

5/31/16 Instructions for Ki67 Reproducibility Study Phase 3b: Whole Sections

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It may be easier for you to read these instructions in their entirety first. Although they appear long, much space is taken up by graphics.

The following instructions describe a standardized procedure for Ki67 scoring on surgical specimens (whole section slides). Thirty (30) surgical specimens will be assessed in this manner by each participant. Both the 1) hotspot and 2) global Ki67 score will be captured for each specimen.

For those who will be scoring the additional 15 matched core biopsies (for the whole section – core biopsy comparison study), the instructions are exactly the same as described in this document.

Slide number:

Whole section slides (n=30)	# 1 to 30
Core biopsy slides (n=15)	# 41 to 55

Please kindly verify that you have received all slides. If any of them is missing, please contact Samuel Leung (Samuel.Leung@vch.ca or phone: 1-604-875-4111 x62649) as soon as possible.

⚠ Please note: if you would like to pause in the middle of a case, please save your work (e.g. save nuclei selection) and log out. When you re-log in and enter the scoring session, you will be exactly where you've left off. Logging out/in will allow the server to know that the scoring session is being paused and will enable an accurate accounting of the time spent to score a case.

Key materials and equipment for scoring:

- A Bright Field Microscope, a range of objectives, a 10x ocular eyepiece
The objectives can include 4x, 10x, 20x and 40x. The resulting magnification would then be 40x, 100x, 200x and 400x.
A High Power Magnification (HPM) is derived by multiplying 10x ocular and 40x objective = 400x.
- Either a slide micrometer or clear ruler (for measuring microscope field diameter)
- Adjacent computer with the following system requirements:
 - internet connection
 - sound/speakers activated
 - mouse/trackpad (touch screen will not work)
 - supported web browser: Internet Explorer (11), Edge, Firefox, Chrome, Safari (please make sure your browser is updated to the most current version)
 - no specific requirement on operating system (PC or Mac) as long as the aforementioned web browser is installed

- **no need** for Java (however, a system with Java installed will not interfere with this web application i.e. no need to uninstall Java)
- **Appendix 1, at the end of this document:** *“What counts as ‘positive’ Ki67 staining?”* This Appendix describes and illustrates the staining intensities that should be considered positive/negative (same as used in the calibration exercise: <http://www.gpec.ubc.ca/calibrator>).

Instructions for the optional practice exercise

Two “beta” cases are available for you to familiarize (or refresh your memory) with the web interface for entering the scores. These cases are **completely optional**.

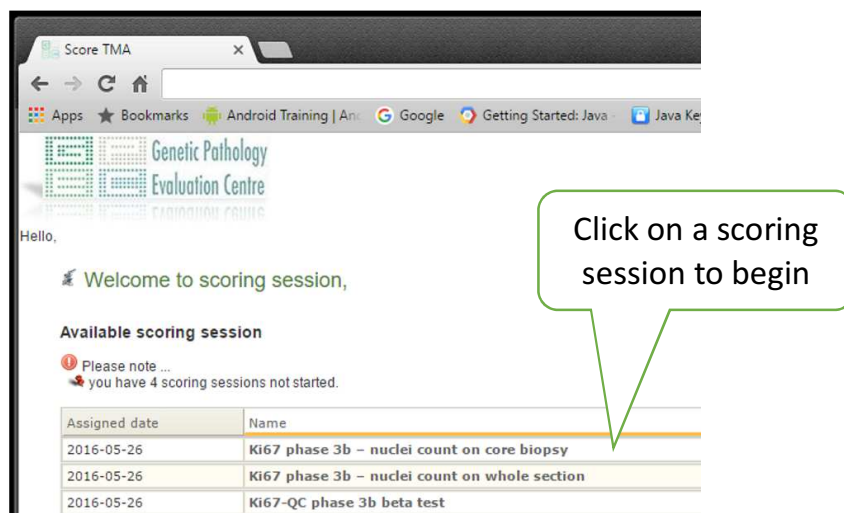
1. Connect to the scoring website at <http://www.gpec.ubc.ca/tmadb> (**Figure 1**) and sign in using the username and password sent to you by Samuel Leung

Figure 1. Sign-in screen

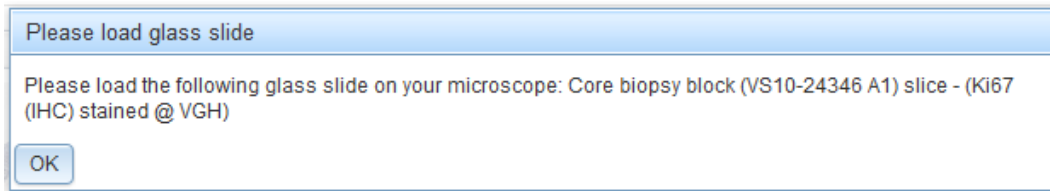


2. In the “Welcome to scoring session” screen that appears (**Figure 2**), click on “Ki67-QC phase 3b beta test”.

Figure 2. Selecting your scoring session



3. If you receive messages asking if you only want to see secure content, click “No”.
4. Click “OK” when you receive the following prompt. (There is no actual glass slide for this practice session. Instead, you will use a virtual slide for practicing.)



5. To call up the virtual slide for the first “beta” case, click on the following link. At the prompt, enter the same username and password you used earlier.

First beta case: Patient VS10-24346 A1:

http://www.gpec.ubc.ca:8080/tmadb-0.1/whole_section_slices/show/4

Make sure the virtual slide opens in a **separate browser window** so that you can easily consult both windows. This separate window will be called “Whole section slice” on the window tab. (Please ignore the link called “Staining detail: Ki67 (IHC) stained @ VGH”.)

6. Thus, **in the first window**, you will see a low-resolution image of the slide with fields beside it for you to input percentages.

In the second window, you will see the high-resolution “whole section slice” Aperio 20x image, which is your “virtual slide” and permits you to zoom in to see detail. **You can zoom in by clicking on the image.**

In this practice session, it is this high-resolution image that you will consult for choosing your fields to score. (In the actual session, you will use glass slides.)

7. Now, please follow the “**Scoring instructions for the actual “phase 3b” exercise**”, which begin on the next page, pretending that the Aperio 20x high-resolution image is an actual glass slide.

NOTE: For scoring during this practice session, you do not actually have to count hundreds of nuclei per core. Please do, however, count enough cells to get used to the nuclei counter web application.

8. Follow the same steps as above, this time for the second “beta” case. The virtual slide for the second “beta” case is found at the link below. Use the same username and password you used earlier:

Second beta case: Patient VS13-29105 A1:

http://www.gpec.ubc.ca:8080/tmadb-0.1/whole_section_slices/show/6

9. You are now done with the practice session. Please now go to “**Scoring instructions for the actual “phase 3b” exercise**”, below, to begin the actual scoring on glass slides.

Scoring instructions for the actual “phase 3b” exercise

1. First, look closely at **Appendix 1** at the end of this document (“*What counts as ‘positive’ Ki67 staining?*”).

Appendix 1 contains visual examples demonstrating the intensities of Ki67 staining that should be considered “positive” or “negative”.

NOTE: Any amount of brown nuclear staining should be considered positive.

2. If you would like to go directly from the practice session to the actual scoring exercise, click on the “**Genetic Pathology Evaluation Centre**” logo in the upper left corner of the browser, then click “**Home**” (**Figure 3**). (You can do this any time you wish to return to the Welcome page.)

Figure 3. Returning to the Welcome page

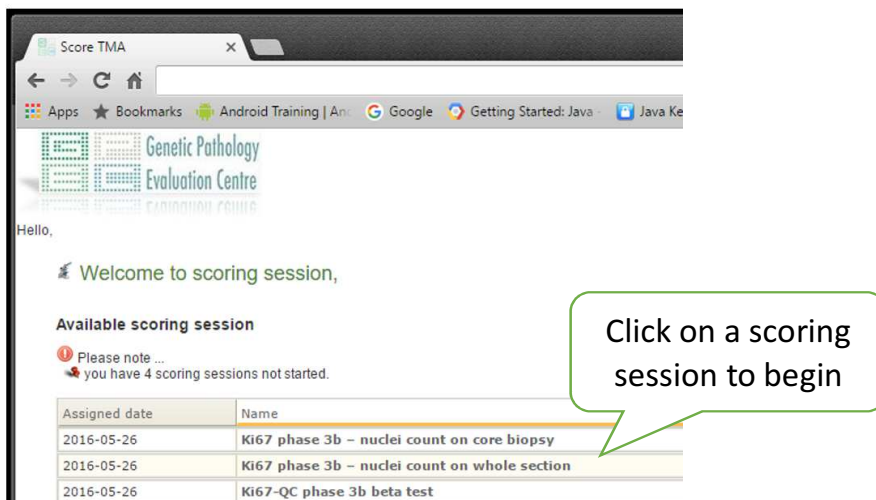


Or, if you have signed out, you can return to the scoring website by:

- Going to <http://www.gpec.ubc.ca/tmadb>
- Signing in (same username and password as in the practice session).

3. In the “Welcome to scoring session” screen that appears (**Figure 4**), click on “**Ki67 phase 3b – nuclei count on whole section**”.

Figure 4. Selecting your scoring session



4. The next screen that appears will show a low-power view of the Ki67-stained slide you are going to score (**Figure 5**).
5. Click the “Slide label” tab. **Confirm that the label on the digital Ki67 image matches the label on the Ki67-stained core on your glass slide.**

If they do not match, please contact Samuel Leung at Samuel.Leung@vch.ca or Phone: 1-604-875-4111 x62649.

The Slide View tab, which provides a macroscopic image of the slide, can also help with verification.

Figure 5. Web screen with low-power image of whole section slide, and where you note the 40x field diameter of your microscope (an example diameter is shown below). *Please note, the example image shown in this figure is for illustration purposes only and does not correspond to the image of the actual study slide.*



6. Enter the **40x field diameter** of your microscope (**Figure 5**).

If you do not have a slide micrometer to measure the 40x field diameter of your microscope, you can easily use a clear ruler instead to measure your low-power field diameter, and then calculate your 40x High-Power Field diameter by following the steps below:

Determining the size of your High-Power Field (HPF) with a clear ruler:

First, use a clear plastic ruler to measure the microscopic field diameter of your *low-power* field. Place the ruler on the stage as if it were a slide, and look through your ocular lenses.

Then calculate the 40x high-power field diameter as follows:

$$\frac{(\text{Low-Power Objective Magnification} \times \text{Microscopic Field Diameter})}{40} = 40x \text{ field diameter}$$

Example of calculating 40x field diameter:

At 2.5x, the field of view diameter is measured with the clear plastic ruler to be 8.8 mm. Plugging into the formula gives the following 40x field diameter:

$$\frac{(2.5x) \times (8.8\text{mm})}{40x} = 0.55 \text{ mm}$$

7. Click “save and continue”.

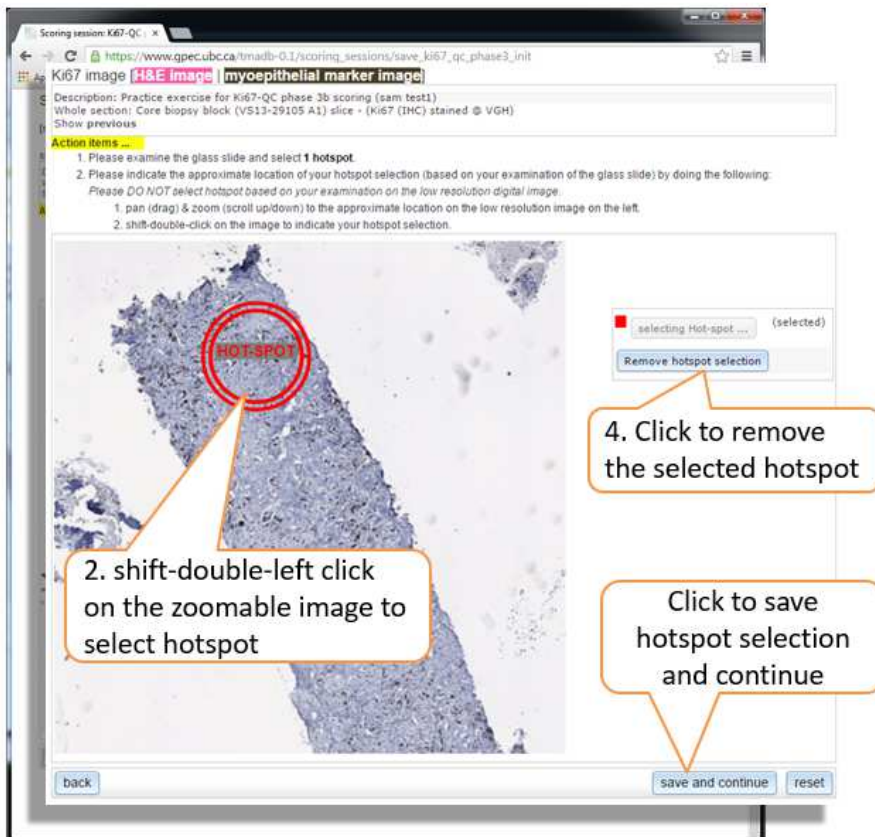
8. Selection and scoring of the hotspot

SELECTING THE HOTSPOT:

The next screen (**Figure 6**) will ask you to choose a hotspot on the **glass slide**, and to indicate its location on the digital image.

You will always be asked to select a hotspot field, even in homogenous cases.

Figure 6. Selecting the hotspot. *Please note, the example image shown in this figure is for illustration purposes only and does not correspond to the image of the actual study slide.*



- a) Examine the entire physical **glass slide** using low-power magnification (with 4x, 10x objectives).

Identify the area you consider to represent the highest Ki67 on the **glass slide**.

If you wish to view images of the H&E and myoepithelial marker-stained slides, click the “H&E image” and “myoepithelial marker image” links at the top of the browser window. In those images, you can zoom/magnify.

- b) Then, go back to the digital image on the website (**Figure 6**). You will see the “Hotspot” button reads “selecting Hotspot”.

On the area in the digital image that corresponds with the hotspot you identified on the glass slide, **double-(left)-click** while holding down the **Shift key**.

This will mark the area with a **red circle** matching the 40x field diameter of your microscope. This area will represent the hotspot.

Red circle = Hotspot

(NOTE: Circle will also be labeled “Hotspot”.)

- c) Click “**save and continue**”. A message will appear: “ok to save hotspot selection?” Press “**OK**” to continue.

IMPORTANT: Please do not select the hotspot based on your examination of the digital image. Rather, base your selection on your examination of the actual glass slide.

Notes on marking the digital image:

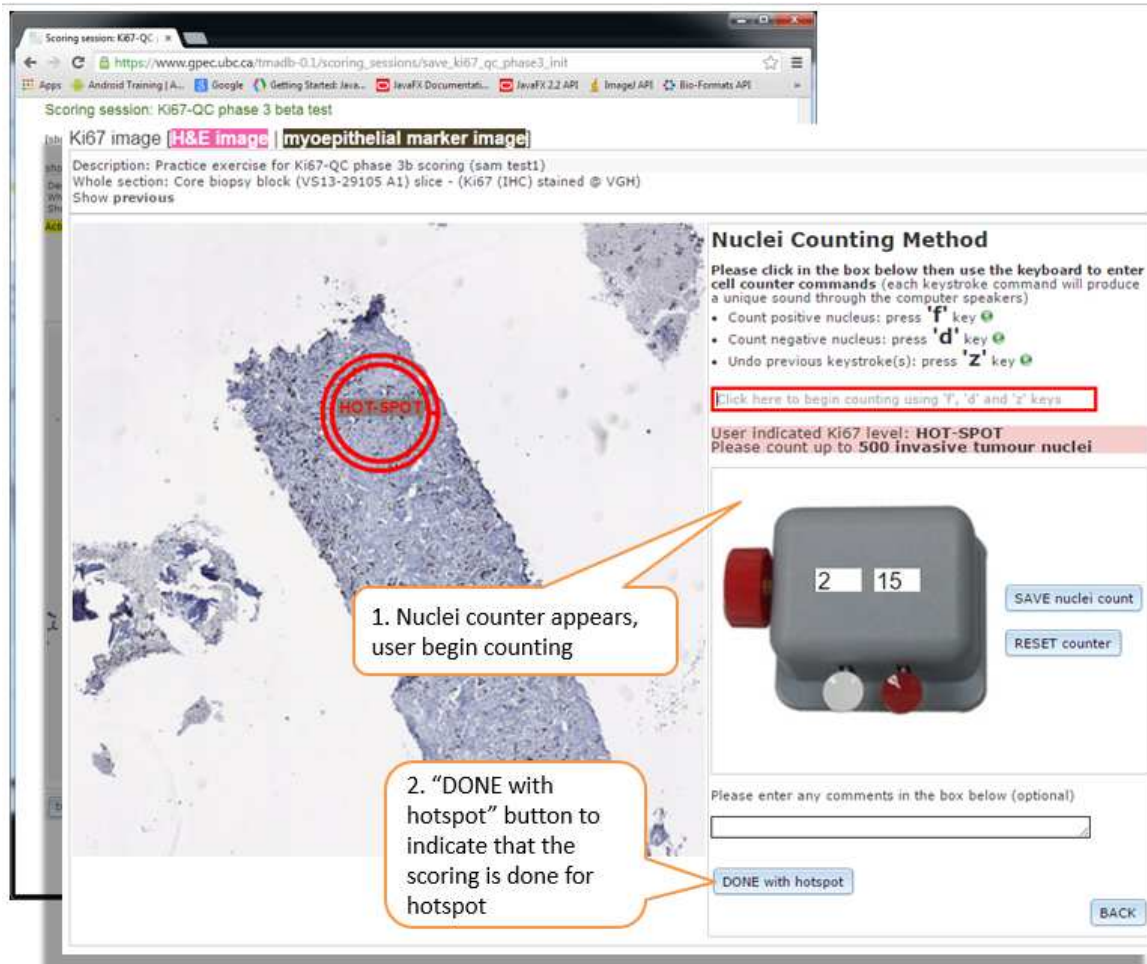
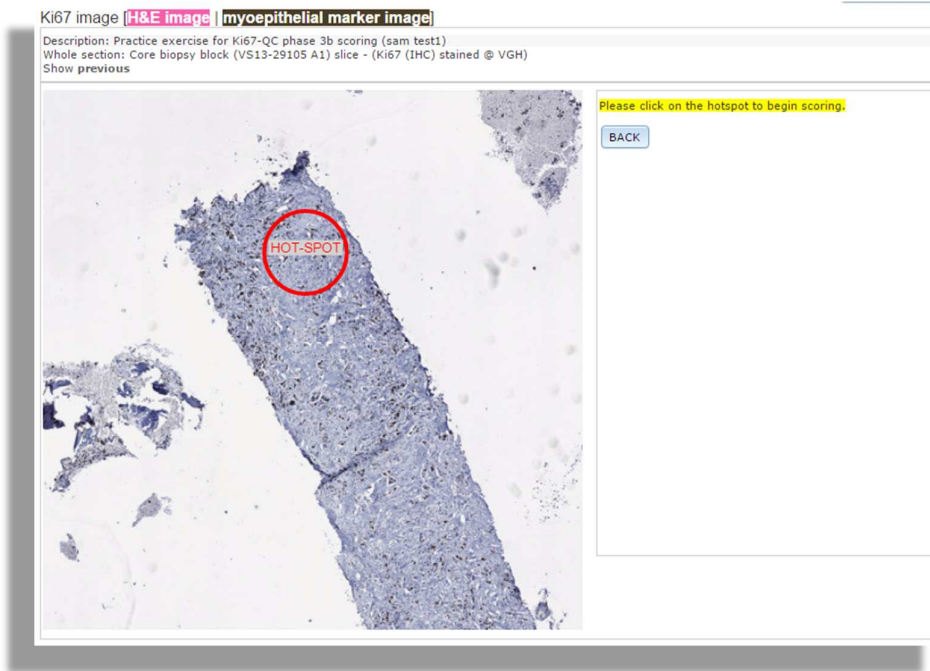
- You can move the image by dragging it.
- You can zoom by using your mouse’s scroll wheel. Since the digital image is low resolution, so you can only zoom to a certain extent.
- The website will also give you options to remove your selections (“Remove hotspot selection” or “reset”).

SCORING THE HOTSPOT:

Make sure your computer’s sound is turned on.

- d) In the next screen that appears (**Figure 7, next page**), click inside the HOTSPOT circle (red circle). This will activate the nuclei counter web application (**Figure 7, next page**).

Figure 7. Clicking on the circle (top figure) will activate the nuclei counter (bottom figure). Please note, the example image shown in this figure is for illustration purposes only and does not correspond to the image of the actual study slide.



- e) Go back to your **glass slide**. Position your high-powered (40x objective) microscope field to the area on the actual whole section glass slide that corresponds with the HOTSPOT circle on the digital image.
- f) Start scoring the glass slide at the top of the hotspot field of view. Count nuclei in a “typewriter” pattern (**Appendix 2**), until **either** 500 invasive tumour nuclei in total are scored **or** the entire field of view has been scored, whichever comes first.

Please remember to use the nuclei counter application as you score the glass slide. This records your scoring data.

You do this by placing your cursor inside the red box labelled “**Click here to begin counting using ‘f’, ‘d’ and ‘z’ keys**” (**Figure 7, above**), and then pressing the following keys as appropriate:

Press the “**f**” key for a positive nucleus.

Press the “**d**” key for a negative nucleus.

Press the “**z**” key to undo a score.

Each of these keystrokes will produce a different sound.

Saving your work:

Make sure to click “SAVE nuclei count” periodically. If you do not save after three hours, you will lose your work. A message will warn you 1 minute before the time-out.

Saving the nuclei count of the hotspot may take up to a minute.

If, as you count, you wish to re-do your counting of the field, click “RESET counter”.

If you have any comments, please put them in the comment field.

When you reach 500 nuclei, the software will make a dinging sound, and a message will appear:

“500 nuclei have been counted. Please click on the ‘DONE with hotspot’ button to indicate that you have finished scoring the hotspot.”

- g) If you are done scoring the hotspot, click “**DONE with hotspot**”.

9. Selection and scoring of representative fields (for global Ki67 score)

Now you will select and score representative fields (for global Ki67 score).

In the next screen that appears (**Figure 8**), you will specify the percentages of invasive tumour in the glass slide that exhibit various levels of Ki67 scores.

SPECIFYING THE PERCENTAGES

- a) Examine the entire **glass slide** section using low-power magnification (with 4x, 10x objectives).

If you wish to view images of the H&E and myoepithelial marker-stained slides, click the “H&E image” and “myoepithelial marker image” links at the top of the browser window (**Figure 8**). In those images, you can zoom/magnify.

- b) **Estimate the percentages** of the invasive tumour in the **glass slide** that exhibit the following Ki67 scores:
- Negative i.e., contains invasive cells but a very low (including zero) percentage of positive invasive cells
 - Low
 - Medium
 - High

Figure 8. Screen where you fill in the percentages. *Please note, the example image shown in this figure is for illustration purposes only and does not correspond to the image of the actual study slide.*

Ki67 image **[H&E image]** **[myoepithelial marker image]**

Description: Demo session for the Ki67-QC phase 3 study web application
Whole section: Core biopsy block (VS13-29105 A1) slice - (Ki67 (IHC) stained @ VGH)
Show previous

Action item ...

- Please estimate (from the glass slide) the percentages of the invasive tumour that exhibit the following Ki67 scores: negative, low, medium and high.

Thumbnail Slide view Slide label



Percent high:	<input type="text" value="percentage (0-100)"/>	%
Percent medium:	<input type="text" value="percentage (0-100)"/>	%
Percent low:	<input type="text" value="percentage (0-100)"/>	%
Percent negative:	<input type="text" value="percentage (0-100)"/>	%

back save and continue reset

If staining is homogeneous across all invasive tumour (i.e., equal Ki67 across the entire invasive tumour component), you can enter 100% in the appropriate Ki67 level, and 0% for the other Ki67 levels.

Areas with increased Ki67 positivity, including areas towards the edge of the tumour, as well as non-proliferating areas of invasive tumour cells, must be included in your assessment.

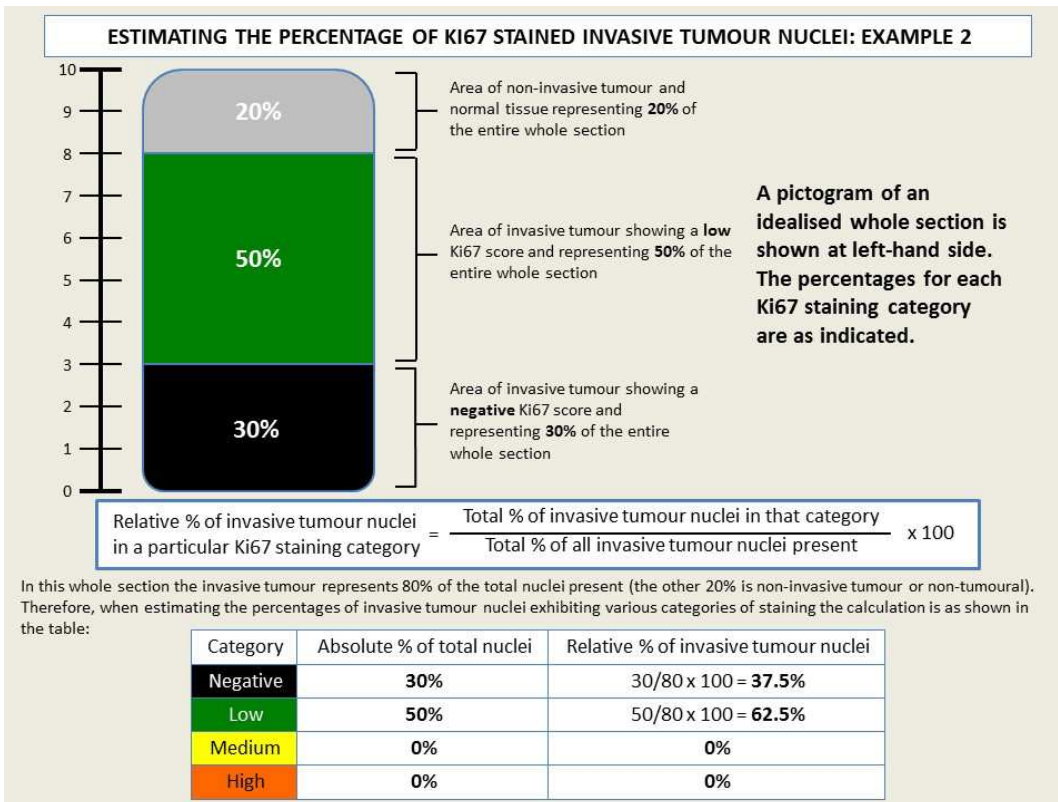
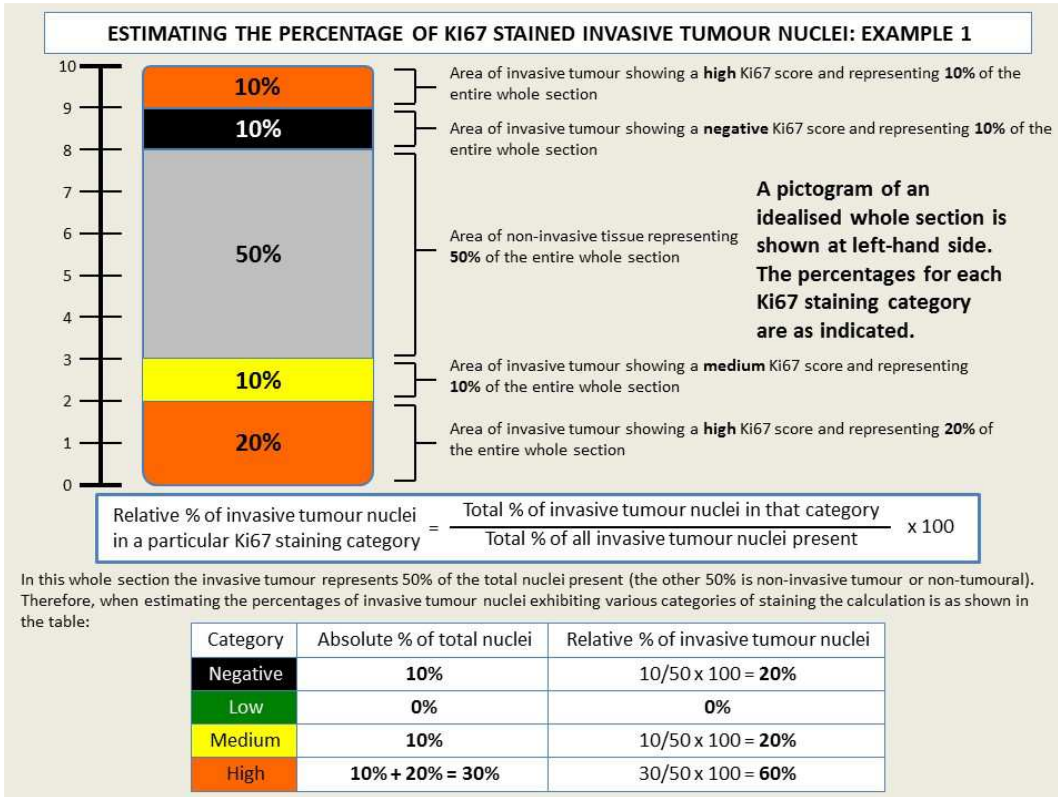
IMPORTANT: Heterogeneity of percentage of cells staining positive frequently occurs across a section.

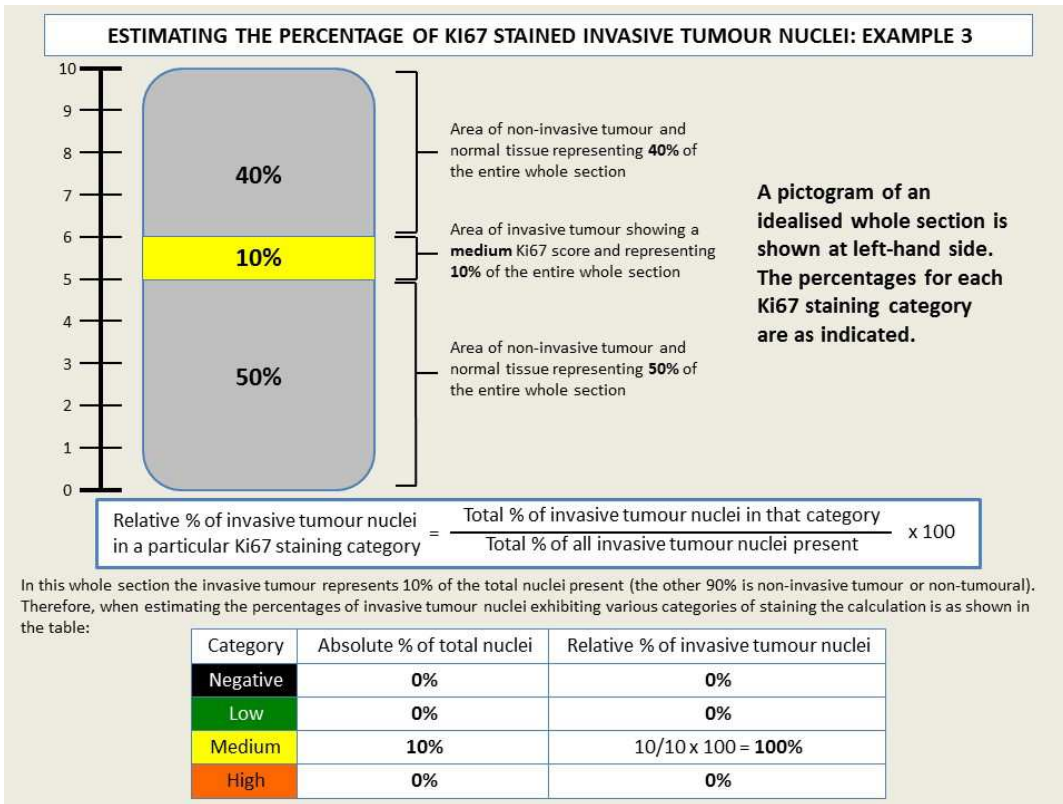
Therefore, scorers should select regions for scoring that are High, Medium, Low, or Negative in relation to the overall percentage positivity.

Thus, “Negative”, “Low”, “Medium”, and “High” are meant to be relative determinations, based on each particular case, and do not reflect specific absolute values.

Please see the examples provided below, which illustrate this concept.

Examples illustrating how to estimate the percentage of Ki67-stained invasive tumour nuclei:





c) Return to your computer screen.

Now, **fill in your percentages into the boxes** labelled “Percent high”, “Percent medium,” “Percent low”, and “Percent negative”, which appear beside the image of the whole section slide (**Figure 8, above**).

d) If you have any comments on the slide, please enter them into the comment field.

e) Click **“save and continue”**.

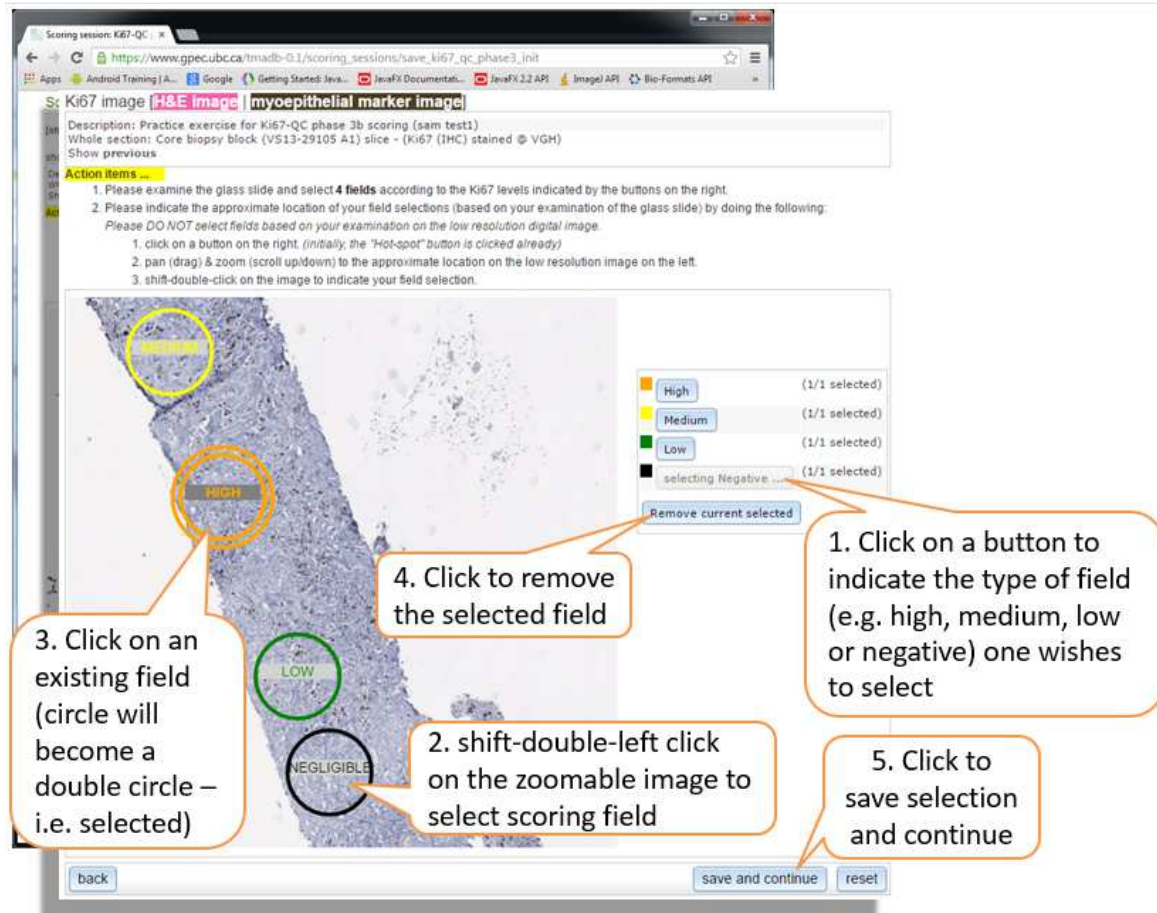
A box will appear that asks, “ok to save percentages of Ki67 scores?”

If you are ready to save your percentages, click **“OK”**. If you are not ready, you can click “Cancel”.

SELECTING THE REPRESENTATIVE FIELDS

In the next screen that appears (**Figure 9**), you will click on the regions in the digital image that you believe are representative of the percentages you indicated for “High”, “Medium”, “Low”, and “Negative”.

Figure 9. Selecting the representative fields. *Please note, the example image shown in this figure is for illustration purposes only and does not correspond to the image of the actual study slide.*



- f) First, examine the entire physical **glass slide** to choose the fields you believe are representative of your percentages.
- g) Then, on the web screen, click on the button indicating the Ki67 level of the field you want to select (i.e., “High”, “Medium”, “Low”, or “Negative”) (**Figure 9, above**).

To indicate the location of the field, **double-left-click** on that area in the digital image while holding down the **Shift** key.

These areas will be marked with colour circles, as follows:

Orange circle = High

Yellow circle = Medium

Green circle = Low

Black circle = Negative (NOTE: Negative fields will also be scored, even if they contain zero or very few positive cells)

(NOTE: Circles will also be labelled accordingly.)

The computer will show you how many fields per Ki67 level you will need to select. (The computer determines these numbers based on the algorithm shown in **Appendix 3**.)

For example, if your percentage for “High” cues the computer to assign 2 fields to “High”, and you have so far specified the location of 1 of those 2 fields, then “1/2 selected” will show beside the “High” button.

- h) When you are done selecting your fields, click **“save and continue”**.
- i) A box will appear that asks, “ok to save field selections?” If you are ready to save your field selections, click **“OK”**. If you are not ready, you can click **“Cancel”**.

SCORING THE REPRESENTATIVE FIELDS

Make sure your computer’s sound is turned on.

- j) In the next screen that appears, click inside one of the circles. This will activate the nuclei counter web application (**Figure 10, next page**).
- k) Go back to the actual whole section **glass slide**. Position your high-powered (40x objective) microscope field to the area on the glass slide that corresponds with the circle you just clicked.
- l) Start scoring **the glass slide** at the top of the field of view. Count nuclei in a “typewriter” pattern, until **either** 100 invasive tumour nuclei in total are scored **or** the entire field of view has been scored, whichever comes first.

Please remember to use the nuclei counter application as you score the glass slide. This records your scoring data.

You do this by placing your cursor inside the red box labelled **“Click here to begin counting using ‘f’, ‘d’ and ‘z’ keys” (Figure 10)**, and then pressing the following keys as appropriate:

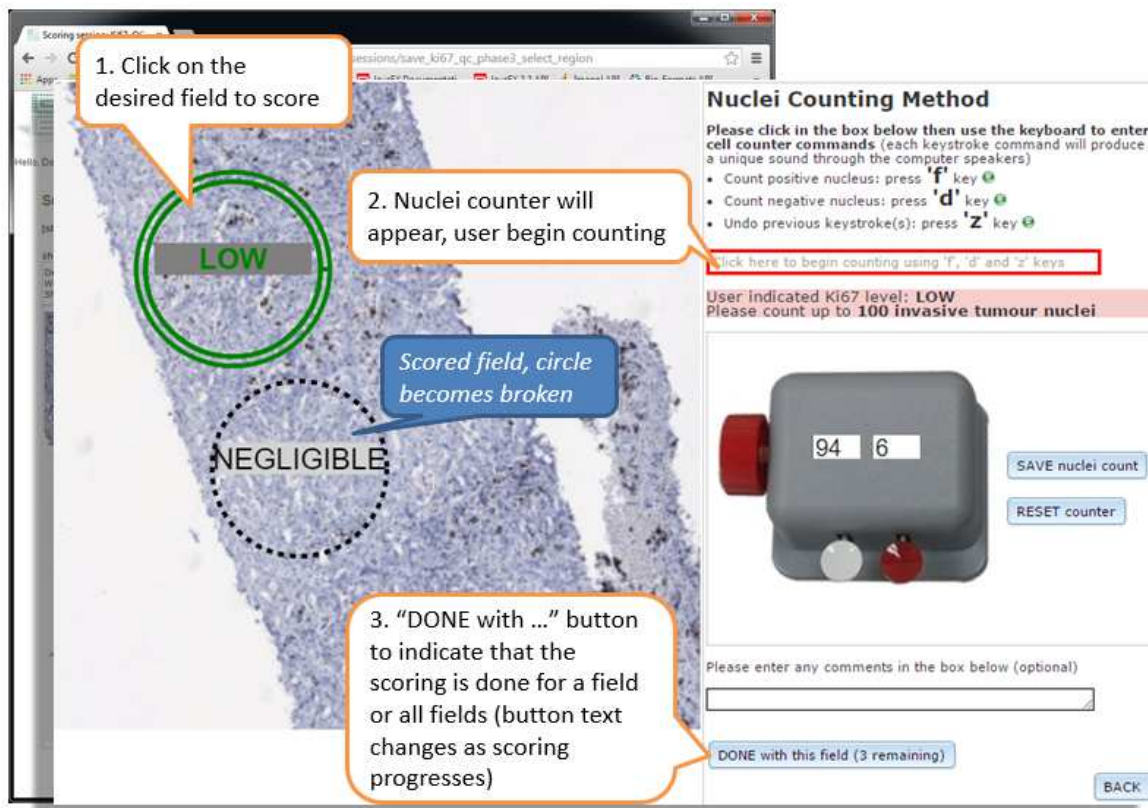
Press the **“f”** key for a positive nucleus.

Press the **“d”** key for a negative nucleus.

Press the **“z”** key to undo a score.

Each of these keystrokes will produce a different sound.

Figure 10. Scoring the representative fields. Please note, the example image shown in this figure is for illustration purposes only and does not correspond to the image of the actual study slide.



Circle formats:

- A **solid circle** indicates the field has not been scored yet.
- A **double-circle** indicates that you have selected / are scoring that field.
- A **broken (dashed) circle** indicates that the field has been scored.
- Each circle is also labelled with the Ki67 level it represents.

Saving your work:

Make sure to click “SAVE nuclei count” periodically. If you do not save after three hours, you will lose your work. A message will warn you 1 minute before the time-out.

Saving the nuclei count of a field may take up to a minute.

If, as you score, you wish to re-do your scoring of the field, click “RESET counter”.

If you have any comments, please put them in the comment field.

When you reach 100 nuclei, the software will make a dinging sound, and a message will appear:

“100 nuclei have been counted. Please click on the ‘done with this field’ button to indicate that you have finished scoring this field.”

- m) If you are done scoring the field, click **“DONE with this field”**.
- n) Repeat the steps above until all circles have been scored. They will all appear as broken circles when scored.
- o) Click **“done with all fields”**. You have now scored the representative fields for this case. (The global Ki67 score for the whole section slide will be calculated as shown in **Appendix 4.**)

10. Submitting your scores

- a) Once you have finished scoring all cases, a “Scoring session report” will appear (**Figure 11**).

If you are ready to submit your scores, click **“Submit scores”**.

If you wish to review or revise the score for a slide, you can click on that slide’s description in the “Description” column to return to that slide.

- b) To sign out, click on the **“Genetic Pathology Evaluation Centre” logo** at the top left of the window, and choose **“Sign out”**.



Figure 11. Reviewing, and then submitting, your scores

The screenshot shows a web application interface for the Genetic Pathology Evaluation Centre. At the top left, there is a logo and the text 'Hello, Doris (Dongxia) Gao'. Below this is a 'Scoring session report' section with a blue notification bar that says 'Ki67-QC phase 3 beta test ... scoring completed! Please review and submit scores.' A table below the notification provides details about the scorer (Doris (Dongxia) Gao), the description (Session for beta testing the Ki67-QC phase 3 study web application), and the start and end dates. Below the report is a 'Scoring result' section with a table of results. Two orange callout boxes with arrows point to specific elements: one points to the 'submit scores' link in the 'Scoring result' section, and the other points to the description of the first case in the table.

2. Click “submit scores” to submit.

1. Click to go back to a particular case to review scoring result

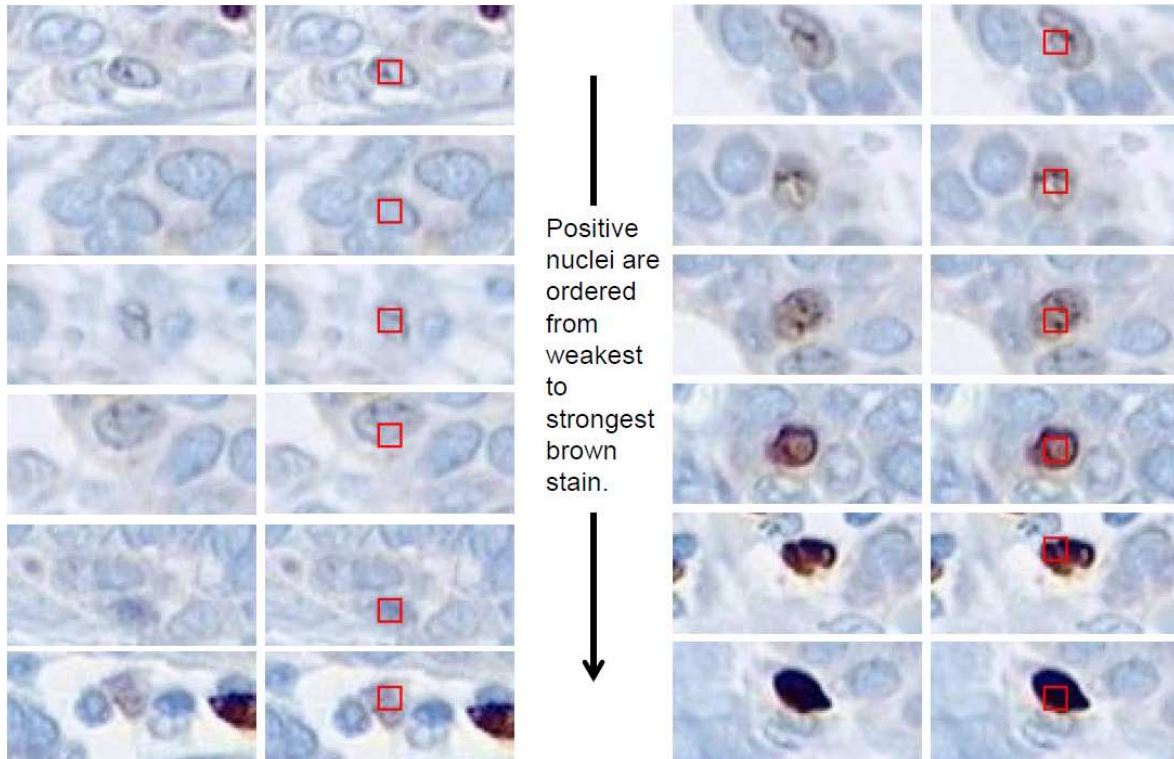
Specimen #	Description	Score type	Score	Scoring date/time
VS10-24346 A1	Core biopsy block (VS10-24346 A1) slice - (Ki67 (IHC) stained @ VGH)	Ki67-QC phase 3	HOT-SPOT:486/500(97%+ve,log2=6.60); HIGH:86/100(86%+ve,log2=6.43); MEDIUM:56/100(56%+ve,log2=5.81); LOW:14/100(14%+ve,log2=3.81); NEGATIVE:4/100(4%+ve,log2=2.00)	2014-04-16 16:25:35
VS13-29105 A1	Core biopsy block (VS13-29105 A1) slice - (Ki67 (IHC) stained @ VGH)	Ki67-QC phase 3	HOT-SPOT:485/500(97%+ve,log2=6.60); HIGH:94/100(94%+ve,log2=6.55); MEDIUM:36/100(36%+ve,log2=5.17); LOW:6/100(6%+ve,log2=2.58); NEGATIVE:3/100(3%+ve,log2=1.58)	2014-05-16 17:18:27

APPENDIX 1. What counts as “positive” Ki67 staining?

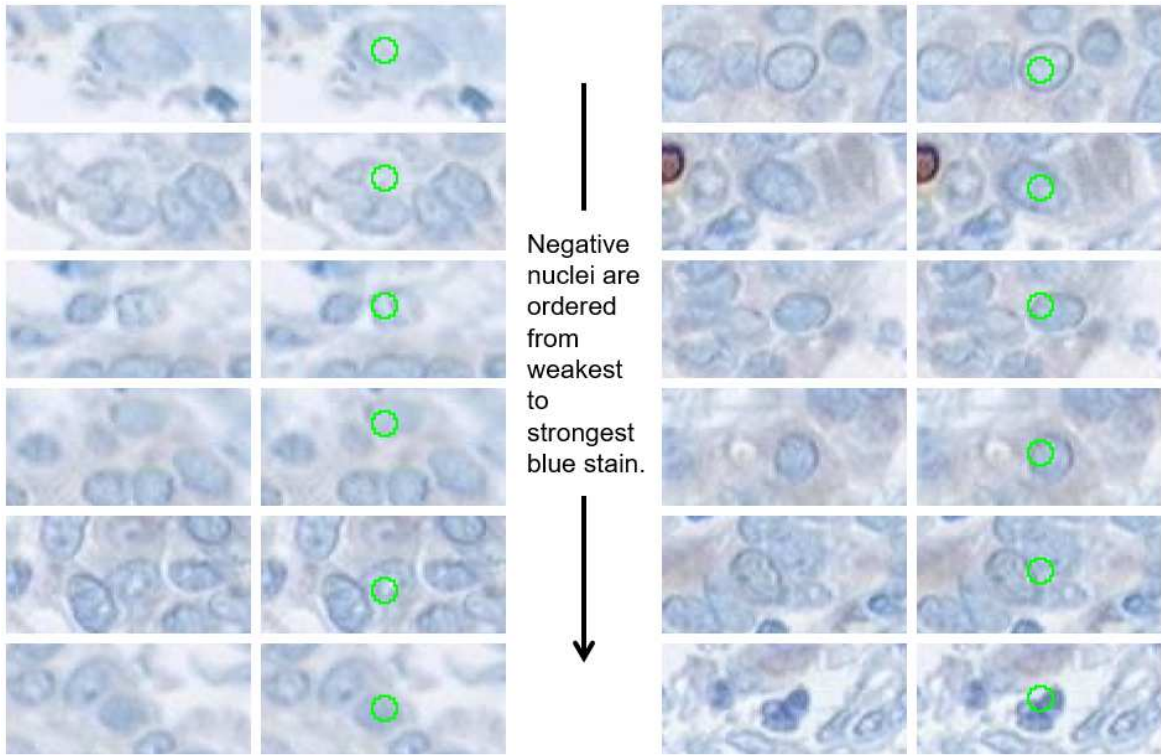
“Positive” = any definite brown staining in the nucleus of an invasive breast cancer cell, above the surrounding background in cytoplasm and extracellular matrix.

- Cells with any degree/intensity of brown nuclear staining are considered positive.
- Cells showing only blue haematoxylin counterstain (i.e. an absence of brown nuclear staining) are considered negative.

The following are example images of range of staining levels that should be considered **positive (red squares)** for Ki67 (unmarked & marked side by side).

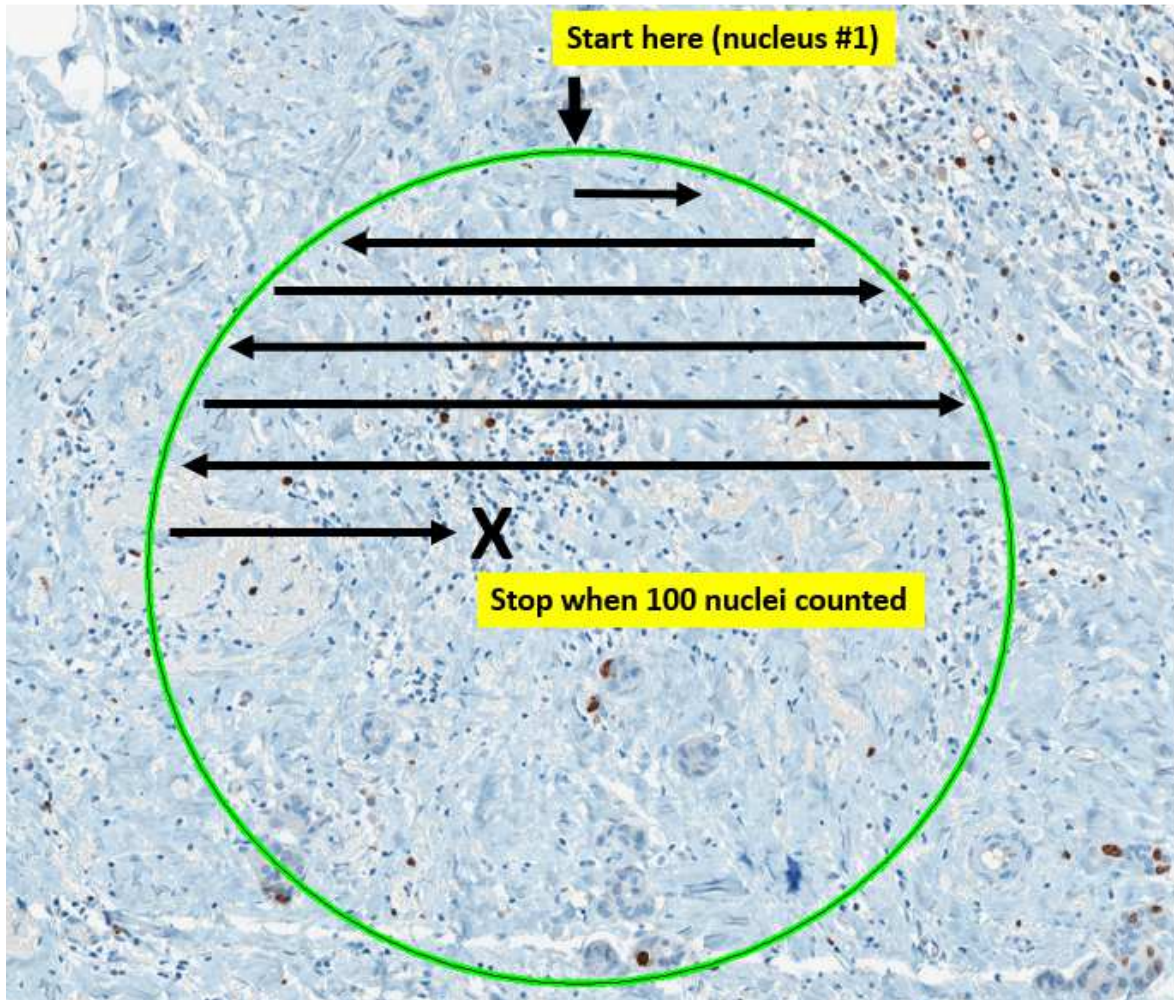


The following are example images of range of staining levels that should be considered **negative (green circles)** for Ki67 (unmarked & marked side by side).



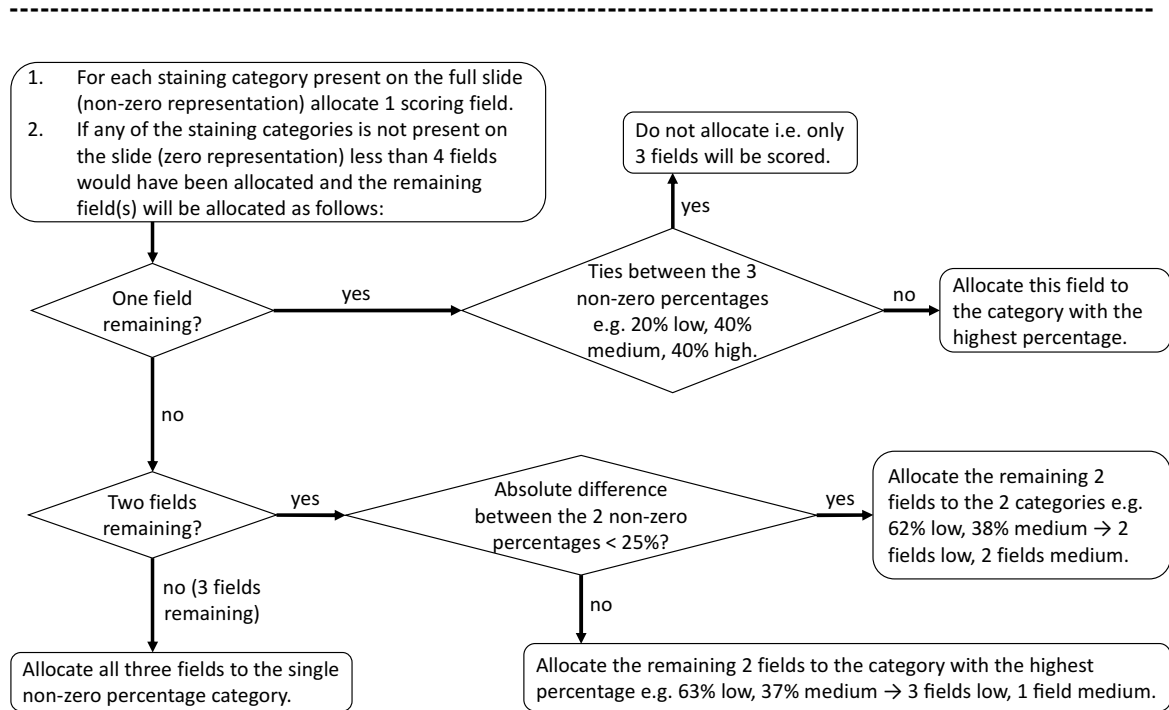
APPENDIX 2. Typewriter Pattern

The following image shows a typewriter nuclei counting pattern. The green circle indicates the selected scoring field.



APPENDIX 3. Algorithm for assigning number of representative fields

For your information, below is a description of how the computer will select the number of fields per Ki67 level for the “selection of representative fields” step.



APPENDIX 4. Global Ki67 Score

The global Ki67 score will be calculated as follows:

$$Ki67\ score = \frac{total\ number\ of\ positive\ tumor\ nuclei\ counted\ in\ all\ fields}{total\ number\ of\ tumor\ nuclei\ counted\ in\ all\ fields} \times 100$$

APPENDIX 5. Known issue with the web application

No sound on Internet Explorer on Windows 10. The nuclei counter will not be able to generate any sound on Internet Explorer (any version) on Windows 10. This is a known issue with Internet Explorer. Therefore, if you are using Windows 10, please use other web browsers such as Chrome, Firefox or Edge.