Report on the Cytology EQA Module: Review of sub-optimal immunostaining in Runs 106—109

Neil Bilbe and Irena Srebotnik Kirbis

Introduction

This small study looks at the outcomes of the 4 Cytology Runs for the 2014/2015 EQA year, with particular emphasis on the levels and reasons for slides with sub-optimal marks.

Overview

There were a maximum of 79 laboratories registered with the scheme for the Cytology module during the year, from 15 individual countries, of which approximately 60% were from the UK and Ireland. For three out of the four runs the submission rate* was 100% (107, 108, 109), but with some labs withdrawing, and some new registrants at the start of the EQA year, the submission rate for run 106 was 97%.

* When a participant returns at least one slide for a run.

Table 1: Overview of submissions and slides with sub-optimal staining (unacceptable)

Run	Markers	No of participants/ slides	Failed slides (%)	Neqas slide - R	In house slide -S	Neqas slide - T	In house slide - U
106	CD45, Ki67	73/285	8 (2.8 %)	3	2	2	1
107	CD45, Calretinin	79/305	10 (3.6 %)	0	5	2	3
108	CD45, Melanoma	78/303	7 (2.3 %)	1	3	1	2
109	CD45, CK	79/310	3 (1 %)	1	1	1	0

The majority of participants in the UK NEQAS ICC & ISH Cytology module are able to produce a good quality of ICC staining on standard NEQAS preparations, as well as on in-house slides (82% had no issues). However, there were a small number of slides where the quality of ICC staining was sub-optimal and assessed with low marks (4 - 9/20). In order to find any specific problems or common themes, we analysed the frequency and reasons for low marks during 2014 - 2015.

There were a total of 28 slides assessed with low marks by the assessors.

- 11 NEQAS slides
  - 5 - Gold CD45 (R)
  - 6 - 2nd antigen (T)
- Preparations
  - 10 cytopsin preparations
  - 1 cell block section
- 17 in-house slides
  - 11 - CD45 (S)
  - 6 - 2nd antigen (U)
- Sample type (in-house)
  - 6 FFPE
  - 5 cell block sections
  - 3 cytospins
  - 2 liquid basec cytology samples
  - 1 FNA
- 17 individual participants
  - 6 UK or Ireland labs
  - 9 from an EU country
  - 2 from outside of the EU

As CD45 was the Gold antigen for the year, (requested 4 times) numerically, you would expect this to have the largest number of low scoring slides; but in fact this was only true for the in-house submissions (S = 11, U = 6) and the number of failed slides for the NEQAS CD45 Gold samples was slightly less than those for the 2nd antigens (R = 5, T = 6).

Reasons for low marks

The main or primary reason/comments given by assessors for low marks were (all slides n=28):

- Background and/or non-specific and diffuse staining 12 (43%)
- Weak or inappropriate demonstration of antigen 13 (46%)
- Uneven staining 1 (4%)
- Poor quality or inappropriate in-house control material 2 (7%)

Sub-optimal immunostaining on NEQAS slides

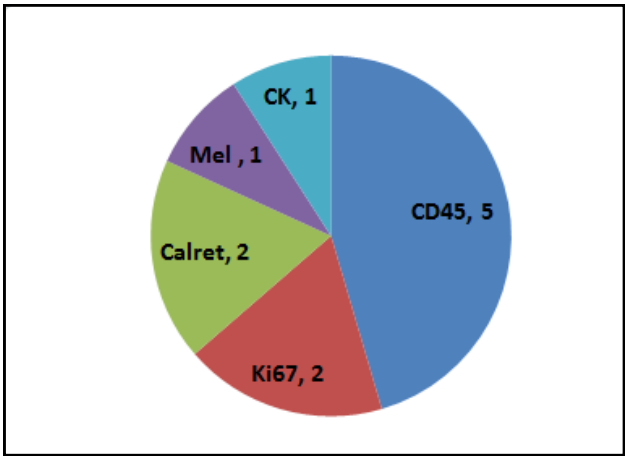
Cytospin (CS) v Cell Block (CB) samples

From Run 107 onwards, labs were able to choose between receiving a cytospin (CS) or cell block (CB) slide.

All cytology module participants were surveyed prior to the Run (107) to ask for their preferred preparation. Numbers have remained fairly consistent with 63% of participants submitting CB and 37% CS slides (average = 49 and 29 labs respectively), for the three runs.

The most interesting finding was that after the introduction of the cell block samples the number of labs failing the Gold (CD45) dropped from a total of 3 labs for Run 106 to only 2 labs for the rest of the year, and these were all on CS slides. Indeed out of the 6 slides assessed as unacceptable for Runs 107 - 109 only one was on a cell block section, and 5 were on cytospins.

Graph 1: NEQAS slides with sub-optimal staining by antigen



These figures represent levels of only:

1% Melanoma and Cytokeratin
2% CD45
3% Ki67 and Calretinin

With a range of between 70 - 79 slides submitted for R and T.

Table 2: Methodolgies resulting in sub-optimal staining on NEQAS slides:

	Run	Primary	Dilution 1:	Platform
CD45	106	Dako monoclonal	500	Bond Max
CD45	106	Dako monoclonal	10	Dako Autostainer
CD45	106	Dako monoclonal	RTU	Dako Autostainer
CD45	108	Dako monoclonal	RTU	Dako Autostainer
CD45	109	Dako monoclonal	RTU	Ventana Benchmark
Ki67	106	Dako MIB1	200	Ventana Benchmark
Ki67	106	Dako MIB1	50	Leica Bond III
Calret	107	Novocastra	100	Dako Autostainer
Calret	107	Cell Marque	40	Lab Vision Autostainer
Melanoma	108	Dako Melan A	100	Dako Autostainer
CK	109	Dako AE1/AE3	200	Dako Autostainer

For the CD45 Dako monoclonal, some labs did not give a dilution. This was listed as RTU in the above table.
N.B. Antigen retrieval details are often unreliable, participants often ignoring this field in data entry, or giving conflicting data, therefore it is not included in the above table (see page 4).

Table 3: Reasons for sub-optimal staining on NEQAS slides

Run	Antigen	Reason	Sample
106	CD45	Background and non specific staining	CS
106	Ki67	Excess background and insufficient block	CS
106	Ki67	Non specific staining	CS
106	CD45	Very weak demonstration	CS
106	CD45	Antigen not demonstrated	CS
107	Calretinin	Diffuse and non specific staining	CS
107	Calretinin	Very weak demonstration	CS
108	Melanoma	Very weak demonstration	CS
108	CD45	Backkground and weak counterstain	CS
109	CK	Very weak demonstration	CB
109	CD45	Very weak demonstration	CS

Images of sub-optimal staining slides on NEQAS samples

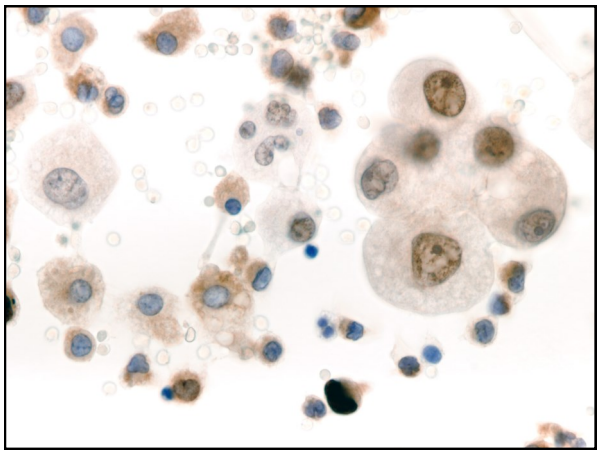


Fig 1: Failed Ki67 on the NEQAS cytospin Run 106 T - there is pronounced cytoplasmic and other non-specific staining

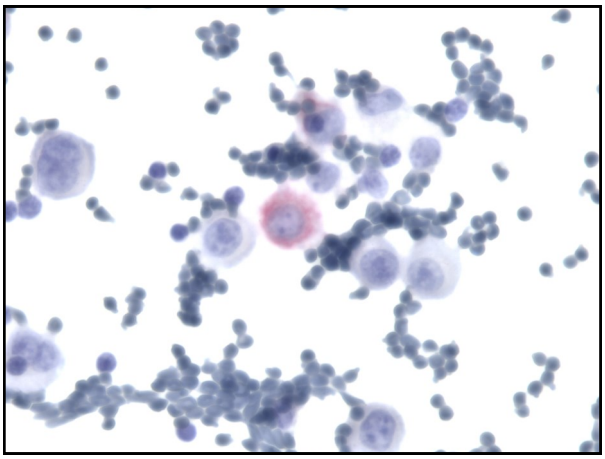


Fig 2: Failed Calretinin on the NEQAS cytospin Run 107 T - staining is very weak with only the occasioanl cell stained

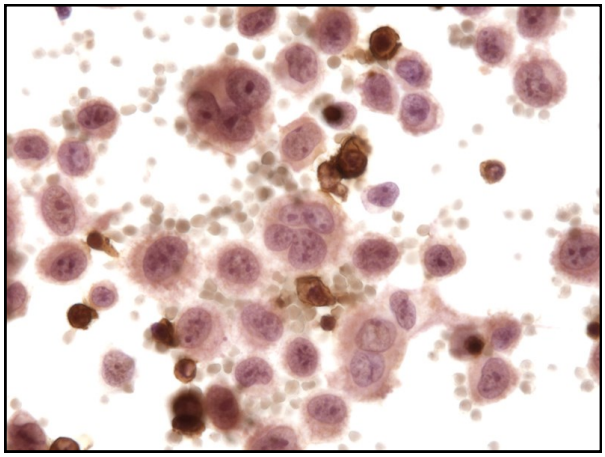


Fig 3: Failed CD45 on NEQAS cytospin Run 108 T – excessive background and non-specific staining of epithelial cells

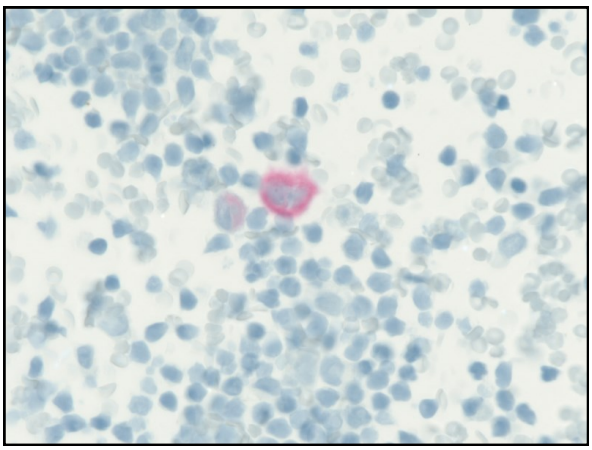


Fig 4: Failed CK on NEQAS cell block section Run 109 R - poor demonstration of the antigen

Sub-optimal immunostaining on in-house slides

Although in-house samples are not included in participant (UK) poor performance reviews, slides are assessed and scored in exactly the same way as for the NEQAS slides. The main difference is that feedback is also provided on the choice or quality of submitted in-house controls.

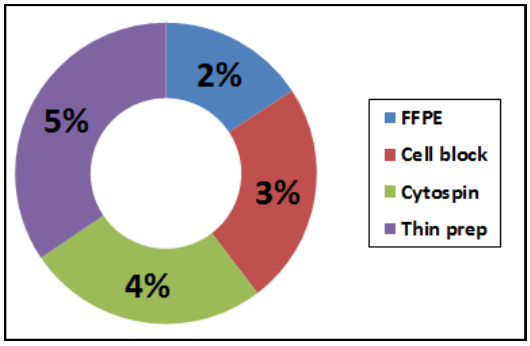
Furthermore, marks are not deducted if non-cytological in-house samples are submitted, even though cytological material is preferable, as long as labs are running the same control that is routinely used.

Table 4: Type of submitted in-house control slides (S & U)

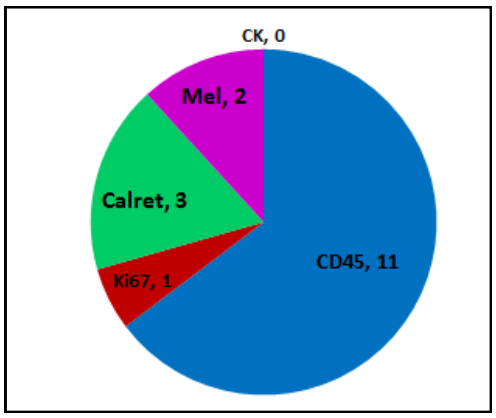
	106	107	108	109	Total
FFPE	58 (41%)	71 (48%)	71 (48%)	69 (45%)	269
Cell block	34 (24%)	37 (25%)	35 (24%)	42 (28%)	148
Cytospin	33 (24%)	24 (16%)	27 (18%)	25 (16%)	109
TP/LBC	9 (6%)	12 (8%)	10 (7%)	10 (7%)	41
Smear	6 (4%)	5 (3%)	4 (3%)	6 (4%)	21
Totals	140	149	147	152	588

The most notable trend seen here is the slight increase in the use of FFPE and cell block slides, and the reduced levels of cytospins after Run 106. Levels of smears or liquid based samples remained relatively unchanged.

Graph 2: Type of in-house slides with sub-optimal staining



Graph 3: In-house slides with sub-optimal staining by antigen



Just looking at antigen numbers does not really tell us much about samples, and reasons for these poor scores. Much more of interest is the combination of sample type, antigen and reasons for low marks.

Table 5: Reasons for sub-optimal staining on in-house slides

Run	Antigen	Letter	Comment /Reason	Sample type
106	CD45	S	Very weak	FFPE
106	CD45	S	Uneven staining	CB
106	Ki67	U	Weak and inappropriate control	CB
107	CD45	S	Poor material and morphology	CS
107	Calretinin	U	Very weak	FFPE
107	CD45	S	Diffuse and background staining	CB
107	Calretinin	U	Poor control material	CB
107	CD45	S	Diffuse and poor quality	LBC
107	CD45	S	Background and insufficient PT	CS
107	CD45	S	Background and poor tissue quality	FFPE
107	Calretinin	U	Weak, background, and poor quality	FFPE
108	Melanoma	U	Very weak	FFPE
108	CD45	S	Backkground and non specific staining	CB
108	CD45	S	Background and weak counterstain	CS
108	CD45	S	Diffuse staining and poor quality of material	LBC
108	Melanoma	U	Weak and poor control material	FFPE
109	CD45	S	Weak and poor control material	FNA

There were 9 comments concerning 'poor in-house material'. Two of these were given as the as the primary reason for failing the slide. In practice though, where a slide has been given a low score for weak or absence of staining, this is also likely to be attributable to the quality and type of in-house control sample used.

Images of in-house samples

The majority of images taken of in-house slides in the Journals are of good examples. We use these to illustrate what can be achieved from a variety of samples, given that we have no direct comparisons, as we do for the NEQAS slides which we assess against the validation slides.

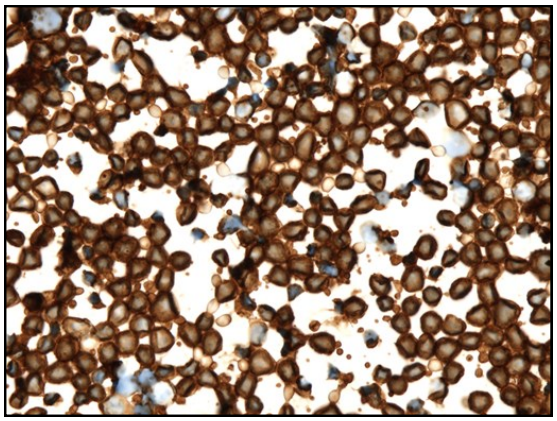


Fig 5: Example of a sub-optimal CD45 staining on an in-house cytospin Run 108 S

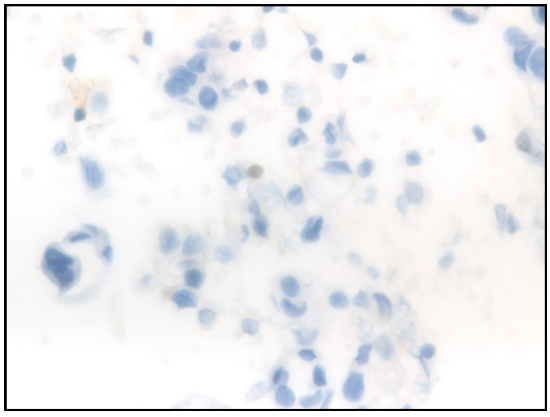


Fig 6: Sub-optimal Ki67 in-house CB section –staining is very weak and only a few cells are stained Run 106 U

Report on the Cytology EQA Module: Runs 106—109

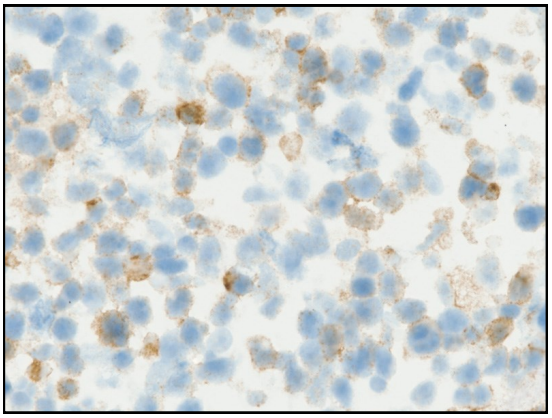


Fig 7: Sub-optimal CD45 in-house FNA sample –staining is very weak and the morphology is poor Run 109 S

Summary

This report confirms that the majority of participants in the Cytology module are able to demonstrate at least acceptable quality of immunostaining on the NEQAS, as well as on in-house slides, irrespective of the type of slides, the requested antigen, or origin of participants (UK, EU, OS).

A very important finding is that sub-optimal immunostaining was only a single or sporadic event for the majority of participants receiving low marks, indicating that participants took appropriate measures to improve the quality of their immunostaining.

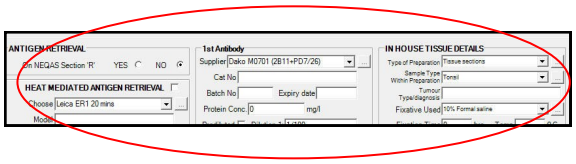
The NEQAS distributed slides (R & T) which were subsequently returned and then assessed as inadequate for diagnosis, were though, more likely to be on cytospin requested samples. As we have reported in previous Journals *sub-optimal methodologies /protocols* on cytospin samples, where no retrieval is employed, are more prone to non-specific staining of some cells in the sample 'cocktail' which contains a variety of cell types, which stain both positively and negatively for the chosen antigen.

For the NEQAS slides, the actual reasons for low marks were split between weak or absence of staining (6), and background/non-specific staining (5).

Of the 4 individual labs that received low marks for Run 106 (all received cytospins) the two that subsequently changed to cell blocks, did not produce a low score thereafter, whereas both the labs who continued to receive cytospins submitted slides with sub-optimal staining for at least one further run.

Given that the development of antibodies and their intended use is targeted towards FFPE and tissue samples, this is also a factor in these findings. But it is worthy of mention that we consistently see a high standard of immunocytochemistry staining on cytospins, and for some of the markers requested, the ***best examples*** of antigen demonstration have indeed been on cytospins.

In terms of any protocols relating to antigen retrieval, as mentioned above, we find the data to be unreliable. For the slides in Table 3, all labs had indicated that no retrieval had been used during the staining. Given that most of these are cytospins this is understandable. But when looking at the data for other labs using cell block sections and a FFPE in-house control, for example on CD45, where the recommended retrieval is 20 minutes, the button for: ANTIGEN RETRIEVAL ON NEQAS SECTION is often left as NO (see below). This is something that UK NEQAS ICC & ISH is addressing.



Further reading

- Journals Run 106 -109 Cytology Summary
- Journal Run 107 Cytology Summary: cell block v cytospin comparison
- Cytology cell block trial Journal Run 102

If you have any comments or queries email: n.bilbe@ucl.ac.uk