the microliter stop condition.

Tip: In order to prevent pollen loss in the Eppendorf tube or filter, you can do the pollen extraction with half the final volume. Then pipet the remaining volume into the emptied Eppendorf tube to recover more pollen. Combine the recovered suspension with the other part of the sample by pouring it through the same filter into the FACS tube.

C) Pollen from stigma after manual pollination

- Collect pollinated stigmas into Eppendorf tubes.

Tip: If the pollen count per stigma is very low, you can pool multiple stigmas and calculate the average number of grains per stigma.

Please make sure you collect and process the stigmas soon after pollination, i.e. before the pollen tubes emerge, as this can lead to biased recovery.

- Suspend the cells and filter the sample based on the instructions in the *Sample Preparation Quick Guide*.

Important: The total buffer volume used for pollen extraction should match the microliter stop condition.

Tip: In order to prevent pollen loss in the Eppendorf tube or filter, you can do the pollen extraction with half the final volume. Then pipet the remaining volume into the emptied Eppendorf tube to recover more pollen. Combine the recovered suspension with the other part of the sample by pouring it through the same filter into the FACS tube.

Measurement and Data Analysis

- Click *Start Measurement*
- A popup message will ask you to make sure that no sample is attached at the sample holder. Remove any tube from the holder. After confirming this request, the fluidic system will be purged with air.
- After purging, another popup message will ask you to attach the sample to the sample holder. The sample will be loaded and measured after confirming the message.
Note: In the first few seconds of the measurement the sample is loaded and therefore no cells will be detected. The measurement will automatically stop when all the sample is consumed, and no more cells are detected for 10 seconds.
- The **Accepted** number indicates the total number of particles measured
- The **Concentration** indicates the number of cells per milliliter of sample, calculated using the **Accepted** number, the microliter stop condition and the dilution factor.
- Use polygon gatings and the *Hide Cells* feature to hide unwanted datapoints (e.g. debris, air bubbles...).