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The Validation of Routine Analytical Methods in Histotechnology: A Practical Approach

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Abstract. The present studies have the purpose to in house validation process in histotechnology for routine methods. The samples used were different types of tissue for quality control (QC) including positive and negative control. For calculation of the performance parameters has establish a grading scale for staining quality and an general quantification scale of the elements in slide samples. For proper internal control for quality assurance/ quality control (QA/QC) can be used: kidney, liver, skin and small intestine tissue and specific positive control and negative tissue control, when it is applicable. Regularly using of these samples and monitoring of theirs characteristics through control chart can assure the QA/QC and fitness to purpose of the methods.

Key words: validation, quality control, histotechnology

INTRODUCTION

To assure a valid result from a measurement test and to reduce sources of error as much so as they not affect the results of analysis, thus ensuring that these parameters reflect animals health status are mainly purpose of the laboratories. Because in the course of the test, many uncertainty factors can be involved, and many of them cannot be appreciated, a validation is mandatory.

Analytical method validation is an important process and treated as such both international and national agencies and industry committees (ISO, CE, U.S. FDA, EPA, EMEA, AOAC, COFRAC, etc.). This process must provide that the analytical methods used for a specific test are suitable for use again. Results obtained during validation of the method can use to assess the quality, reliability and consistency of analytical results. This process is part of good laboratory practice (GLP). (9, 13, 14) In histotechnology the validation is very different related with the chemical methods because of its particularity.

Providing certainty that laboratory tests are accurate require the use of appropriate reference materials to validate the method (developed by laboratory methods), calibration equipment, ensure traceability, measurement uncertainty estimation (depending on the analytical method), and quality control / quality assurance . Thus, in choosing and using an analytical method, it must meet the desire of "fitness for purpose", which means that the result obtained by this parameter and test time is comparable with the results of any laboratory, any time, demonstrating that it ensured measurement traceability. Analytical method should be validated before its introduction into routine use, and revalidated whenever there were

changed the initial conditions (ex changes to equipment / software, staff changes, changes in the type of matrix or their sampling process and thus the species, changes in the concentration or volume of matrix available, demonstrating sensitivity –including selectivity, inclusivity and exclusivity- in the presence of concomitant potential sources of interference) or when the method is changed and this change is outside the scope initially or make significant changes in measurement. (15)

MATERIAL AND METHOD.

The validation was done for the histological processing of tissue samples (formaldehyde fixation) and for the following staining methods: haematoxylin - eosin (HE), Mann (M), Ziehl-Neelsen (ZN), Perls(P), haematoxylin eosin – methyl blue (HEA), haematoxylin eosin – light green (HEV), alcian blue (AA), alcian blue periodic acid Schiff (AAPAS), Brown and Brenn (BB), Levaditi (L), Pappenheim (PP) and Gridley (G).

Table 1.

The tissue used for the staining methods

NR CRT	TISSUE	HE	HEA	HEV	PP	AA	AA PAS	M	P	ZN	BB	G	L
1.	Brain negative Babes- Negri body	X		X				X					
2.	Brain positive Babes- Negri body		X		X			X	X				
3.	Epididymis		X									X	
4.	Heart positive Sarcocystis	X	X										
5.	Kidney	X	X	X	X								X
6.	Liver negative AF bacilli									X			
7.	Liver positive AF bacilli									X	X		
8.	Liver positive Perls	X					X		X				
9.	Lung		X									X	
10.	Mammary tumor		X		X								
11.	Skin	X	X	X			X						
12.	Small intestine		X			X	X			X	X		

The validation process includes four main phases: development method, validation / optimization, validation itself, complete and check the report. There was selected the types of samples and sites, and their number of subsamples, as well as equipment and reagents to be used (available in sufficient quantities, clearly identified and sufficient to establish the exact composition and purity check their respect), and environmental conditions required. Equipment must be identified, calibrated in advance, as well as glassware that has an impact on measurement. (9, 13, 14)

The validation criteria used for the staining methods (2, 3, 4, 5).

NR CRT	ELEMENT	HE	HEA	HEV	PP	AA	AAPAS	M	P	ZN	BB	G	L
1.	Nuclei	blue -violet (HM) violet-reddish (HH)	violet dark	violet dark	Violet, tumoral elements blue-violet to violet dark	red	Grey/ blue		red		Different nuance of pink - red to blue		
2.	Cytoplasm	pink	pink to red	pink to red	blue				pink				
3.	Pericarion							blue					
4.	Neuropil							pink					
5.	Red blood cells	carmine	red- carmine	red- carmine	red			orange		carmine			
6.	Fundamental substances	pink	blue light	blue light	blue pal to violet								
7.	Cartilage				violet								
8.	Hyaline	pink- red	blue-pal, pink, pink-red, red	blue-pal, pink, pink-red, red						red			
9.	Connective tissue fibers	pink dark			blue- violet								
10.	Collagen fibers		blue nuanced	blue nuanced									
11.	Elastic fibers											blue	
12.	Muscle fibers	red	red	red	pink- red								
13.	Fibrin	pink											
14.	Fibrinoid	pink- red	blue-violet nuanced	blue-violet nuanced									
15.	Acid fast bacilli									red light			
16.	Bacterium		blue-violet dark	blue-violet dark				Blue dark					
17.	Gram negative Bacterium										red		
18.	Gram positive Bacterium				blue						blue		
19.	Conidia											pink- purple	
20.	Fungi				pink- bluest violet							blue	
21.	Parasites		blue-violet-pink nuanced	blue-violet-pink nuanced	blue- violet dark (Sarcosporidia, Toxoplasma-nuclei red)								
22.	Spirochetes												black
23.	Russell body									red			
24.	Babes- Negri body							red magenta to red light, with blue dark granulations					
25.	Acidophil granulations		red -pink- red	red -pink- red	red								
26.	Basophile granulations		blue< blue - violet	blue< blue - violet	violet pal								
27.	Basophile viral inclusions		blue pal	blue pal									
28.	Oxiphile viral inclusions		pink pal- red	pink pal- red									
29.	Arsen	green											
30.	Calcium		violet nuanced light	violet nuanced light									
31.	Iron (free or in siderocyte)								blue Prussia				
32.	Keratin		red	red						red			
33.	Mucin											blue	
34.	Acid mucins					blue	blue						
35.	Neutral mucins						magenta						
36.	Other tisular elements									blue	yellow	yellow	yellow

Classification of analytical methods based on performance parameters to be determined (7)

		Detection limit CCbeta	Decision limit CCalpha	Trueness/ Recovery	Precision	Sensitivity /Specificity	Applicability/ Stability
Qualitative method	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative method	S	+	-	-	+	+	+
	C	+	+	+	+	+	+
S= screening methods; C= confirmatory methods; += determination is mandatory							

For each method in hand, analytical requirements and characteristics of corresponding elements of its performance were established. The main elements assessed for validation are different from each type of stain but the most are: nuclear details- nucleoli, chromatin (either vesicular and hyperchromatic nuclei)-, cytoplasm, collagen, muscle (to see the form of fibrillar pattern), red blood cells, mucin, bacterium, iron, and Babes- Negri body (0). For establishing the appropriate acceptable responses range for either positive and negative samples, were used internal positive sample control and internal negative sample control (brain positive for Babes- Negri body, brain negative for Babes- Negri body, tuberculoses positive sample, tuberculoses negative sample, Perls positive liver samples, Perls negative liver samples, bacterium positive samples, bacterium negative samples, small intestine – positive control for AA and AAPAS-, tissue negative for AA and AAPAS, skin, kidney), or was done with arbitrary internal control samples. (2, 3, 4, 5)

For validation and quality control (QC) the following were used: kidney- identification of basement membranes, dense chromatin cells (glomerular), sparse stain chromatin cell (collecting tubules); small intestine- mucin staining, vesicular nuclei (epithelial cells), nuclear chromatin specific arrangement, bacterium-, skin- keratohyaline granules, collagen, degree of definition of the reticular/ papillary border of the dermis, form of fibrillary pattern of muscle, red blood cells- liver- Perls positive granules/ siderocytes, nuclear membrane, fast acid bacilli- brain Babes- Negri body, negative control for Perls, Babes- Negri body, mucin and bacterium. In all kind of tissue the arteriole can be use like a key regarding of staining quality because its structure: endothelium, basement membrane, internal elastic lamina, media with one/ two layers of smooth muscle cells, external elastic lamina, adventia with a few elastic and collagen fibers, and the itralumenal red blood cells.

The sample tested was in 12 replicas for each type of stain. Two different operators had tested each a lot with six replicas from each type of samples (0). For each method, validation had been used comparation of its performances with two or more techniques for same samples (0).

The performance parameters differ depending on the analytical method, and mainly used are following accuracy, precision, specificity, detection limit, quantification limit, linearity, robustness, recovery. (0, 0). In the veterinary laboratories, not all mentioned parameters have significance in the evaluation of analytical methods. Within-laboratory, the most commonly used parameters are: repeatability (precision), intermediate precision, accuracy, Chi-square, sensitivity, specificity, positive predictive value, negative predictive value, false positive rate, false negative rate, detection limit and limit of quantification, CCalpha, CCbeta and coefficient of variation. (1, 9, 11, 12, 13, 14) In histotechnology can be use almost all that had been specified previously if the analyte can be quantified. For this purpose values for expression of each element were given. There was assigned for the absence of staining for element like false logical value and for the

characteristic staining of element like true logical value. The logical value was converted in numeric value: each logical false value became value 0 and each true value become value 1. For different grading of positivity, arbitrary values in the interval 0 to 1 were assigned For monitoring the analytical performance, can be used a control chart for the representative elements of each stain with their values according to grading scale (0).

The fitness for purpose of analytical methods a laboratory guide to method validation and related topics - modified-(9)

Type of method: qualitative, semi-quantitative, quantitative/ screening or confirmation	Confirmation of identity, sensitivity/specificity Limit of detection Limit of quantification Ccalpha CCbeta
The distribution of the analyte: dispersed / localized, homogeneous / heterogeneous	
The presentation form of the analyte (analytes) as one / more than one form, the importance of its form: extraction / free / total, etc.	Confirmation of identity, Recovery
Analyte identification (analytes) of interest and the most likely to present (%, μgg^{-1}, ngg^{-1}, etc)	Confirmation of identity, sensitivity/specificity Limit of detection Limit of quantification Ccalpha CCbeta Limit of quantification Working & linear ranges
The result: the precision required, level of uncertainty acceptable, way of expressing the precision / uncertainty	Recovery Accuracy / trueness Repeatability precision Reproducibility precision
Matrix and characteristics: chemical, biological or physical; analyte interference likely	sensitivity /specificity
Sampling and sub sampling (if necessary, and how they can affect the outcome)	
Restrictions (if any) related to the size / availability of sample	
Prerequisites related to resources, (how), staff time, financial, equipment and reagents, laboratory equipment	
The results are comparative inter-laboratory	Ruggedness/robustness Reproducibility precision
Performance comparable to external specifications	Accuracy Reproducibility precision

The results were divided in true positive N_{11}; false positive N_{21}; false negative N_{12}; true negative N_{22}. The performance of the method was appreciated through repeatability, intermediate precision, accuracy, sensitivity, specificity, Chi square, linearity, detection limit and quantification limit. For mathematical evaluation, MS Office Excel software was used

The grading scale for staining quality

SCORE	DESCRIPTION
0	The structure have not characteristic shape and/or stain
0.25	The structure have not characteristic shape, and no characteristic stain at X1000 field but the gross shape is almost specific and/ or have low distinct stain
0.50	The structure have characteristic shape, and no characteristic stain at X1000 field but the details are specific in shape and have distinct stain
0.75	The structure have characteristic shape, and no characteristic stain at X100 field but at 400X field the details are specific in shape and have appropriate stain

1	The structure are characteristic shape and stain
The + / - can be add before the value for abnormal hyperchromatic/ hypochromatic staining	

RESULTS AND DISCUSION

Repeatability (r) expresses the precision under the same operation condition over a short interval of time, and represents the closest extreme in an independent measurement with 95% confidence level. [1].

$$r = t_{5\%}^{n-1} * S_D \quad [1]$$

$\mathbf{t_{5\%}}$ = Student coefficient
$\mathbf{S_D}$ = standard deviation

$$S_D = \sqrt{\frac{\sum (Xi - X)^2}{n-1}} \quad [2]$$

$$\overline{X} = \frac{\sum Xi}{n} \quad [3]$$

The logical value were converted in numeric value: each logical false value became value 0 and each true value become value 1. For all methods the $S_{D\ (}$standard deviation) =0.00, implicit r (repeatability) =0.00, for each set, cu *repeatability limit = 1* (true value) ±0.

Intermediate precision (R) had been calculated by multiplying the repeatability with 1.6 an accepted coefficient. The results are qualitative and expressed like positive and negative. The logical value had transformed in numeric value: each logical false value became value 0 and each true value become value 1. The technique had $S_{D\ R(}$standard deviation) =0,00, implicit R (intermediary precision) =0,00, for each set, cu *intermediary precision limit = 1* (true value) ±0.

The accuracy (AC) is sometimes termed trueness, and result from the comparison of de values to the true value for the sample/ elements and had 1 (100%) in all tested methods [4].

The general classification of the samples

The samples status	The obtained results		Total
test	POSITIVE	Negative	
POSITIVE	N_{11}	N_{12}	$N_{1.}$
Negative	N_{21}	N_{22}	$N_{2.}$
Total	$N_{.1}$	$N_{.2}$	$N= N_{1.} + N_{1.}$ or $N_{.1} + N_{.2}$
$N_{11=}$ true positive; N_{12} = false negative; N_{21} =false positive; N_{22} =true negative			

$$AC = \frac{N_{11} + N_{22}}{N_{11} + N_{12} + N_{21} + N_{22}} \quad [4]$$

The Chi-square (^{2}) [5] reveal whether hypothesized results are verified by an experiment, and in our case it are almost absolute 0.00, (must be <3.84) in all tested methods.

$$\text{Chi - square}(\ ^2) = \frac{\left(\left|N_{12} - N_{21}\right| - 1\right)^2}{\left(N_{12} + N_{21}\right)} \qquad [5]$$

The general quantification of the elements in slide samples

Nr. crt.	Score	Description
1.	0	no elements found in the tissue
2.	0.2	≤ 10 elements per 100x field (5 fields average)
3.	0.4	11 ≤ elements ≤ 100 per 100x field (5 fields average)
4.	0.6	> 100 elements per 100x field but < 1 elements per 1000x oil immersion field (5 fields average)
5.	0.8	> 100 elements per 100x field but 1 to ≤ 10 elements per 1000x oil immersion field (5 fields average)
6.	1	> 11 elements per 1000x oil immersion field (5 fields average)

The sensitivity (p+/SE) [$P(T^+|D^+)$]calculated had been 1 for all methods, or can be expressed like 100% and reveal the probability that a true positive sample/ elements will be tested positive. p+=1**,** SE=100% [6]

The specificity (p-/SP) [$P(T^-|D^-)$]calculated had been 1 for all methods ,or can be expressed like 100% and reveal the probability that a true negative sample/ elements will be tested negative. p-=1, SP=100% [7]

$$\text{p+} = \frac{N_{11}}{N_{1\bullet}} \qquad [6]$$

$$\text{p}- = \frac{N_{22}}{N_{2\bullet}} \qquad [7]$$

Positive predictive value (Ppv)($D^+|T^+$)=$N_{11}/(N_{11}+N_{21})$ is the proportion of positive test sample/ element it is true positive, and it is 1 for all methods.

Negative predictive value (Npv)($D^-|T^-$)=$N_{22}/(N_{22}+N_{12})$ is the proportion of negative test sample/ element it is true negative, and it is 1 for all methods.

False positive rate pf+ is the proportion of negative instances that were erroneously reported as being positive .It is equal to **1** minus the specificity of the test: pf+=0 for all methods.[9]

False negative rate pf- is the proportion of positive instances that were erroneously reported as being negative .It is equal to **1** minus the sensibility of the test: pf-=0 for all methods.[8]

$$\text{pf-} = \frac{N_{12}}{N_{\bullet 1}} \qquad [8]$$

$$\mathrm{pf}+ = \frac{N_{21}}{N_{\bullet 2}} \qquad [9]$$

The detection limit has not been established. It can be done for the methods in the case it will give value using an arbitrary fashion like a scaling for quantification.

The quantification limit had been not established. However, arbitrary can be appreciated that it can be between up to 0.20.

In general, for routine histotechnology methods, the quantifications parameters are not mandatory, but they can be used for quality control (QC). If it used the value criteria, can be calculated the CCalpha, CCbeta and coefficient of variation.

$$CV = \frac{S_D}{\overline{X}} \qquad [10]$$

CV= coefficient of variation

CCalpha decision limit (CC α) represent the limit above which it can be concluded with type α error probability as the sample is not conform (positive).(7)

$$CC\alpha = LOD * 2.33 * \mathrm{STD\,R} \qquad [11]$$

LOD= detection limit

STD-R= standard deviation of reproductibility

CCbeta detection capability (CCβ) is the smallest amount that can be detected by measuring, identified and / or quantified in a sample of type β error probability**.** (7)

For monitor the quality of the methods can be used the control chart with reporting by the grading scale and elements quantification the representative tissue for each methods, and the positive and negative control if are applicable (M, ZN, P, BB, G, L). The following tissue for quality control can be used: kidney, liver, skin and small intestine. The quality control samples must be used each time when the stains solutions or equipment are changed, but not less than monthly for routine assays. The three replica of each kind of tissue/ slides can be enough for internal control. For the validation process in histotechnolgy the mainly performance parameters are repeatability, reproducibility, sensitivity, specificity. (3) The others parameters can be used if will be attributed value to the histological elements.

CONCLUSION

The validation process used in histotechnology and offer the advantage to assure an accurate result and it certify the fit for purpose of the method. During the validation process and quality monitoring of the assay can be establish the critical points of the methods. For proper internal control for QA/QC can be used: kidney, liver, skin and small intestine tissue and specific positive control and negative tissue control, when it is applicable.

BIBLIOGRAPHY

1. Adrian I. Ardelean, Monica Salagean, Duma Mihaela, 2008-The Validation Protocol Used In Haemagglutination Inhibition Tests For Avian Influenza And Newcastle Disease Diagnosis: A Practical Approach, Lucrări Ştiinţifice – vol. 51, ISSN 1454-7406,seria Medicina Veterinara, Iasi

2. Alexandru N.,1995 - Morfopatologie: Investigaţia histologică în diagnostic, tipo. L.C.S.V.D., Bucureşti
3. Alexandru Nicolae, Florica Barbuceanu, 2008 Ghid de morfopatologie: instructiuni de prelevare, validare metode, diagrame de diagnostic; Buletin Informativ de Morfopatologie nr.14, IDSA Bucuresti
4. Alexandru Nicolae, Florica Ristea, 2005 Buletin Informativ de Morfopatologie nr.7, IDSA Bucuresti
5. Alexandru Nicolae, Florica Ristea, 2007 Ghid de morfopatologie: instructiuni, metode, evidente; Buletin Informativ de Morfopatologie nr.12, IDSA Bucuresti
6. ANSVSA- ORDIN nr.17/ 30.03.2011 pentru aprobarea Normelor metodologice de aplicare a Programului acţiunilor de supraveghere, prevenire, control şi eradicare a bolilor la animale, a celor transmisibile de la animale la om, protecţia animalelor şi protecţia mediului, de identificare şi înregistrare a bovinelor, suinelor, ovinelor şi caprinelor pentru anul 2011, precum şi a Normelor metodologice de aplicare a Programului de supraveghere şi control în domeniul siguranţei alimentelor pentru anul 2011
7. Commission Decision of 12 August **2002** implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results,
8. EAL- G12 1995 Trasabilitatea rezultatelor masurarilor si a mijloacelor de incercare la etaloane nationale,
9. EURACHEM – 1998 The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics,
10. http://www.aoac.org/RI/ap5.pdf, consultat, 2011
11. ILNAS 2008 Guide sur la vérification et la validation des méthodes d'essais et d'étalonnage selon l'ISO/CEI 17025 ,
12. ISO/IEC 17025: 2005 General requirements for the competence of testing and calibration laboratories,
13. U.S. EPA, 1995 Guidance for methods development and methods validation for the Resource Conservation and Recovery Act (RCRA) Program, Washington, D.C..,http://www.epa.gov/sw-846/pdfs/methdev.pdf
14. U.S. FDA – 2000 Guidance for Industry (draft) Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls and Documentation,
15. U.S. FDA – 2001 Guidance for Industry, Bioanalytical Method Validation,