


<p>Micronuclei and other nuclear abnormalities in mussel haemocytes: Inter-laboratory calibration</p> <p>FINAL REPORT 22/05/2019</p>	 <p>BEQUALM Biomarker Programme Office NIVA, Gaustadalléen 21, NO-0349, Oslo, Norway</p>
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1. Introduction

The Biological Effects Quality Assurance in Monitoring (BEQUALM) programme was original started in 1998 as an EU funded research programme. The main aim of the programme was to develop a quality assurance (QA) system for biological effects techniques that are used in national and international monitoring programmes. 'Biomarkers' was one of the three QA systems under the BEQUALM programme, which is currently managed by the Norwegian Institute for Water Research (NIVA).

Biomarkers to be used for national or international monitoring programmes should be subject to appropriate internal and external Analytical Quality Control (AQC), to ensure results produced are comparable with other laboratories and particularly since AQC is a requirement for submitting data to the ICES database. This report describes an inter-calibration exercise on nuclear abnormalities in prepared haemocyte samples of the blue mussel (*Mytilus edulis*). The nuclear abnormalities included, micronuclei formation as well as nuclear buds and bi-nucleated cells. Micronuclei formation is a core biomarker within the ICES integrated monitoring and assessment strategy and used widely within European environmental laboratories.

2. Participating laboratories

There were 9 participating laboratories. This report presents the results submitted by all 9 laboratories. The laboratories were identified by Lab code in order to keep the intercalibration anonymous.

3. Approach

3.1. Test material

Test material, consisting of mussel haemocytes, were prepared by the University of Stavanger. The mussel haemocytes used in the assessment were from the Norwegian offshore Water Column Monitoring (WCM) programme of 2017, where mussels were transplanted around oil and gas platforms (Statfjord A and B) in the North Sea and exposed for 6 weeks to produced water. However, the images taken for the assessment did not represent a particular exposure group but were selected randomly from different mussel samples.

3.2. Haemocyte preparation

Mussel haemolymph (ca. 400 µl) was extracted from the posterior adductor muscle with a syringe filled with 200 µl of seawater + EDTA solution. The needle was removed, and samples transferred to Eppendorf tubes on ice. Using a cytospin (2 min at 800 rpm), the mussel haemolymph was transferred to labelled microscope slides and left to dry at room temperature. The haemocytes were fixed in methanol for 15 min, air dried at room temperature and stored in a microscope slide box until required for staining. The slides were stained with 3 % (v/v) Giemsa solution for 10 min and rinsed twice with tap water. Cover slips were attached to the slides using DPX Mounting Media. The slides were left to dry for 24 h at room temperature and were ready for scoring.

3.3. Microscopic images

The photographic images of haemocytes were taken with a digital camera connected to a light microscope (Olympus IX71 inverted microscope, 1000 x magnification). A total of 179 images were taken, with each image containing between 20 and 100 cells.

3.4. Instructions to participants

An email was sent out to each participant, which included a link to the location of the 179 images. In addition, an excel sheet template was included to score the following observations for each image: 1) number of cells assessed (viable cells); 2) number of micronuclei in viable cells; 3) number of nuclear buds in viable cells; and 4) number of bi-nucleated cells in viable cells.

Guidelines on the assessment of viable cells and the different nuclear abnormalities were available from the scientific literature. However, participants were advised to read Bolognesi and Fenech, (2012) which included information on the criteria for identifying and scoring different cell types and nuclear abnormalities in haemocyte preparations.

4. Data assessment

For a statistical comparison between the participating laboratories for the three nuclear abnormalities, individual z scores were calculated. The z scores were calculated using the formula:

$$z \text{ score} = \frac{(\text{measured value} - \text{mean value from all laboratories})}{\text{standard deviation from all laboratories}}$$

An assessment criterion for each z score was based on the ISO/IEC 17043:2010 guidelines:

z score < 2	satisfactory
2 < z score < 3	questionable
z score > 3	unsatisfactory
z score > 6	extreme

5. Results and discussion

The main results of the inter-calibration are presented, with each laboratory submitting data on the number of 1) viable cells, 2) micronuclei, 3) nuclear buds and 4) bi-nucleated cells in all 179 images. The total number of these features in all images and the total number normalised to 1000 cells, as typically presented in the scientific literature, are displayed in Table 1.

The data showed differences between the number of viable cells that were assessed by the different laboratories. Highest numbers of cells that were considered viable and assessed were by LAB3 at 6772 cells, whilst LAB9 assessed only 498 cells from the 179 images. Differences in the total number of micronuclei ranged from 18 (LAB8) to 459 (LAB6), whilst nuclear buds and bi-nucleated cells ranged from 14 (LAB9) to 379 (LAB3) and 7 (LAB1, LAB9) to 41 (LAB4).

Table 1. The total number of nuclear abnormalities reported by each laboratory.

Lab code	# cells assessed	# Micronuclei	# Nuclear Buds	# bi-nucleated cells	MN/1000 cells	nuclear buds/1000 cells	bi-nucleated cells/1000 cells
1	4442	160	28	7	36.02	6.30	1.58
2	6431	301	350	33	46.80	54.42	5.13
3	6772	218	379	27	32.19	55.97	3.99
4	2191	110	79	41	50.21	36.06	18.71
5	2537	132	124	33	52.03	48.88	13.01
6	3200	459	103	25	143.44	32.19	7.81
7	964	74	33	20	76.76	34.23	20.75
8	687	18	37	24	26.20	53.86	34.93
9	498	53	14	7	106.43	28.11	14.06

Despite the large range in the number of cells classified as viable between laboratories (6772 to 498), all z scores were satisfactory (<2 , Table 2). For the total number of micronuclei in all images, only one laboratory (LAB6) recorded a z score outside ± 2 indicating questionable results. Whilst for the total number of nuclear buds and the total number of bi-nucleated cells, all z scores were satisfactory ($<\pm 2$).

When micronuclei were normalised to 1000 cells, LAB6 had a z score indicating questionable results ($>\pm 2$, Table 2, Figure 1). Seven of the nine labs had micronuclei/ 1000 cells with a z score within ± 1 .

When nuclear buds were normalised to 1000 cells, LAB1 had a z score indicating questionable results ($>\pm 2$, Table 2, Figure 2). Seven of the nine labs had nuclear buds/ 1000 cells with a z score within ± 1 .

When bi-nucleated cells were normalised to 1000 cells, LAB8 had a z score indicating questionable results ($>\pm 2$, Table 2, Figure 3). Once again, seven of the nine labs had bi-nucleated cells/ 1000 cells with a z score within ± 1 .

It should be noted however, that the z scores described as questionable had z scores only marginally outside the benchmark of ± 2 .

Table 2. Z scores calculated for the values reported by each laboratory. Values in yellow highlight those laboratories outside ± 2 for the different endpoint, indicating questionable results.

Lab code	Cells assessed	# micronuclear	# Nuclear Buds	# bi-nucleated cells	Micronuclei/ 1000 cells	nuclear buds/ 1000 cells	bi-nucleated cells/ 1000 cells
1	0,58	-0,07	-0,71	-1,49	-0,70	-2,01	-1,12
2	1,42	0,95	1,60	0,77	-0,42	0,96	-0,78
3	1,56	0,35	1,80	0,25	-0,80	1,05	-0,89
4	-0,38	-0,43	-0,35	1,47	-0,34	-0,17	0,51
5	-0,23	-0,27	-0,02	0,77	-0,29	0,61	-0,03
6	0,05	2,09	-0,18	0,08	2,06	-0,41	-0,53
7	-0,90	-0,69	-0,68	-0,36	0,34	-0,29	0,71
8	-1,01	-1,09	-0,65	-0,01	-0,95	0,92	2,07
9	-1,09	-0,84	-0,81	-1,49	1,11	-0,66	0,07

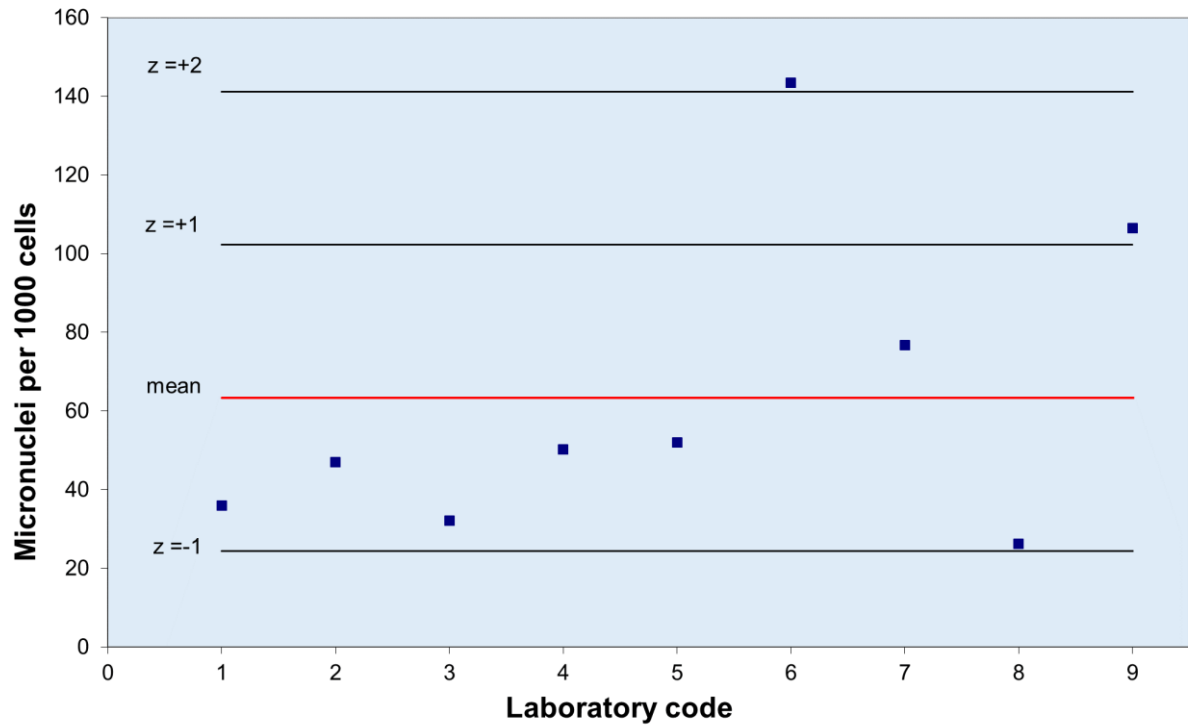


Figure 1. Micronuclei per 1000 cells reported by each laboratory from microscopic photographic images. Red line represents the mean value, whilst the black lines denote the appropriate Z score values.

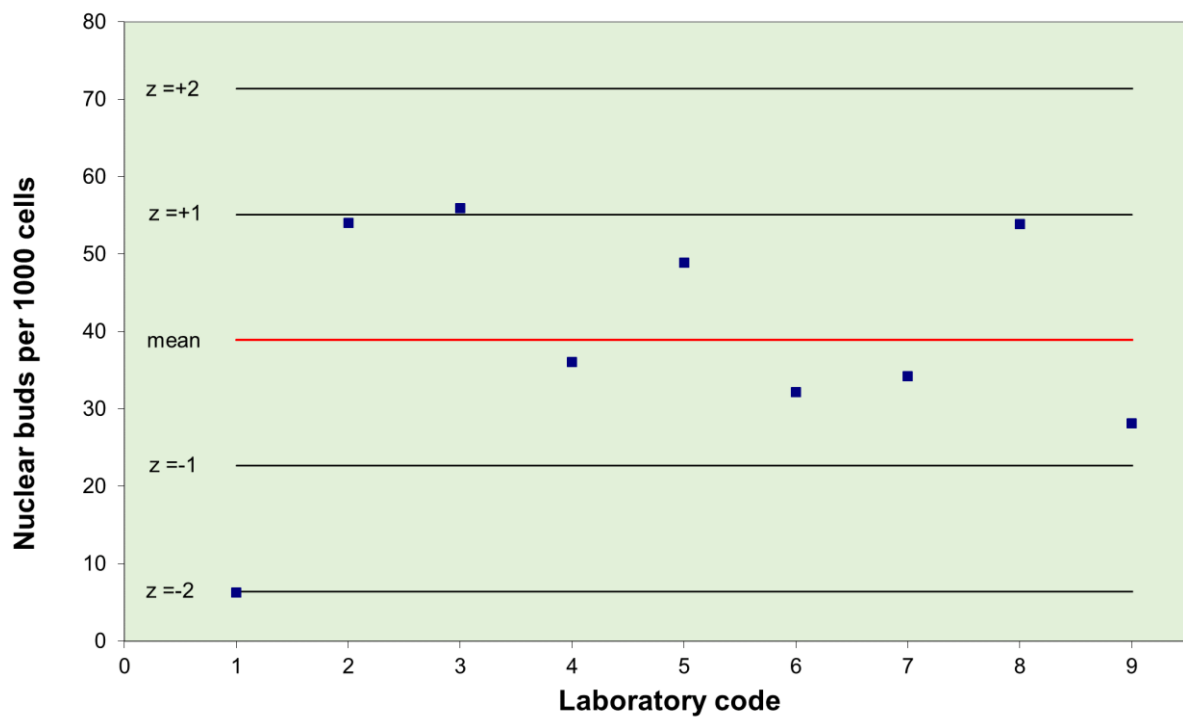


Figure 2. Nuclei buds per 1000 cells reported by each laboratory from microscopic photographic images. Red line represents the mean value, whilst the black lines denote the appropriate Z score values.

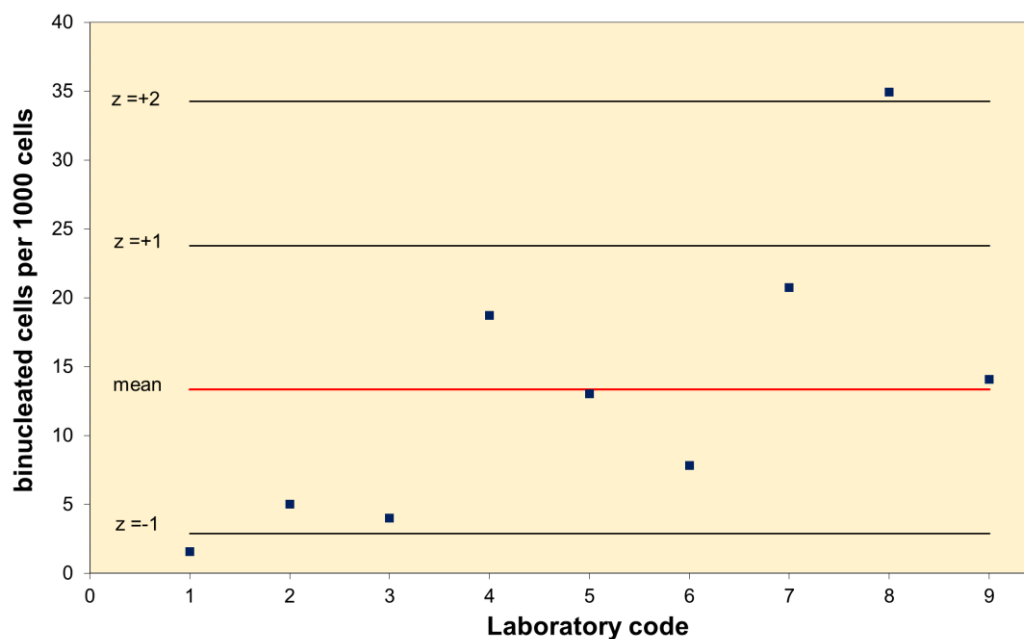


Figure 3. Binucleated cells per 1000 cells reported by each laboratory from microscopic photographic images. Red line represents the mean value, whilst the black lines denote the appropriate Z score values.

6. Preliminary conclusions

- This inter-calibration exercise showed that inter-laboratory differences exist.
- Large variation between the number of viable cells, indicated that identifying granular (non-viable) from agranular cells was not achieved by all laboratories. However, despite this, z scores indicated satisfactory results for this assessment.
- There was reasonable agreement between laboratories in the frequency of micronuclei, nuclear buds and binucleated cells when normalised to 1000 cells, although in all cases one laboratory was found to have a z score marginally outside ± 2 , indicating questionable results.
- The results should be used by participating laboratories to assess their internal protocols.
- No statement of performance will be issued for this inter-calibration.

7. References

- Bolognesi C, Fenech M. 2012. Mussel micronucleus cytome assay. *Nature Protocols* 7: 1125-1137.
- ISO/IEC 17043: 2010. Conformity assessment -- General requirements for proficiency testing.

APPENDIX

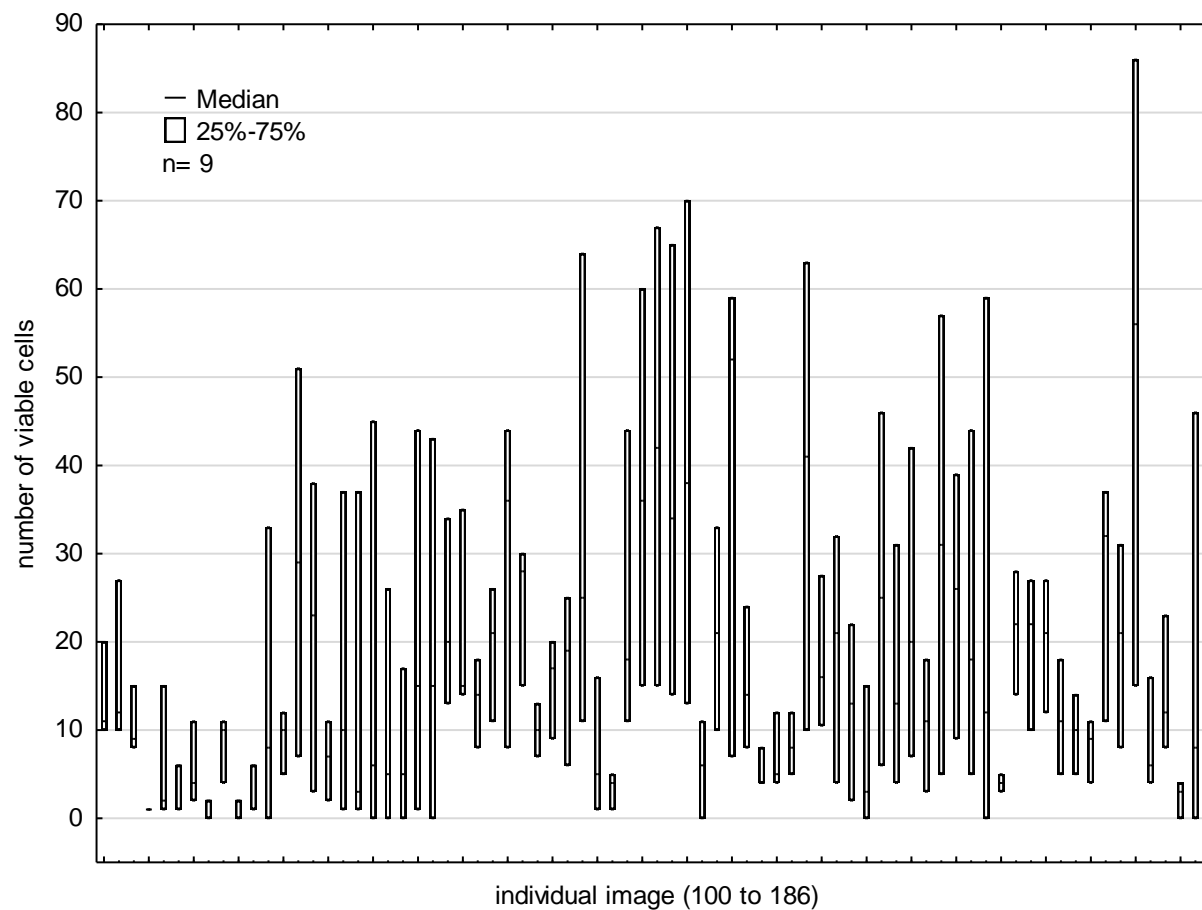


Figure A1. The number of viable cells recorded for each photographic image (image 100 to 186) for all nine laboratories (median, quartile, n=9).

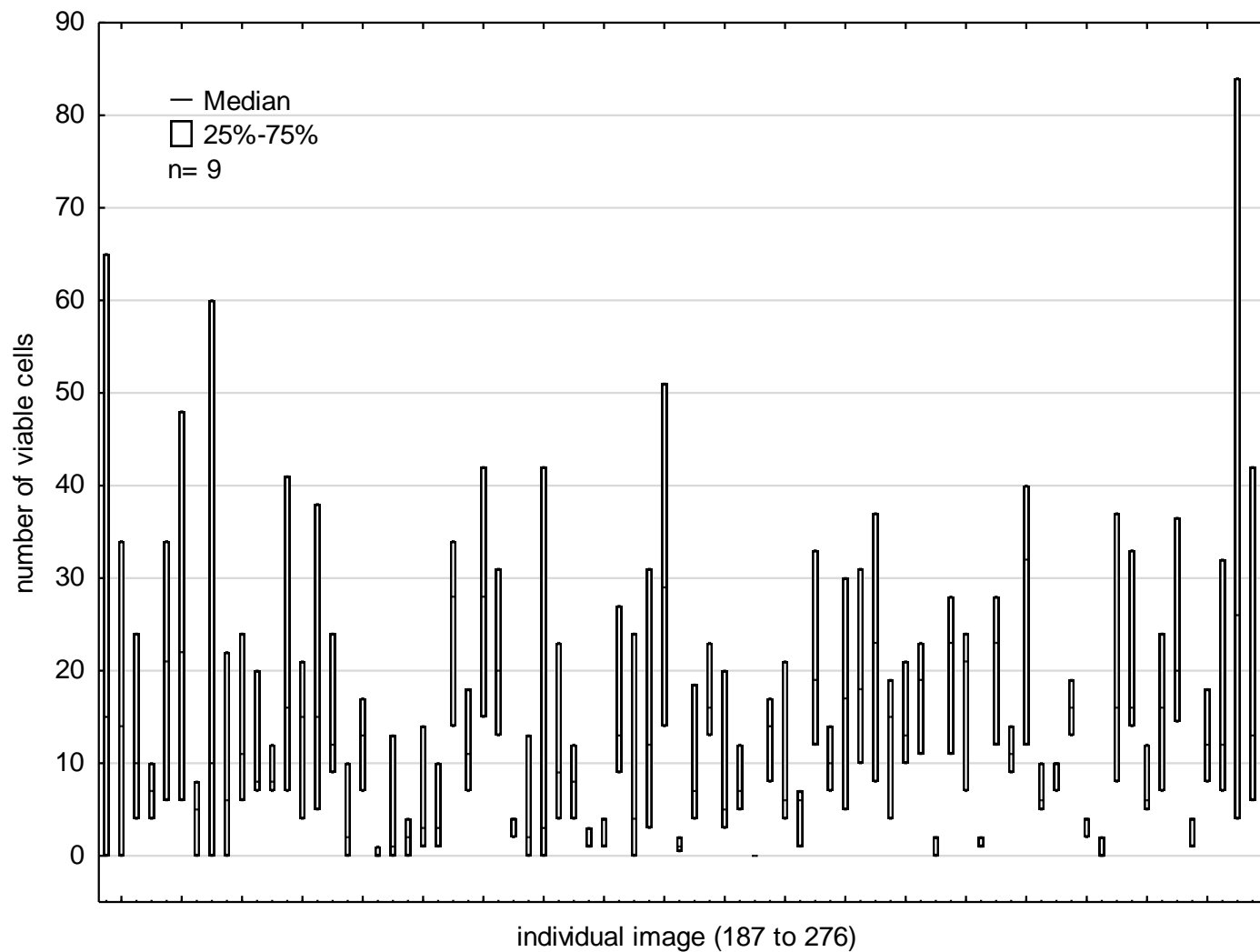


Figure A2. The number of viable cells recorded for each photographic image (image 186 to 276) for all nine laboratories (median, quartile, n=9).

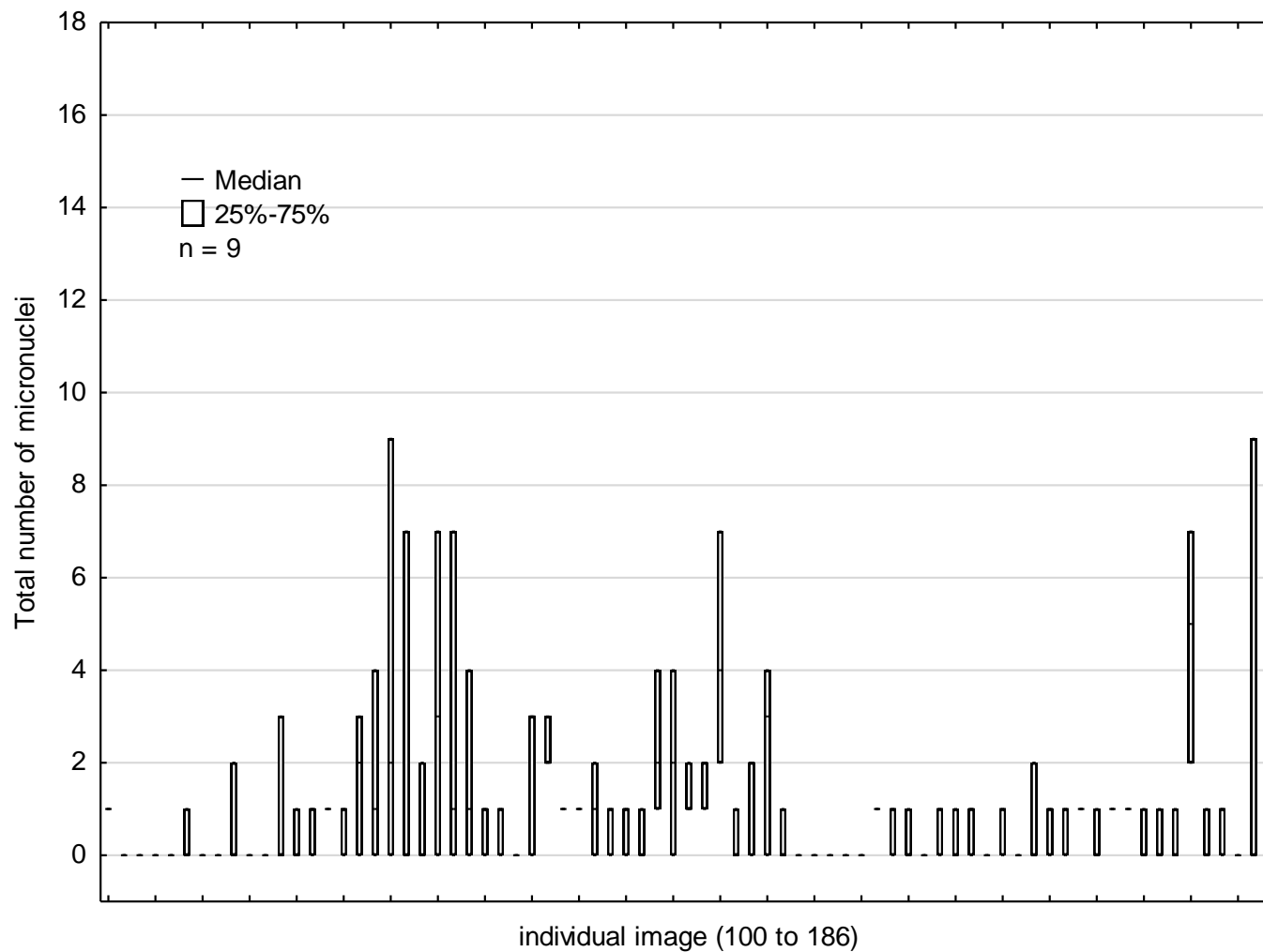


Figure A3. The total number of micronuclei recorded for each photographic image (image 100 to 186) for all nine laboratories (median, quartile, n=9).

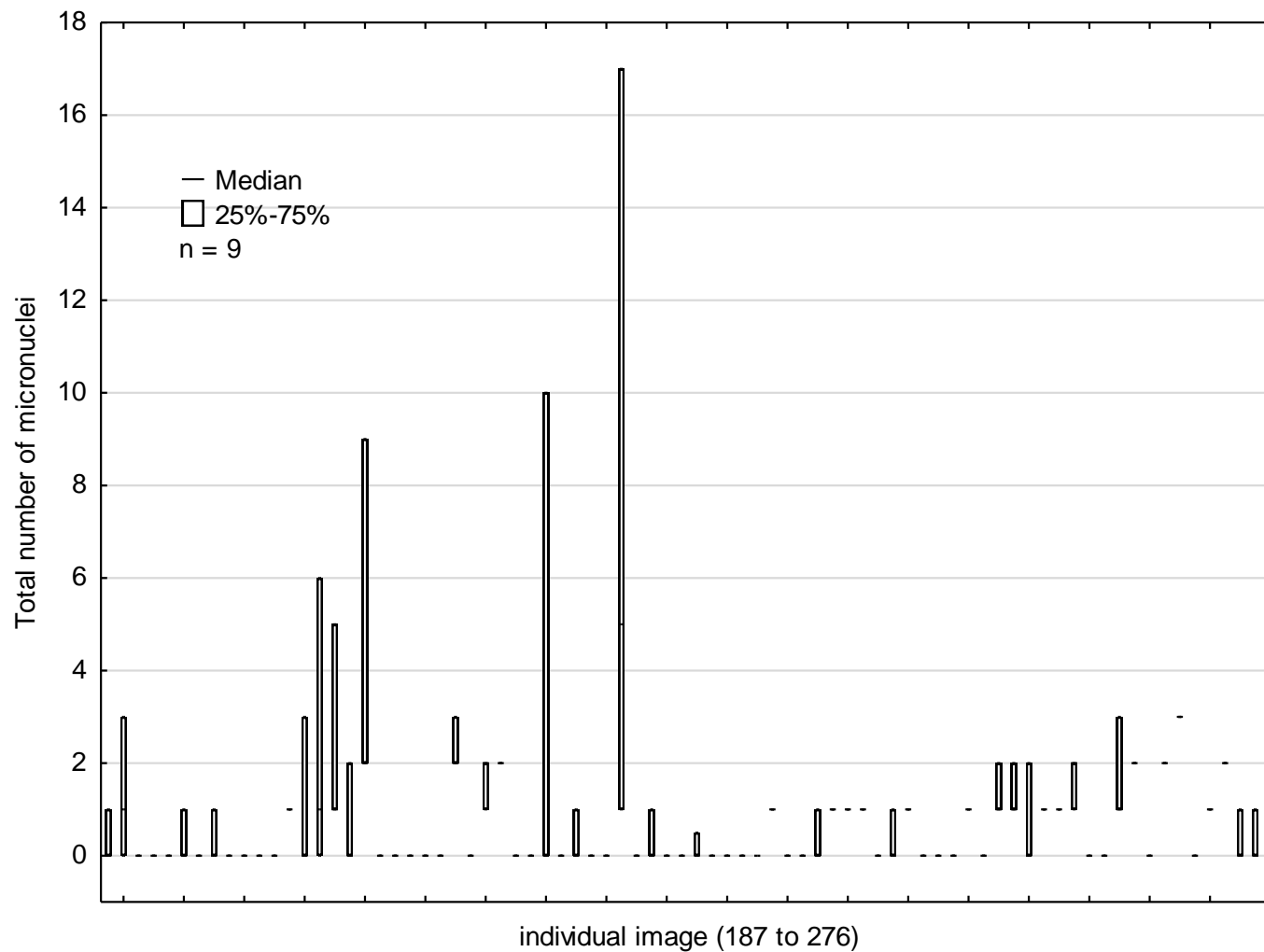


Figure A4. The total number of micronuclei recorded for each photographic image (image 187 to 276) for all nine laboratories (median, quartile, n=9).

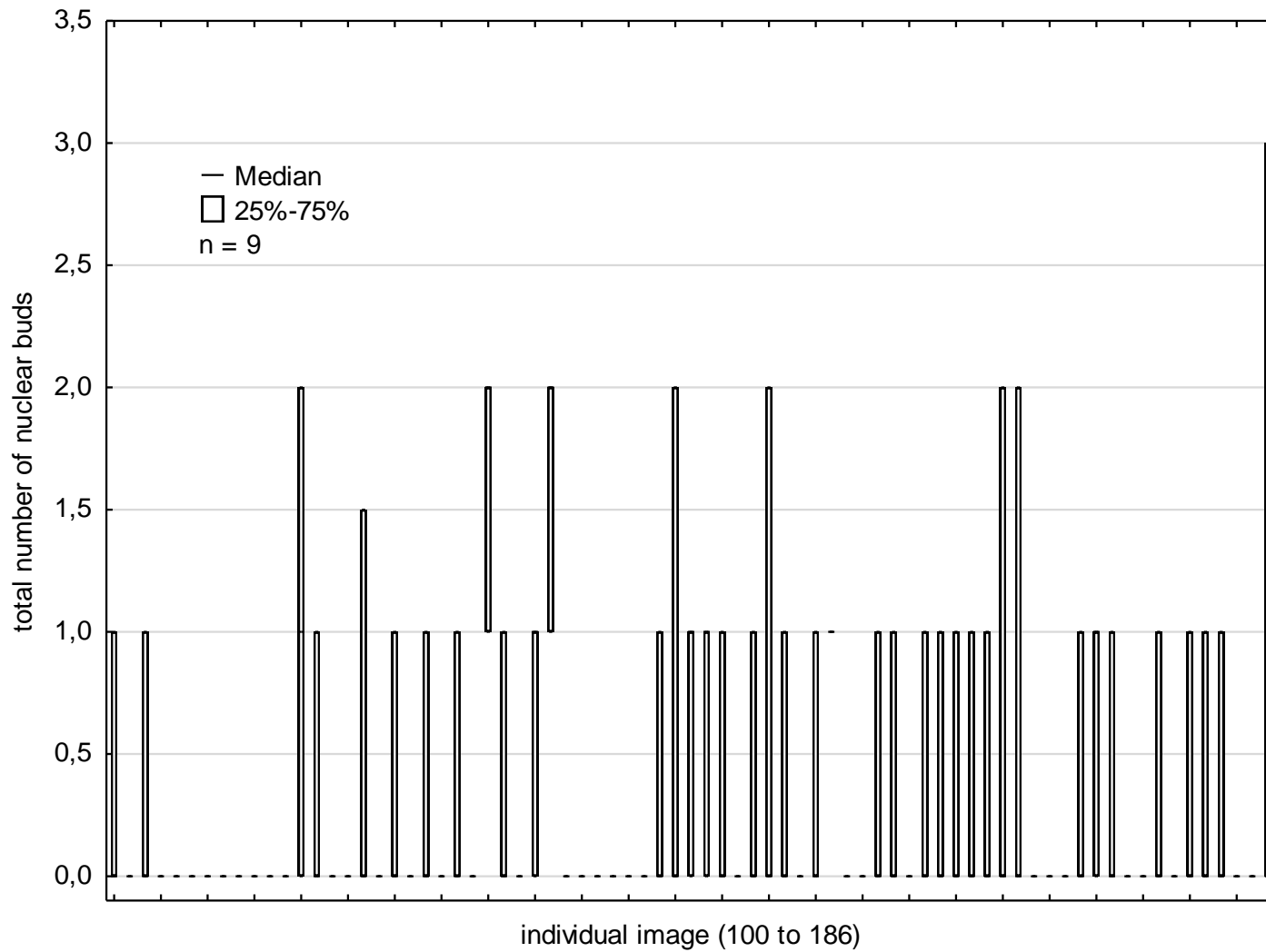


Figure A5. The total number of nuclear buds recorded for each photographic image (image 100 to 186) for all nine laboratories (median, quartile, n=9).

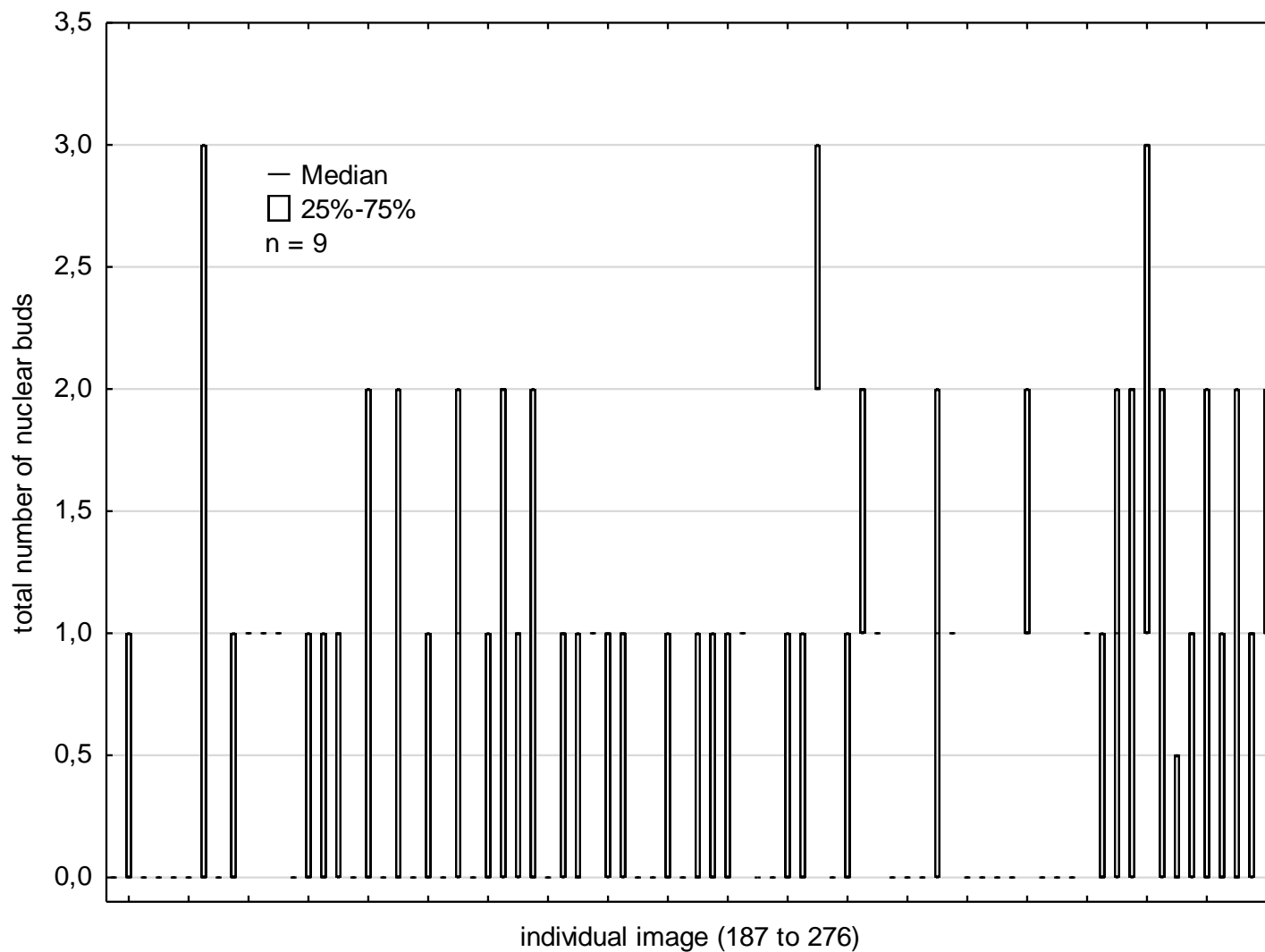


Figure A6. The total number of nuclear buds recorded for each photographic image (image 187 to 276) for all nine laboratories (median, quartile, n=9).

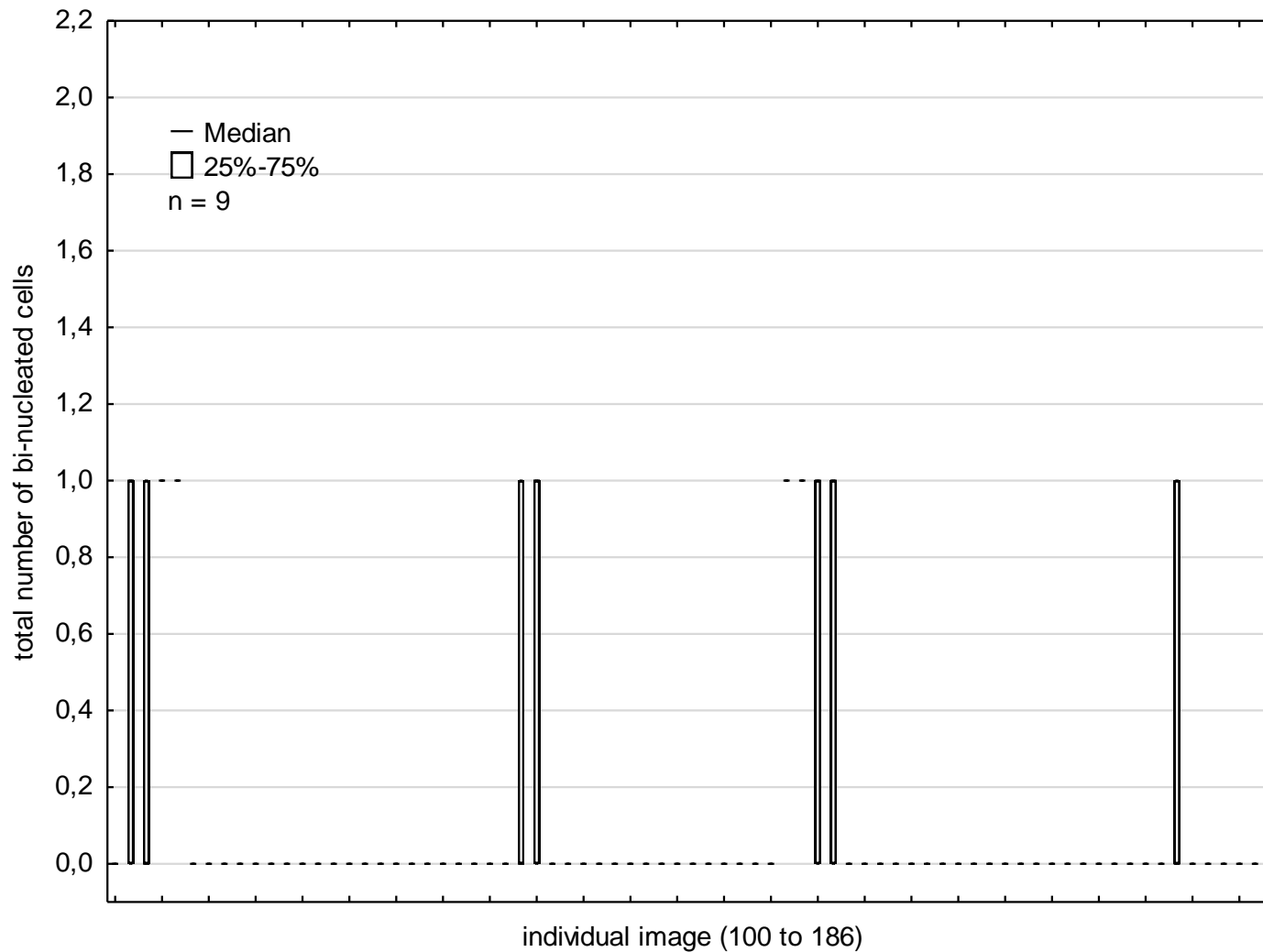


Figure A7. The total number of bi-nucleated cells recorded for each photographic image (image 100 to 186) for all nine laboratories (median, quartile, n=9).

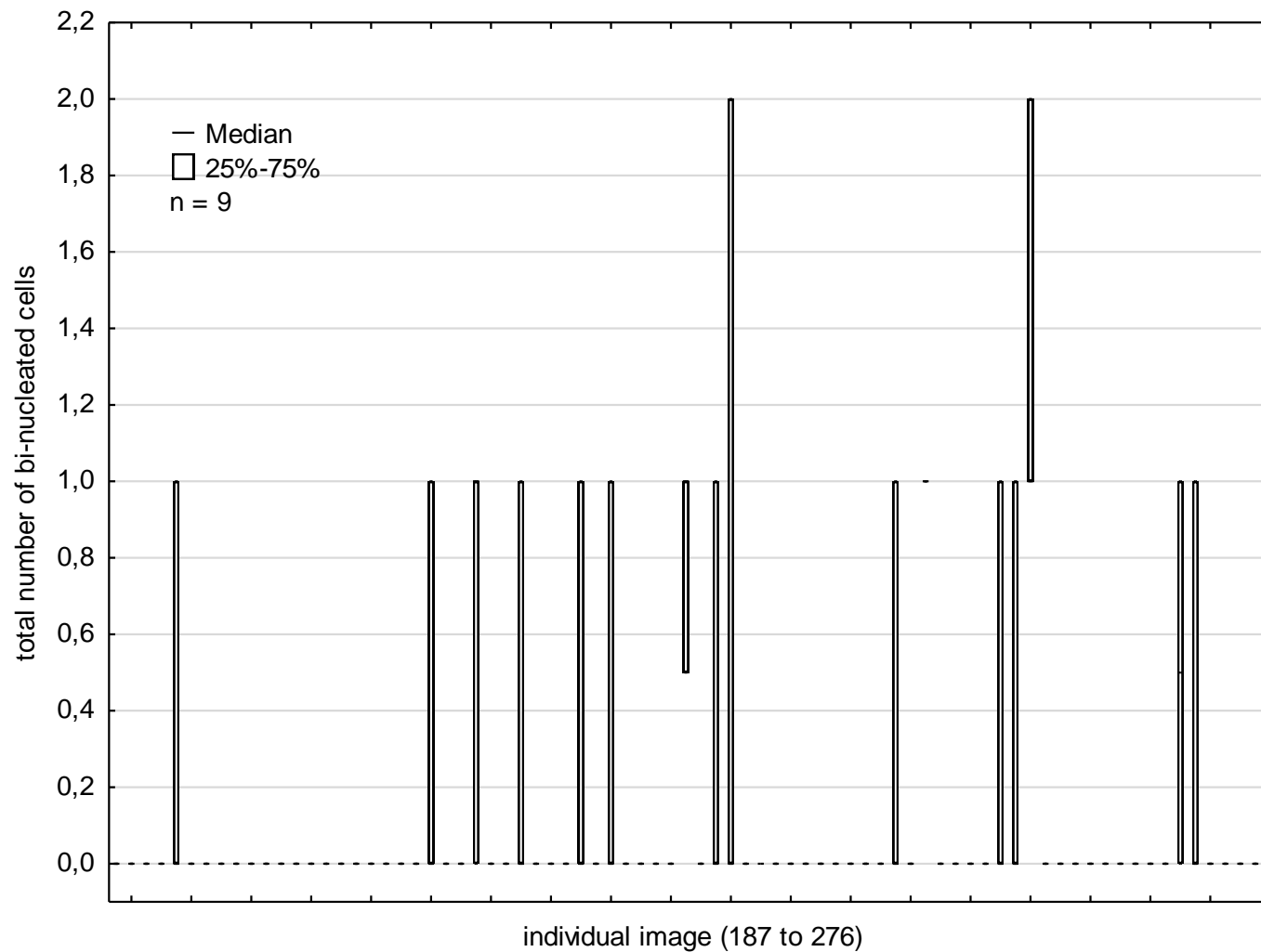


Figure A8. The total number of bi-nucleated cells recorded for each photographic image (image 187 to 276) for all nine laboratories (median, quartile, n=9).