

TITLE:

A Web Platform for Automated Image-Based Quantification of Human and Rodent Isolated Pancreatic Islets

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SUMMARY:

This protocol describes standardized sampling, staining, image acquisition, and automated web-based quantification of isolated human and rodent pancreatic islets using deep-learning-assisted image analysis and visual quality control.

ABSTRACT:

The purpose of this publication is to provide a written and live-action video step-by-step protocol for standardized use of a web-based platform dedicated to automated quantification of isolated pancreatic islets, starting from sampling and image acquisition to image upload, visual validation, and automatic report generation. The platform was developed for standardized, automated assessment of two-dimensional microscopic images of isolated pancreatic islets to support clinical and experimental islet isolation workflows. Deep-learning-based image segmentation is used to identify islets and exocrine tissue and generate contour-based image analysis. Computational algorithms subsequently quantify islet count, volume, and purity. The visual Quality Control tool supports expert validation of automatic image analysis by allowing users to accept, manually correct, or exclude invalid images. The Clinical Islet Isolation module supports purity-fraction batch analysis and generates automatic PDF reports together with downloadable image and tabular data files for further analysis. The Simple Comparison module compares automated measurements with locally obtained reference submitted with test images to evaluate the suitability of locally standardized images for automated analysis. The protocol also outlines recommendations for retraining using locally generated standardized images when necessary. Representative data from human and rodent islet isolations, including potential pitfalls, demonstrate improved automated analysis performance after appropriate retraining.

INTRODUCTION:

Transplantation of isolated pancreatic islets into diabetic recipients reduces or eliminates the need for external insulin administration¹. The process of islet isolation from cadaveric or living donors is subject to biological and procedural heterogeneity, reflected in variations in yield and quality. Total islet volume represents the primary input for dose determination and clinical decision-making regarding transplant suitability². The islet size histogram and the islet index number account for differences in size-related effectiveness³. Therefore, attention is paid to individual islets in addition to the total volume, which currently serves as a surrogate marker for beta cell content. Isolated human islets are less dense than rodent islets and are often not well separated from exocrine tissue. Consequently, they are more difficult to distinguish unless stained. A rapid and simple zinc staining method using dithizone was adopted, in which intense red light is reflected from zinc-enriched insulin granules⁴. Given the opacity of the tissue, only light reflected from the surface is collected, allowing the use of simple stereo- or inverted-microscopy instruments equipped with a light source and a camera.

Numerous attempts have been made to reduce interlaboratory variation by employing digital image analysis tools^{5–12}; however, these approaches have several limitations. In these methods, segmentation of islets and exocrine tissue either relies on manual thresholding, which is prone to subjective error^{5–10}, or uses fully automated segmentation approaches^{11,12}. In addition, local software installation is often required^{5–11} and may be restricted to specific hardware^{10–12}, limiting general accessibility and standardization.

The overall goal of the present protocol is to provide a standardized workflow for automated quantification of isolated pancreatic islets using microscopic images and a deep-learning driven web-service with human expert oversight. We present recommended guidelines for sampling islet grafts, generating suitable microscopic images, and properly using the *IsletNet* tools. The protocol introduces the concept of human supervision of automatically generated results and presents workflows for prospective and established users (**Figure 1**). The Simple Validation tool allows prospective users to compare *IsletNet* results directly with locally obtained reference methods (**Figure 1A**). Because locally generated standardized images may differ substantially among centers, *IsletNet* may require retraining before reliable analysis can be achieved (**Figure 1B**). To facilitate this process, recommendations for standardized staining and image acquisition are provided. For established users, the Clinical Islet Isolation tool is designed to support the human islet isolation workflow (**Figure 1C**). Downloadable reports together with additional tables and visualizations generated by *IsletNet* are described in **Table 1**, while the key calculated parameters are summarized in **Table 2**.

[Place **Figure 1** here]

[Place **Table 1** here]

[Place **Table 2** here]

PROTOCOL:

Animal procedures were approved by the Ministry of Health of the Czech Republic (Approval no. 49/2023) in accordance with European Communities Council Directive 86/609/EEC. Human islet isolation and use procedures were approved by the State Institute for Drug Control (Approval no. suks111286/2023). Standardized microscopic images of rat and human islet preparations were analyzed using the Simple Comparison and Clinical Islet Isolation tools to compare automated analysis with manual counting. The standard short conversion table¹³ was used for both manual and automated islet equivalent calculations.

1. Human supervision

1.1. Initial view of primary results (**Figure 2**)

1.1.1. Wait for the automatically generated values for islet count, volume, and purity, together with the graphical visualization of the corresponding automatic annotations (e.g., contours or segmentations), to appear in the Result Overview window.

1.1.2. View summarized results for the current sample image in the left information panel (**Figure 2A**).

[Place **Figure 2** here]

1.1.3. View replicate values (**Figure 2B**) and batch averages (**Figure 2C**) in the right-hand table.

1.1.4. Browse Previews (**Figure 2D, 2E**) using the links (**Figure 2F**). Right-click Preview and follow the pop-up menu to download a single image if needed.

1.1.5. Browse sample images using the navigation arrows (**Figure 2G**) or select directly from the table.

1.1.6. Open the Inspection window by clicking inside the current image (**Figure 2E**) to evaluate the quality of the automatically generated contours and validate the results.

1.2. Visual quality control

1.2.1. Move around the image and zoom in or out to inspect the contours for potential errors (**Figure 3A**).

[Place **Figure 3** here]

1.2.2. Toggle between the **Contour** and **Original Islet** views and, if available, additional visualization modes.

NOTE: The available views are listed in the legend on the right side of the window (**Figure 3B**) and can be accessed using keyboard shortcuts or the links in the top navigation bar (**Figure 3C; Table 3**).

[Place **Table 3** here]

1.2.3. Proceed to the next image by pressing the down-arrow key if the contours are acceptable (**Table 3**).

1.2.4. Correct contouring errors manually when possible. If undetected, partially detected, or spurious islets remain present, classify the image as invalid and exclude it from the analysis.

NOTE: Excluded and corrected images are marked in the **Result Overview** window (**Figure 2H,J**) and in all downloaded files.

1.3. Correctable errors: Unseparated adjacent islets

1.3.1. Separate adjacent islets manually when necessary.

1.3.2. Right-click outside the islets to initiate a separation line (**Figure 3D**).

1.3.3. Add intermediate clicks to guide the separation line between adjacent islets (**Figure 3E**).

1.3.4. Right-click outside the islets to complete the separation line (**Figure 3F**).

1.3.5. Submit the separation line to update the segmentation result (**Figure 3G**).

NOTE: Toggle the visualization of added separation lines on or off as needed (**Figure 3H**).

1.4. Correctable errors: Spurious islets

1.4.1. Use the separation tool to divide a spurious islet into multiple objects smaller than the islet size threshold.

1.4.2. Avoid counting small islet fragments by setting the predefined minimum islet size threshold (conventionally 50 μm). Adjust the threshold according to local laboratory practice if necessary.

1.4.3. Right-click any point on the line and select the appropriate option from the pop-up menu to remove one or all manually added separation lines (**Figure 3I**).

1.5. Uncorrectable errors: Missing, partially segmented, or spurious islets

1.5.1. Toggle the **Segmentation** view to verify whether the islets were detected correctly.

1.5.2. Exclude from evaluation any invalid image containing undetected, partially detected, or spurious islets (**Figure 3J**).

1.6. Uncorrectable errors: Misclassified objects

1.6.1. Exclude the image from evaluation if exocrine tissue, background artifacts, or bubbles are incorrectly classified as islets (**Figure 3J**).

2. Using the platform

2.1. Accessing version and technical information

2.1.1. Review the current software version and the latest training date displayed in the top navigation bar (**Figure 2K**).

2.1.2. Open the Home section to access the technical description and associated reference information.

2.2. Getting started

2.2.1. Determine whether locally generated standardized images can already be analyzed correctly by the platform, based on the error criteria described in steps 1.2.4 and 1.5.2, or whether retraining is required for local standardized images (**Figure 1A,B**).

3. Simple comparison and local training

3.1. Simple comparison

NOTE: Use the Simple Comparison tool to compare automated analysis results with a locally established reference method, typically standard manual counting¹³ or alternative methods^{5–12}. Upload locally generated standardized images together with the corresponding reference values and reference method type. The output includes downloadable tables, images, and PDF reports (**Table 1**).

3.1.1. Open the Simple Comparison dashboard from the top navigation bar (**Figure 4A,B**).

[Place **Figure 4** here]

3.1.2. Review the Hints section for the required image properties (**Figure 4C**).

3.1.3. Download the form.xlsx template (**Figure 4D**).

3.1.4. Count islets in multiple samples using a locally established reference method.

3.1.5. Acquire standardized microscopic images corresponding to the samples analyzed by a local method, usually by manual counting.

3.1.6. Ensure that all counted islets are fully included within the image frame.

3.1.7. Enter the exact image filenames and the corresponding parameter values into the form.xlsx template using English decimal points (**Figure 4E**).

3.1.8. Upload the completed spreadsheet and the corresponding image files by drag-and-drop or using the upload buttons (**Figure 4F**).

3.1.9. Select the method used for islet volume estimation (**Figure 4G**).

3.1.10. Click **Submit batch** to generate the side-by-side comparison.

3.1.11. Wait for the analysis results to appear.

3.1.12. Open the Inspection window to perform visual validation of the contours.

3.1.13. Exclude images from the analysis if the contours are incorrect (**Figure 3J**).

NOTE: Manual contour editing is not available in the Simple Comparison tool.

3.1.14. Download the results as tables, images, and PDF reports (**Table 1**).

3.2. IsletNet training for locally standardized images

3.2.1. Contact the development team at the Institute for Experimental Medicine (IKEM) at info@isletnet.com.

3.2.2. Share representative images covering the entire range of image qualities currently obtained locally.

3.2.3. Discuss image quality requirements with the development team.

NOTE: Key image-quality parameters include sufficient contrast, complete inclusion of islets within the image frame, and minimal overlap between adjacent islets. Islet coloration may vary from red to pink or violet, provided that the distinction from exocrine tissue remains clear.

3.2.4. Create a training set of standard images covering the expected range of islet purities,

magnifications, and color variation.

4. Sampling

NOTE: Islets sediment rapidly and differentially according to size and shape, which can bias sampling results.

CAUTION: Perform all islet-handling procedures inside a laminar flow hood while wearing appropriate personal protective equipment.

4.1. Use 50 mL or 250 mL tubes for the islet suspension.

4.2. Determine the required sampling fraction according to the observed pellet volume and purity using local standard operating procedures, aiming at 20–80 islets per image.

4.3. Mix the islet suspension thoroughly by gently inverting the tube five times immediately before sample collection.

4.4. Draw the sample immediately using a large-bore pipette tip to prevent blockage by exocrine tissue and minimize islet damage.

5. Optimizing islet image acquisition

5.1. Dithizone preparation

NOTE: Consistent dithizone staining is required to achieve sufficient contrast between islets and exocrine tissue. Two commonly used preparation methods are described below.

CAUTION: Dithizone is a suspected reproductive toxin. Dimethyl sulfoxide (DMSO) is toxic and skin penetrant. Sodium hydroxide is highly corrosive and can cause severe chemical burns. Handle all chemicals while wearing appropriate personal protective equipment, avoid skin contact, and dispose of waste according to institutional hazardous waste procedures.

5.1.1. Resuspension in DMSO

5.1.1.1. Weigh 50 mg of dithizone powder.

5.1.1.2. Dissolve the dithizone in 5 mL of DMSO.

5.1.1.3. Incubate the solution at 37 °C and vortex vigorously for 1 min every 5 min until dissolution is complete.

5.1.1.4. Dilute 1 mL of the stock solution with 20 mL of Hank's Balanced Salt Solution containing

2% fetal bovine serum to prepare the working solution.

5.1.1.5. Filter the solution through a 0.22 μm syringe filter.

5.1.1.6. Wrap the tube in aluminum foil to protect the solution from light.

NOTE: Use the working solution within approximately 12 h.

5.1.2. Resuspension in alkaline alcoholic solution

5.1.2.1. Weigh 50 mg of dithizone powder.

NOTE: Prepare aliquots in advance if desired.

5.1.2.2. Add 800 μL of 70% ethanol to the dithizone.

5.1.2.3. Add 400 μL of 1 M sodium hydroxide solution and vortex thoroughly until dissolution is complete.

5.1.2.4. Dilute the solution with phosphate-buffered saline containing 2% human albumin to a final volume of 20 mL.

5.1.2.5. Filter the solution through a 0.22 μm syringe filter.

5.1.2.6. Wrap the tube in aluminum foil to protect the solution from light.

NOTE: Use the working solution within approximately 12 h.

5.2. Islet staining

5.2.1. Add 25 μL of dithizone working solution to the center of a 35 mm dish.

5.2.2. Add 50 μL of islets suspended in Hank's Balanced Salt Solution containing albumin.

5.2.3. Pipette the suspension in and out once slowly to mix the solutions gently while avoiding bubble formation.

5.2.4. Incubate the sample at 20–25 $^{\circ}\text{C}$ for 1–2 min while visually monitoring the staining under the microscope until the islets appear bright red. Some preparations may contain poorly stained islets for which IsletNet is also trained.

5.2.5. Add 4 mL of phosphate-buffered saline slowly around the drop while leaving the islets in the dithizone solution undisturbed.

5.2.6. Swirl the dish gently in a circular motion to mix the solutions while maintaining the islets near the center of the dish.

OPTION: Spread the dithizone-islet drop into a thin layer using a pipette tip or needle instead of dilution to reduce reflections.

5.2.7. Inspect the sample under the microscope and return any displaced islets to the camera field gently using a pipette.

5.2.8. Start image acquisition immediately to minimize cytotoxicity and islet volume changes.

5.3. Image acquisition

NOTE: Use a stereo or inverted microscope equipped with a low-magnification objective and a stable light source. Shield the dish from additional light sources, such as room lighting, or standardize ambient lighting conditions.

5.3.1. Turn on the light source and allow the halogen bulb to warm up for 5 min.

5.3.2. Set a standardized light intensity to avoid glare and underexposure. The exact range depends on the imaging hardware used.

NOTE: The optimal light intensity may vary according to magnification and sample purity.

5.3.3. Select the objective and zoom setting that allows all islets to remain within the camera field of view.

5.3.4. Maintain the same magnification for all samples within a batch and preferably throughout the entire imaging session.

5.3.5. Calibrate the white balance using a normalized white slide if necessary. If image quality remains unsatisfactory because of unique characteristics of the islet preparation, adjust RGB values and/or gamma settings.

5.3.6. Adjust the exposure time and, if necessary, the illumination intensity or gain using the color histogram as guidance to avoid oversaturation.

NOTE: Samples containing abundant exocrine tissue may require different camera or illumination settings because of reflected light from non-islet tissue. To ensure reproducibility, save these parameters as a separate configuration file for future reference.

5.3.7. Capture the image and save the file in a supported format, including JPG, PNG, TIFF, or

BMP.

NOTE: Include the date, purity fraction, magnification or pixel size, and dilution in the filename to facilitate future reanalysis (e.g., iz260105_H1_obj1.25x_1000x.jpg).

5.3.8. Acquire an image of the stage micrometer using the identical magnification and zoom settings used for sample imaging.

6. Clinical islet isolation tool

NOTE: The key workflow steps are summarized in **Figure 1C**. Analyze images in purity-fraction batches as samples become available.

6.1. Setting the pixel size

NOTE: The calculated pixel size remains valid only when the stage micrometer image is acquired using the identical magnification and zoom settings as the analyzed samples.

6.1.1. Open the **Clinical Islet Isolation** dashboard.

6.1.2. Click **Upload Stage Micrometer** and select the stage micrometer image.

6.1.3. Draw the endpoints of the red measurement line across a known distance on the digital ruler.

NOTE: Use a longer calibration distance when possible to improve measurement accuracy.

6.1.4. Enter the known distance in micrometers (μm).

6.1.5. Accept the calculated pixel size.

NOTE: The platform retains the calculated pixel size until modified manually.

6.2. Batch upload of purity-fraction images

NOTE: Use at least 3–4 replicate samples to ensure reliable calculation of the batch mean and standard deviation values. Use the same dilution for all samples within a batch.

6.2.1. Drag and drop images representing replicate samples of the same purity fraction into the upload area.

NOTE: The platform prevents repeated use of the same image based on filename recognition only.

6.2.2. Modify the pixel size if necessary.

6.2.3. Enter the minimum islet size threshold.

6.2.4. Enter the sample dilution value (e.g., 1000).

6.2.5. Enter the pellet volume in milliliters recorded from the original flask.

6.2.6. Enter the operator initials.

NOTE: The operator initials appear in the generated report.

6.2.7. Enter the Session ID representing the current isolation procedure.

6.2.8. Enter the Batch ID (e.g., 1 High Purity).

NOTE: The report orders fractions alphanumerically according to Batch ID.

6.2.9. Click **Submit Batch** to start the analysis.

6.3. Review and finalize batch results

NOTE: Close the previous batch window by clicking the batch header if necessary (**Figure 2K**).

6.3.1. Wait for the numerical and graphical results to appear in the **Result Overview** window.

6.3.2. Open the **Inspection** window by double-clicking the image in the **Results** window.

6.3.3. Review and, if necessary, correct the contours as described in step 1.

6.3.4. Proceed to the next image using the down-arrow key if the contours are acceptable.

6.3.5. Add another batch or proceed to the session summary using the buttons in the bottom navigation bar (**Figure 2M**).

6.4. Batch editing

NOTE: The platform prevents inadvertent repeated use of the same image. This includes removed images. Rename images before reuse if necessary.

6.4.1. Click **Edit Batch** to add or remove images, reset sample specifications, or rename the batch (**Figure 2L**).

6.4.2. Click **Remove Batch** to delete the batch entirely (**Figure 2L**).

6.5. Viewing the summary and generating reports

6.5.1. Click **Show Summary** to display the session overview (**Figure 2M**).

NOTE: Return to previous batches and edit them if necessary.

6.5.2. View the total islet count, volume, and pellet volume values for the session together with the corresponding mean batch values.

6.5.3. Download the report.

NOTE: The short report includes total islet count, volume, purity, pellet values, histograms, and purity-fraction summaries. The complete report additionally includes graphical information for each sample image, with accepted values displayed in red and excluded values displayed in black.

REPRESENTATIVE RESULTS:

Pure rat islets

In the first experiment, 36 images representing 12 samples containing a total of 249 purified rat islets were acquired during four independent imaging sessions. Images were generated using a stereomicroscope under three standardized but distinct illumination conditions employing halogen and light-emitting diode (LED) light sources together with different camera settings. Ground-truth (GT) segmentations were generated manually for three representative images by *IsletNet* training expert.

When the images were analyzed using the publicly available version of the Simple Comparison tool, none of the automatically generated contours passed the visual quality control (vQC). The platform was subsequently retrained using representative local images, and all contours generated by the retrained experimental version passed vQC.

The ratio of automated to manual islet volume assessments demonstrated consistent performance across the three imaging conditions after retraining, with median values of 1.03, 0.98, and 0.94 for illumination conditions A, B, and C, respectively (**Figure 5A**). These results represented a substantial improvement compared with the original training model (**Figure 5B**).

Pixel-wise comparison against GT segmentations demonstrated close agreement between automated and manually generated contours after retraining (**Figure 5D,G**), corresponding to accurate contour placement in representative images (**Figure 5C,F**). In contrast, the original training model showed an increased proportion of false-negative pixels and contour misplacement,

resulting in a shift toward smaller islet categories in the corresponding islet size histograms (**Figure 5E,H**).

[Place **Figure 5** here]

Clinical human islet preparations

In the second experiment, 17 images representing a single human islet isolation procedure with three purity-fraction pairs were analyzed using the publicly available version of the platform. One sample was excluded because of sample loss during processing. After vQC, all images representing medium- and low-purity fractions were excluded because of spurious islet detection (data not shown). The platform was subsequently retrained using representative local images.

Following retraining, all images passed vQC after the addition of three manual separation lines. Batch-wise comparison with manual counting demonstrated an overall underestimation of $\leq 5\%$ for total islet count and volume assessments. Samples diluted between 2,000- and 4,000-fold contained between 80 and 4 islets, depending on purity fraction. The relative contribution of individual purity fractions to the final preparation is shown in **Figure 6A**. High-purity fraction 1VC2 contributed predominantly to smaller islets compared with medium-purity fraction 2SC1. The overall islet size histogram generated automatically by the platform is shown in **Figure 6B**.

[Place **Figure 6** here]

Automated and manual measurements were subsequently compared for all individual images (**Figure 6C**). Median ratios between automated and manual measurements were 1.00 for islet count and 0.93 for islet volume, with several outliers observed. In one representative low-purity sample (3NC1), the platform identified the same number of islets as manual counting (five islets) but estimated a 29% lower islet volume. These data points are indicated by arrowheads in **Figure 6C**.

The largest islet from the sample 3NC1 is shown in **Figure 6D**, including the original image and the automatically generated contour overlay. Pixel-wise comparison against GT segmentations generated independently by two experts is shown in **Figure 6E**. The corresponding islet size histograms demonstrated a shift in size distribution between the two experts GT segmentations (**Figure 6F**).

Operational performance of automated analysis

Operational performance of the Simple Comparison and Clinical Islet Isolation tools was evaluated under standard internet conditions with average download and upload speeds of 50 Mbps and 30 Mbps, respectively. Five image batches containing 12 images each were analyzed using image sizes ranging from 0.5 MB JPEG files to 20 MB TIFF files. Download duration was measured for complete ZIP result packages.

Analysis visualization times and download durations increased progressively with image size for

both tools (**Table 4**). The Clinical Islet Isolation tool required longer processing times than the Simple Comparison tool for equivalent image sets. These results demonstrate the practical relationship between image resolution, file format, and analysis throughput during routine operation.

[Place **Table 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow for prospective and established users of the platform. (A) Local validation workflow using the Simple Comparison module. (B) Retraining workflow for locally standardized image sets. (C) Routine use workflow using the Clinical Islet Isolation module. Key workflow steps are summarized for image upload, analysis, visual quality control (vQC), and report generation. Scale bars are not applicable.

Figure 2. Result Overview window. (A) Current sample result summary. (B) Batch replicate samples. (C) Mean batch values. (D, E) Contours, segmentations, and histogram previews. (F) The corresponding navigation links. (G) Replicate sample navigation arrows. (H, I, J) Corrected, unmodified, and excluded image marks. (K) Top navigation bar. (L) Batch title and navigation controls. (M) Bottom navigation bar for batch addition and summary generation.

Figure 3. Inspection window for vQC and manual contour correction. (A) Image zoom display. (B) Legend and navigation hints. (C) Top navigation bar of the Inspection window. (D–G) Construction and submission of a manual separation line for adjacent islets. (H) Toggle for displaying or hiding manual separation lines. (I) Removal of manual separation lines. (J) Toggle for excluding an image from analysis. Scale bars are not shown in this interface view.

Figure 4. Interface and workflow of the Simple Comparison tool. (A) Top navigation bar. (B) Simple Comparison dashboard. (C) Upload hints and instructions. (D,E) Form.xlsx template download and uploaded parameter visualization. (F) Drag-and-drop upload area for images and spreadsheets. (G) Islet size-to-volume conversion method selection.

Figure 5. Assessment of platform retraining using three imaging setups and rat islet preparations. (A) Comparison of *IsletNet* and manual islet equivalent (IEQ) measurements after retraining using three imaging setups. (B) Comparison of *IsletNet* and manual IEQ measurements using the original public training model. (C–H) Contours and pixel-wise comparison. (C,F) Representative contour placement using the vQC tool after retraining and with the original training model, respectively. White arrowheads indicate correct contour placement, and magenta arrowheads indicate incorrect contour placement. (D,G) Pixel-wise comparison against ground truth after retraining and with the original training model, respectively. True positive, false positive, false negative, and true negative regions are indicated in the legend. (E,H) Representative islet size histograms after retraining and with the original training model, respectively, compared with manual measurements. Imaging setup 1 = LED illumination; imaging setup 2 = halogen illumination; imaging setup 3 = LED dark-field illumination. Scale bars = 100 μ m. Boxplots are shown ac-

according to Tukey convention; whiskers extend to data points within $1.5 \times$ interquartile range from the first and third quartiles.

Figure 6. Quantification and analysis of human islet isolation samples. (A) Islet count and islet equivalent volume measured using *IsletNet* and manual counting methods across purity fractions of the complete graft. (B) Final islet size histogram generated by *IsletNet* using batch mean values. (C) Comparison of *IsletNet* and manual measurements for count and volume across 17 individual images. (D) Representative image detail from sample 3NC1 showing the original image and contour placement generated by retrained *IsletNet*. (E) Pixel-wise comparison of *IsletNet* segmentation against the ground truth generated by the manually-counting expert vs. *IsletNet*-training expert (GT1 vs. GT2). True positive, false positive, false negative, and true negative regions are indicated in the legend. (F) Representative islet size histogram for the same image detail comparing *IsletNet* and manual measurements. Magenta arrowheads indicate contour discrepancies. Scale bars = 100 μ m. Boxplots are shown according to Tukey convention; whiskers extend to data points within $1.5 \times$ interquartile range from the first and third quartiles.

Table 1. Downloadable result files generated by the platform. List of downloadable output files generated by the Clinical Islet Isolation and Simple Comparison tools, grouped according to file format and output category. The table includes report files, segmented image outputs, visualization files, and spreadsheet exports for islet count, volume, purity, and histogram analyses.

Table 2. Key parameters included in downloadable reports and spreadsheet outputs. Detailed list of metadata, user-defined settings, and quantitative analysis parameters included in downloadable XLSX and CSV files generated by the Clinical Islet Isolation and Simple Comparison tools. Parameters include session metadata, image acquisition settings, islet quantification metrics, volume estimation methods, purity measurements, size histogram outputs, and individual islet descriptors.

Table 3. View modes, keyboard shortcuts, and navigation controls available in the Inspection window. Summary of image visualization modes, keyboard shortcuts, and navigation functions available during vQC and manual review of segmentation results in the Inspection window. The table includes contour overlays, segmentation displays, histogram visualization, and image navigation controls used in the Clinical Islet Isolation and Simple Comparison tools.

Table 4. Analysis visualization times and download durations for the Clinical Islet Isolation and Simple Comparison tools. Comparison of processing times required for result visualization and file download using image batches of different file sizes and formats. Measurements were obtained using batches containing 12 images and are reported as elapsed time from batch submission to result visualization or download completion.

DISCUSSION:

The protocol presented here describes the practical workflow for prospective and established users of *IsletNet*, with emphasis on two critical features of the platform: expert vQC and neural

network retraining. The representative results demonstrate that accurate automated quantification depends not only on image segmentation performance, but also on the user's ability to critically evaluate contour placement and image suitability before accepting the results. Two additional advanced tools, Pixel-to-Pixel Comparison and My Segmentations, were used to generate selected validation results; however, these tools are intended primarily for neural network training support and are therefore not included in the protocol workflow.

IsletNet was developed as a collaborative and broadly accessible reference tool for standardized islet image analysis across laboratories. The neural network was trained and validated using manually annotated GT segmentations of original two-dimensional microscopic images of isolated human and rat islets^{14–17}. Separate training models were generated for dithizone-stained and unstained preparations, and an additional research model for unstained rat islets is available upon request. The current platform version and training date are displayed in the user interface. *IsletNet* automatically segments islets and exocrine tissue and subsequently calculates islet number, volume, and purity using the user-defined pixel size and minimum islet size threshold. The islet size threshold choice depends on image quality and purpose of the analysis (side-by-side comparison with the manual counting vs. study of small islets) rather than species. Given the resolution of conventional microscopy, the meaningful minimum islet size is 50 μm ^{7–9,12,14}, but it can be reduced up to 10 μm for images obtained with an appropriate instrument¹⁰. Islet volume estimations are generated using the standard sphere-based conversion table¹³ as well as alternative conversion models^{7,18,19}, including fitted ellipse approximations^{5,6} and contour-based Spinacle and Sphiracle models¹⁷. The resulting values are reported in islet equivalents and nanoliters, while purity is calculated from either tissue area or estimated tissue volume. A critical step in the protocol is the acquisition of standardized microscopic images suitable for automated segmentation. *IsletNet* was trained using images acquired with stereo and inverted microscopes equipped with a range of complementary metal-oxide-semiconductor (CMOS) cameras and illumination systems. Low-magnification objectives (1 $\times$ –2 $\times$) are advantageous because they allow the complete sample to remain within the field of view while maintaining a pixel size compatible with reliable detection of small islets. Consistent illumination, stable white balance, and avoidance of image overexposure are essential for reliable contour generation. The representative results demonstrate that retraining using standardized representative local images can substantially improve segmentation performance across heterogeneous imaging conditions. As a practical guideline, images are generally suitable for training when islet and exocrine tissue boundaries are clearly distinguishable to an experienced observer. Images containing clustered or overlapping islets should be avoided whenever possible.

The protocol also highlights several limitations and troubleshooting considerations associated with automated image analysis. Although *IsletNet* substantially increases throughput and reduces operator subjectivity compared with fully manual counting, the method remains sensitive to image quality. Incorrect contour placement, spurious islet detection, and failure to separate adjacent islets may occasionally occur and can affect downstream quantification. Consequently, diligent vQC remains essential before accepting automated results. Immediate on-site application to *IsletNet* is advantageous because unsuitable images can be reacquired with adjusted

imaging parameters. Manual correction of unseparated adjacent islets is possible within the Inspection window; however, misplaced automatic separation lines cannot currently be edited. In addition, the automated separation algorithm is based on contour geometry and islet size distribution statistics and may therefore differ from an individual expert's initial interpretation while still remaining analytically acceptable.

The utility of *IsletNet* is determined primarily by consistency between local image quality and the training dataset rather than by species alone. Although the current training set contains human islets of varying purities and purified rat islets, the platform may also perform adequately with additional preparations when image characteristics remain compatible with the training data. For example, porcine islets purified by culture may resemble embedded human islets present in the training dataset. In contrast, extensively cultured islets with weak dithizone staining were not represented during training and may require additional retraining. The Simple Comparison tool, therefore, serves as an important validation step for new users or new imaging configurations. The representative results further demonstrate that differing expert interpretations of embedded islets can influence GT generation and downstream volume estimation. To address the absence of broad expert consensus in future multicenter studies, the IsletSwipe application²⁰ was developed to facilitate consensus-based image annotation.

Compared with conventional manual counting under a microscope, *IsletNet* provides substantially higher throughput, permanent digital documentation, and standardized quantitative outputs while reducing operator-dependent variability. Manual counting may still provide greater confidence in tissue identification in borderline cases, but it is limited by subjective size estimation, lower scalability, and extensive personnel training requirements. As a web-based service, *IsletNet* enables inter-laboratory comparability by centralized analysis. Analysis speed remains dependent on local internet bandwidth, image file size, and image complexity. Larger TIFF files may improve image fidelity but require longer upload and download times compared with compressed JPEG images.

Overall, the results presented here demonstrate that reliable automated quantification can be achieved when local image acquisition remains reproducible and consistent with the training set. Users are encouraged to perform careful vQC and to help retrain the platform whenever substantial changes in local imaging conditions are introduced.

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DISCLOSURES:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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