June 2010

UK NEQAS for Antibodies to Nuclear and Related Antigens Scheme

Commentary on EQA Distribution 102, Samples 1021/1022
With feedback from participants and discussion

Sample 1021

The designated response on this sample was and remains:

- ANA positive with a speckled ANA staining – typical for Ro with positive transfected cells in HEp2000 systems
- dsDNA negative
- Centromere negative
- Ro positive

The returns on this sample were very reassuring, with most participants getting the correct answer and a strong consensus between different laboratory sites.

Indirect immunofluorescence and EIA for ANA Sample 1021

Indirect immunofluorescence for HEp 2 and HEp2000 showed that the vast majority of participants found a positive ANA, with only 6 out of 401 finding false negativity and these individuals should look at the sensitivity of their techniques.

The high range of positive titres reported for these methods would suggest an in-house problem for those centres with false negative results rather than a systematic problem with the reagents used. The sensitivity of your screening systems should be examined if you missed the ANA, which was clearly positive at commonly used screening dilutions. As expected, a considerable portion of users of rat or mouse tissue were negative and this probably reflects the lower sensitivity of this technique. While this lower sensitivity may be entirely appropriate and utilise a locally validated threshold, laboratories which reported a negative should ensure that sensitivity of their system is appropriate for its current clinical application and if necessary check the sensitivity of their techniques.

Enzyme immunoassays for ANA were universally successful in detecting the presence of ANA, but once again there was a tendency to a wide range of apparent titres.
ANA staining pattern Sample 1021

The vast majority of labs reported a speckled antinuclear pattern consistent with Ro positivity. A small number reported the presence of nucleolar antibodies or additional homogenous staining. These have not been scored adversely, but are probably “over-reporting” as no evidence of such patterns at any reportable level can be seen in UK NEQAS internal testing procedures at dilutions of 1 in 20 on HEp2000 cells. Laboratories that found additional patterns should determine if their thresholds for reporting the presence of a pattern are appropriate. Any such patterns if present should have been extremely weak. If they were not, please send us a digital image to demonstrate this or ask for a repeat sample.

On immunofluorescence staining there were no patterns which would be consistent with the coarse speckled pattern of RNP or Sm or Jo1 and those who made such reports should re-examine their systems for reporting. It would not be possible to distinguish Ro from the La staining in the absence of other confirmatory techniques or HEp2000 substrates (Figure 1).

Figure 1:

Fine speckled ANA at 1 in 80 dilution on HEp2000
Keyhole pattern, no homogenous or nucleolar pattern
Enhanced in Ro 60
ENA returns Sample 1021

EIA results for dsDNA and ENA were consistent with the observed ANA pattern.

ENA results on this sample were very satisfactory with a vast majority of enzyme immunoassay users finding Ro positivity and very few individuals reporting additional specificities, most frequently Ro plus La. Counter-current immuno-electrophoresis and double-diffusion methods, whilst less sensitive, performed satisfactorily finding Ro only and no additional specificity. Immunoblotting produced a greater percentage of false positivity for other specificities but performed satisfactorily in detection of Ro. Line immunoassay and fluorescent bead technology were also in consensus.

Therefore anyone who issued a report suggesting the presence of antibodies other than Ro would be out of consensus and should examine their methodologies.

A digital image of sample 1021, suitably annotated, is presented in Figure 1.

Double stranded DNA antibodies Sample 1021

All users of Crithidia luciliae reported negative results, as did the vast majority of users of other immunoassays. This is consistent with the immunofluorescence pattern which did not suggest the presence of any significant dsDNA antibodies.

Anyone who issued a positive dsDNA report should satisfy themselves that their thresholds and methodologies are appropriate.

There is no evidence of heterophile interference in this sample.
Sample 1022

This sample produced much confusion. It was very challenging, but educational. Feedback is summarised in the text:

The most appropriate response on this sample after summation of the results is:

IIF Results:

- ANA positive which gives different patterns in fresh and aged sera and different cell substrates but contains a clear pattern which could reasonably be interpreted as an unidentified speckled or multiple dots ANA or atypical Centromeric staining on IIF – it certainly looks a bit like Centromere at first glance with a variety of different sizes of dots, but perhaps too few and too fine in character, but on balance is probably best fits “multiple nuclear dots” or “possible Centromere A activity”. Its true nature is uncertain and there is really no consensus that it is a typical or definite Centromere pattern. A number of laboratories reported the possibility of Centromere A (or C1) activity, but many recorded the atypical nature of the staining with poor staining of dividing cells, while others regarded this as “pseudocentromere” staining. Some laboratories declined to report it on the basis that it was an unusual pattern and others noted the discrepancies between the various test modalities but often did not make it clear what significance they would place on the result when interpreting.

- Reports of additional weak homogenous or nucleolar staining would be acceptable, and the serum clearly changes with storage, although any homogenous staining is so weak in interphase cells as to be of very doubtful significance and there is a clear case for reporting it as weak, or indeed for not reporting it at all. There is an obvious and astounding difference in the sensitivities of different IIF methods across participants with some revealing obvious staining at much higher apparent titres than others. Nucleolar staining appears to increase with storage at 4 degrees and can appear quite prominent. Some laboratories reported staining of the dividing chromatin but some noted the unusual absence of equivalent homogenous staining on the interphase nuclei.

- Although the speckled pattern undeniably suggests the presence of a possible Centromere pattern, definitive Centromere staining is absent in the majority of dividing cells in the majority of participant reports and there are many features of the staining that would make reporting a typical “Centromere” pattern potentially clinically misleading, unless the report was issued with clear interpretive warnings. Reporting patterns that are so weak that they are hard to definitively identify generally means their positive predictive values are likely to be very low, whether the weak staining relates to titre or apparent physicochemical characteristics like avidity/affinity. Clinically significant antibodies are usually found at higher titre in untreated cases, and clinical significance has often been defined using less sensitive methods historically.

- It would have been appropriate for those few laboratories who felt an atypical, isolated Centromere A antibody was definitely present, to reflect that clearly in their reports. The clinical significance of this finding is debatable, and to report the presence of a typical Centromere or the likelihood of a subset of systemic sclerosis could be a highly misleading interpretive report.
• It is clear that typical Centromere IIF staining is not present in any substantial titre for most participants screening systems. This clinically relevant pattern should be reported to clinicians and has a clear association with particular conditions when present in significant titres. The predictive values of other atypical patterns, extremely weak staining or isolated Centromere A is not well established (even in Systemic Sclerosis) and it is inappropriate to report these in the same way, or with the same significance, as typical Centromere B staining (see comparative pictures supplied). Furthermore the rest of the autoantibody results would not be consistent with scleroderma/systemic sclerosis.

• The fact that only a few of the metaphase or anaphase or telophase cells produced staining which was typical of Centromere should have alerted the laboratory to the possibility of a different staining pattern or led to a consideration of the presence of low affinity antibody (these are generally of doubtful clinical significance) or very low titre antibody. The same is true for the other EIA Vs IIF discrepancies seen in the ENA and dsDNA results.

• If you felt that Centromere antibody was present, you would have to consider the likelihood that typical Centromere staining would be absent from the majority of dividing cells yet be perfectly visible in all the interphase cells. We titred out a known Centromere antibody to determine if we could reproduce the pattern but could not (see Figure 9). Since CENP-A is a histone-like protein and different from CENP-B this is at least a possibility.

Because of the heterogeneity of the responses from participants, we will not score the IIF screen or EIA for ANA returns, as it is impossible to generate a consensus response using the current systems, and the accuracy of any apparent consensus is highly debatable. However, the presence of a typical Centromere IIF pattern is not supported by consensus and there is clear evidence that such a report is potentially very misleading clinically - whether you take the view that there is true Centromere A antibody but is weak, or that this is not a true Centromere staining. Because of the complexity of the responses we will not score the ANA Centromere component on this occasion.

• Centromere – the desired response will remain negative for definitive or typical Centromeric staining, consistent with the majority IIF report and the CENP-B EIA consensus.

• The presence of the typical IIF Centromere pattern is seen where both CENP-A and CENP-B activity co-exist. A & B are usually present together in the vast majority of clinically relevant circumstances. If you were reporting the presence of CENP-A alone, without moderate or strong typical staining, then this should have been clear and unambiguous in your return. Would you report a Centromere F pattern as a “positive Centromere” without qualification?

• Several laboratories criticised this approach, usually on the basis that they felt that there is probably some isolated CENP A reactivity in the serum and because isolated CENP-A has been reported to be associated with Systemic sclerosis. This weak staining might be argued to show the presence of weak low-affinity CENP A reactivity of uncertain significance, but any report should have highlighted the fact that the significance of this combination is uncertain, and should not have suggested the presence of Scleroderma without qualification when the rest of the autoantibody pattern was clearly not that of a systemic sclerosis and there are clearly problems with detecting staining in dividing cells in most systems as illustrated in the digital images appended to this report.
• Few of the digital images submitted by participants show a Centromere pattern sufficiently strong to stain most of the dividing HEp-2 cells in an absolutely typical manner, nor does the pattern mimic a typical Centromere B (probably +C) pattern when diluted to extinction (Figure 9), creating uncertainty about whether this IIF pattern should be reported as a Centromere at all. The vast majority of clinically relevant CENP-A reactivity is found together with CENP-B and with a typical IIF staining. Therefore, most clinically relevant Centromere patterns should be seen on IIF, and both EIA types would be expected to be positive, resulting in consensus positive CENP-B EIA and CENP-A EIA.

• The possibility that there are subtle differences in the pattern of an isolated CENP-A, changes in the apparent pattern related to substrate or screening titre differences or low affinity or low titre antibody need to be borne in mind. It is difficult to envisage a Centromere without CENP-A in any cell line, perhaps suggesting that the latter explanation is more likely?

• What about the reports of CENP-A EIA positivity? Surely this means the sample should be designated positive for Centromere? The CENP-A may be positive, but we are assessing detection of “typical” Centromere activity at levels likely to be clinically significant, and the majority of EIA methods are CENP-B EIA, but the IIF is what is being assessed for the Centromere pattern. Interestingly, only 6 of the 11 users of the CENP (A+B) EIA would commit themselves to a return and 2 of these were negative. One of the CENP-A returns were also negative.

• EIAs can be excessively sensitive and thus their predictive values and specificities can be very different from the reference techniques used to evaluate clinical predictive values of antibody specificities in the past, and often they are not directly comparable to the more specific but less sensitive techniques on which the predictive values of most of the well-established autoantibodies have been established. It does not follow that positive results in an EIA will replicate the IIF report, but when it does not, it is potentially providing information about the serum - further consideration is required.

• Furthermore, the precise sensitivity and specificity of IIF staining for detecting the rarer clinically relevant antibodies (such as isolated CENP-A) is not known. There is no clear evidence (of which we are aware) that IIF is an appropriate screen for isolated CENP-A and this data might be interpreted to suggest that it is not a reliable methodology.

• UK NEQAS will be working with participants to ensure that only typical IIF staining in clinically relevant titres is reported and interpreted correctly. Reporting atypical, or very weak Centromere patterns without interpretation or qualification is not validated as a clinically useful or reliable practice.

• We understand that there may have been some uncertainty about what to report for the speckled element, as some may not have been sure to report speckles or dots or atypical Centromere patterns and a third of participants chose Centromere. We have therefore decided not to score the Centromere element, and the debate about whether the staining is a pseudocentromere, an unusual weak Centromere, unidentified dots or speckles is not relevant – all would have been equally justifiable given the lack of consensus.
dsDNA Results Sample 1022:

- There has been comment that the designation of the sample as a “probable false positive” is not appropriate, in that the Crithidia is certainly not negative (true), and that a positive Crithidia and dsDNA assay is due to dsDNA antibody until proven otherwise (usually true). This reasoning is correct. We didn’t score this element because of the lack of consensus, but have provided a commentary to elucidate the probable lessons from this distribution, and the clues that suggest that this specimen is not behaving typically. A fact that was noted by many participants.

- Double stranded DNA assays were two-thirds positive by Crithidia techniques but only 50% positive by enzyme immunoassays techniques. Several methods were predominantly negative.

- However, EIA and Crithidia positivity conflicts strongly with the lack of strong immunofluorescence staining for chromatin associated patterns on HEp-2 lines (see images) with the exception of some staining of condensed chromatin in some systems – always difficult to interpret on its own. Laboratories should report their results cognisant of the results in other test systems in their own laboratory, and should have highlighted this discrepancy. Any that did not should re-visit their internal protocols for ensuring consistency of reporting.

- Anti-dsDNA antibodies are indeed probably present. But are they the sort of antibodies that appear to be of low specificity in EIA or the sort that are strongly associated with SLE or other CTD. The common finding of a positive EIA in the absence of confirmatory IIF staining is most often of uncertain significance and has a low predictive value for the presence of SLE. Therefore the most likely pre-test probability interpretation in the absence of clinical details is indeed a clinically “false positive” result. The clues are in the lack of moderate or strong homogenous IIF staining in most cells and the slightly atypical Crithidia positivity. The homogenous staining in interphase cells is so weak (even in the digital images submitted) that there is a clear argument for not reporting it at all. The finding of occasional condensed chromatin with apparent positive homogenous staining is not in itself evidence of significant dsDNA activity if the rest of the interphase nuclei do not match. The art of the clinical interpretation is to set the results in the context of the patient’s condition. The art of the laboratory technical validation is to come to a consensus about what the pattern of results are telling us about the sample.

- This inconsistency between the techniques, possibly based on differing sensitivities for high and low affinity antibodies (amongst other variables), should at least have been picked up at the interpretation and validation of the results, and factored into the interpretation.

- However, in the absence of clinical details, the pre-test probability that a clinically significant high titre, high or medium affinity dsDNA antibody is present in the absence of moderate or strong homogenous HEp-2 staining is probably low, and low affinity antibody is often of doubtful clinical significance. This is the most likely scenario until the clinical details are known to modify the interpretation.
• It transpires that the material came from a long-term donor who is thought to have long-standing lupus - probably stable and on treatment. Now the results start to make sense:
  
  ▪ This clinical scenario is entirely consistent with the presence of multiple low-affinity antibodies, and thus the lack of strong dsDNA staining on IIF on both Crithidia and HEp-2/2000 and other cell substrates.

  ▪ The slightly atypical nature of the staining on the Crithidia (lack of prominent and typical homogenous staining and a tendency to stain other focal intra-nuclear structures) suggests that some of this nuclear reactivity is not simply anti-dsDNA.

• On the other hand, if the Crithidia had been negative, then the vast majority of isolated EIA positivity without significant homogenous staining in the average laboratory would be found in people who did not have an obvious autoimmune disease, and thus is truly clinically “false-positive”.

• This scenarios a well documented characteristic of the increase in sensitivity and consequent loss of specificity of EIA versus the older techniques such as immunoprecipitation.

• Those laboratories that were uncomfortable with the apparent disparity between the results were indeed correct, and this emphasises the need for vigilance at technical validation and clinical interpretation of all autoantibody results. Appropriate IQC and EQA procedures should ensure that laboratories investigating autoimmunity have the skills needed to offer sophisticated and clinically relevant interpretation. We will be developing the schemes to support this aspiration in the near future.

• It was noted that one EIA method predominantly gave negative dsDNA responses. This was held to be a potentially bad outcome by some respondents – on the basis that it was out of consensus with other more sensitive EIA. However, for the reasons outlined above, it is equally possible that this actually has a positive effect on assay specificity. Furthermore, as usual there were plenty of methods that produced both positive and negative returns from different users. Hence the UK NEQAS mantra – “Know your assay”.

• You need to know your assay performance characteristics, and understand the clinical use of your assay in order to effectively use it and interpret it for your clinicians. This sample also illustrates the complexity of assessing clinically relevant performance in the nuclear and related antigens scheme.
ENA Results Sample 1022:

- Sm – We stated that this was probably clinically “false positive”, but Sm/RNP reactivity was reported in many systems. This interpretation is reasonable in the absence of clinical details to adjust the pre-test probability. This report could reasonably be finessed to declare the presence of low level or low affinity reactivity of uncertain clinical significance in the absence of any significant immunofluorescence to suggest Sm/RNP, or Ro/La for that matter.

- This situation is no different from the dsDNA assay issues discussed above. The IIF and EIA disparity probably favours the presence of low titre or low affinity antibodies of doubtful clinical significance, until the clinical details are known. Since it transpired that the patient has long-standing SLE (presumably treated or quiescent) then the presence of multiple low affinity or low level autoantibodies, including Sm, is not surprising. However the majority of the reported EIA positives were inconsistent with the IIF picture and this should have been picked up and highlighted by participants.

- Perhaps a more worrying observation is the continuing complete lack of homogeneity and agreement of reported ENA specificities across the various manufacturers for almost any ENA type. There are many potential causes of heterogeneity, from different assay sensitivities to different substrates. Clearly many of these assays pick up low affinity antibody, which is of a different clinical significance to high titre or high affinity antibody. The latter usually produces consistent results across the different assay methodologies. This simple fact underpins the use of test-gating strategies where a less sensitive or more specific test (such as IIF at an appropriate threshold sensitivity) is used to screen for samples requiring further testing by more sensitive methods.

- With regard to ENA typing there was little consensus, although over 100 laboratories reported the presence of RNP or Sm, alone or in combination. However, this was a minority response in <30% of users.

- The huge variety of different combinations reported raises the possibility of an interfering substance (such as heterophile antibodies – likely because this is not thought to be an uncommon problem) or widespread lack of substrate specificity (contaminants or impurities – probably unlikely, especially for recombinant substrate) or different sensitivities (a combination of substrate characteristics, chosen positivity threshold and assay configuration). The lack of consistency between the HEp-2/2000 immunofluorescence pattern and the ENA/dsDNA assay points to EIA interference or low affinity/low titre antibody of uncertain relevance. Immunoblotting appeared equally susceptible to reporting multiple specificities but predominantly detected Sm or RNP.

- In our hands, as an illustration of the unusual characteristics of the sample, utilising a line blot with purified thymus/spleen extract gave positive signal for Sm and RNP but a completely negative western blot using purified antigens from a HEp-2 source. Both assays were from the same manufacturer!

- The majority of CIE and double diffusion ENA methods were entirely negative, but small in number. This observation would fit the presumption that these are low affinity antibodies which are being differentially detected in different systems.

- **Heterophile antibody absorption did not change the EIA results or IIF results, Rheumatoid factor was not present – suggesting that heterophile assay interference is not the problem.**
**Figure 2a:**

Sample 1022 at 1 in 80 on HEp2000 (200 x magnification)
Possible very weak homogenous but nothing to suggest much chromatin staining – appears negative at x 100 and I would have reported it as negative for a significant homogenous ANA, but the speckles are easy to see, but not obviously a Centromere.

Weak cytoplasmic, also present but far below threshold for reporting at 1 in 80 on HEp2000 and not visible here.

Note keyhole, lack of nucleolar staining, multiple speckles of variable morphology or nuclear dots but not Sm/RNP like and probably fewer and finer than you would see with most Centromere antibodies. Possible weak homogenous or fine speckled ANA might be reported by some, but it is very weak and it is doubtful if it is clinically relevant or worth reporting, but there is very little to suggest moderate or strong dsDNA reactivity. Most metaphase cells show no evidence of classic Centromere activity, and you have to hunt hard to find any at all, and these are not typical – see below. The single cell in the LHS corner is tempting, but not enough to make me call the sample a definitive Centromere.
It is very interesting that 2/3 of the Crithidia assays were also reported positive, as this technique is often used to weed out false positivity in the EIA techniques, but it may not be infallible. Laboratories should re-visit their protocols for reporting apparent dsDNA positivity in the absence of HEp-2 immunofluorescence staining to ensure that they are happy that the test results are of clinical significance. Some pictures are provided below which demonstrate the difference between Crithidia staining in known SLE with dsDNA antibodies and sample 1022 and which may suggest differences from the staining seen with higher affinity antibodies (where nucleus and kinetoplast both stain homogenously typically and the pattern matches the HEp-2 homogeneous staining). These differences were noted by some participants.

A positive dsDNA antibody, positive Crithidia, positive “speckled” ANA (possibly weak Centromere) with some homogenous staining and ENA positivity might lead you to suspect that this material came from an individual with SLE, especially if a homogenous pattern was also reported. However the weak or absent homogenous HEp-2 staining is not really consistent with the other assay results (look at the strength of the kinetoplast signal in the Crithidia slide) and suggests lower affinity antibody of uncertain significance.
**Figure 3a:** A positive dsDNA appearance from a patient with SLE – note the positive kinetoplast and nucleus on this Crithidia slide with obvious homogenous staining (which matches the HEp-2 staining).

**Figure 3b:** The Crithidia appearance of sample 1022. The kinetoplast is certainly positive, but any nuclear staining is very different from figure 3a on the same assay run and any homogenous staining is weak, with apparent staining of intranuclear substructures. The basal body is also very prominent, which may lead to confusion. Would you report this as typical dsDNA positivity without comment or examination of the other results?

Both samples in figure 3a and 3b give dsDNA ELISA results >200 IU/ml in our hands.
## Summary of Conclusions Sample 1022

<table>
<thead>
<tr>
<th>Method</th>
<th>Observations</th>
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<tr>
<td><strong>IIF</strong></td>
<td>Speckled positivity - moderately strong&lt;br&gt;No typical course staining for Sm.RNP&lt;br&gt;No evidence Ro/La patterns&lt;br&gt;Nucleolar staining variable and influenced by storage and cell substrate&lt;br&gt;Very weak homogenous, if at all – no convincing evidence of significant titres of anti-dsDNA antibodies&lt;br&gt;Speckled staining suspicious, but not proven to be a significant Centromere, although occasional suspicious cells are seen, nothing typical or strong in most centres, and clear suggestion of substrate specific differences in sensitivity&lt;br&gt;If Centromere A is present it is not typical in most and very low titre or weakly binding in the dividing cells in the majority of IIF assay systems&lt;br&gt;No obvious dominant strong staining present that would have obscured weaker underlying patterns</td>
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<tr>
<td><strong>EIA for ENA</strong></td>
<td>Poor correlation with IIF patterns universally – generally suggesting low affinity antibody of uncertain significance. Many tests pick up Sm or Sm/RNP, but lots of other patterns of positivity without a clear theme&lt;br&gt;Precipitation assays predominantly negative consistent with low affinity antibody&lt;br&gt;Heterophile interference excluded by HAMA adsorption</td>
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<tr>
<td><strong>EIA for dsDNA</strong></td>
<td>Lots of positivity across methods, but 50% of EIA methods negative and most methods produced both positive and negative results – consistent with observations above&lt;br&gt;Immunoprecipitation assays predominantly negative&lt;br&gt;One EIA method predominantly negative</td>
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<td><strong>Crithidia dsDNA IIF</strong></td>
<td>Positive but often with an unusual pattern for high titre anti-dsDNA and less nuclear staining than would be expected from a reasonable titre of avid dsDNA antibodies. At least some of the nuclear staining is apparently due to other specificities - note focal staining of areas of the nucleus</td>
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<td><strong>EIA for CENP-B</strong></td>
<td>Almost universally negative – matches IIF patterns which are not suggestive of strong Centromere B pattern with only a very few positives (and hence the majority of CENP-A reactivity. Mixed assays (containing A+B) were 2/3 positive, but 1/3 negative. Single CENP-A assay consensus positive</td>
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<td><strong>EIA for CENP-A</strong></td>
<td>Positive – but predominantly a single method group and does not match the IIF pattern well in most centres, since the majority of dividing cells show no definitive Centromere-like reactivity in the majority of screening IIF assays&lt;br&gt;The results suggest that IIF is not a good screening test for presence of isolated CENP-A antibodies, or there may be substrate-related differences in sensitivity and it is controversial if the observation of positivity is of any clinical relevance in this material. In this respect this antibody behaves differently from typical Centromere IIF activity</td>
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Enzymes immunoassays for ANA showed a considerable number of negative responses with 32 out of 86 returns being negative. This affects most assays and probably indicates variable sensitivity, substrate differences or assay interferences.

Because we cannot currently score ANA EIA separately from IIF we are forced not to score this positive/negative element at all as the EIA are likely to be affected by the same issues as the dsDNA/ENA EIA detailed above. We will alter our scoring to allow separate assessment in future scheme developments.

Furthermore, we will eliminate the potential to pick up 2 MIS points for a connected ANA report error in the near future (a common complaint), although this will be accompanied by a decrease in the Persistent Poor Performer threshold, as few participants seem to realise that the Persistent Poor Performer threshold is adjusted to compensate for this. We would encourage participants to focus on the lessons of the distribution and not the “score”.

No typical Centromere staining is visible at dilutions as low as 1 in 20 in our hands on HEp cells and the CENP-B EIA was consensus negative, indeed of 11 users of a Centromere EIA containing both recombinant A and B - 4 returned positive reports and two negative, but the others declined to report a result. Only 3 other users of Centromere B EIA reported a positive result. Serial dilution of a Centromere serum fails to replicate the pattern seen in sample 1022. Taking all this into account the desired response remains Centromere negative, but we will withdraw the MIS scoring as the complexity of ensuring that laboratories receive appropriate recognition for their additional reported comments which address some of the issues debated above is too great for the current MIS system.

Repeated analysis in UK NEQAS has failed to demonstrate any pattern that would suggest definitive and reportable typical Centromere activity. This raises interesting questions for all reporting laboratories (amongst others):

- What is the sensitivity of your IIF for the presence of CENP-A antibodies (or other Centromere patterns)?
- Does isolated CENP-A give the same pattern as CENP-B?
- Would all cells express it, and why are the dividing cells not staining in many systems?
- What is the predictive value of an isolated CENP-A EIA in the absence of IIF staining – and do you really want to report it?
- Is it reasonable to suppose substrate differences in expression of CENP-A, when this does not apparently affect CENP-B expression?
- How good is the data suggesting that isolated CENP-A is specific or sensitive for CENP-B negative Systemic Sclerosis?

Many laboratories invoke ANA screening to gate ENA assays – laboratories with inconsistent results should examine their validation/gating practice.

In our hands we cannot find any nucleolar staining in fresh sample, but in aged samples it begins to appear rapidly at RT and 4 degree storage, and this may explain why nucleolar staining was reported by a large number of laboratories. The digital images above clearly shows the lack of nucleolar staining on a HEp2000 substrate using fresh material.

One participant suggested that speckled staining was masked by strong homogenous staining, although all the digital images would argue against that interpretation.
1022 - Images submitted by participants:

*Figure 4a and b:*

The Crithidia here look very strongly positive for kinetoplast and rather weak for the nucleus, similar to Figure 3b in these pictures from a manufacturer.

The Hep 2 staining shows a single rather prominent Centromere-like pattern but it is not typical – see attached pictures of a typical Centromere (Figure 8). There also appears to be some staining of condensed chromatin, seen by some participants. The homogenous staining is very weak and always appears enhanced by digital imaging.

*Figure 5a and b:*

The HEp-2 picture here, sent by a participant, is interesting and quite suggestive of a Centromere at 1/320 dilution on HEp-2 cells. Using a HEp-2 cell line we cannot detect Centromere in dilutions as high as 1 in 20 in UK NEQAS. Clearly there are massive differences in the sensitivity of different lines and IIF systems, and therefore potentially major differences in the clinical predictive values – we need to work on this to promote consensus. One would have to hypothesise that there were considerable differences in CENP-A expression on different HEp-2 cell to explain this, a situation rarely seen for moderate/high titre of clinically relevant Centromere A+B staining. This distribution appears to have produced clear evidence for this. There is also a clear implication that isolated CENP-A staining is not reliably detected by IIF screening, and considerable controversy about its clinical relevance and specificity for Scleroderma.
Figure 6a and b:

Two rather nice pictures from a participant showing a pretty obvious Centromere-like pattern on a HEP-2 at 1 in 80 – but showing massively different sensitivity for this pattern between different HEP or other substrate preparations – however the interphase staining is not obviously typical, being less heterogeneous in size than a typical CENP-A+B positive, confirming the frequent reports from participants that the staining was not typical. Note the absence of staining of condensed chromatin reported by many participants and obvious in some images– suggesting that that staining is not purely anti-dsDNA.
**Figure 7a & b:**

Images from a manufacturer who detected signal in both a CENP-A and CENP-A/B EIA and saw an IIF pattern that was interpreted as Centromere on a HEp-2 Substrate, although these images are much less convincing than Figures 5 and 6. There are few mitotic cells with completely typical Centromeric pattern. Whether this is a reflection of substrate differences in sensitivity for CENP-A or because of the physicochemical properties of the antibody is uncertain. These problems are not usually a major issue for definitive CENP-B positive samples as far as we are aware. Once again there is a staining of condensed chromatin.

![Image of Figure 7a & b](image1.jpg)

**Figure 8a & b:**

This picture from a participant has been taken on very sensitive camera settings, as the cytoplasm is clearly visible. It appears to show staining of condensed chromatin, however the staining is really weak on the interphase cells and this is probably borderline for reporting a significant homogenous pattern – it is unlikely that examination by eye would reveal such a prominent pattern. Note that the anaphase and metaphase cells display none of the obvious characteristics of a Centromere pattern, although the weak speckles are still visible in the nucleus. There is insufficient homogenous staining to truly obscure a Centromere pattern, the interphase dots are clearly visible but the dividing cells don't obviously suggest Centromeric patterns.

The Crithidia nuclear staining looks rather more homogenous than our in-house stain but with definite differential staining of intranuclear structures. Titre, substrate and assay configuration may all influence the appearance.

![Image of Figure 8a & b](image2.jpg)
**Figure 9a:**

A black and white view of sample 1022 stained in UK NEQAS on HEp2000 at 1 in 80 – no obvious Centromere pattern in this cell

![Image of a black and white cell view](image)

**Figure 9b:**

UK NEQAS – two cells with possible atypical Centromere-like staining on HEp2000, one with a set of neatly lined granules seen—again this is not absolutely typical of a Centromere pattern and this is only seen in a tiny minority of the dividing cells on multiple slides at multiple dilutions up to 1 in 20. I would not issue a report of clinically significant titre of typical Centromere activity on that basis.

x 400, 1 in 20 dilution, HEp2000 cells.

![Image of two green stained cells](image)
**Figure 9c:** This different UK NEQAS sample 1022 slide preparation doesn't have a Centromere pattern at all in any dividing cell (as was the case with multiple different slides) x 400, 1 in 40 dilution, HEp2000 cells.

**Figure 10a & b:** Typical Centromere patterns on a different serum (not sample 1022) – note the rather broader/less fine dot and speckles and the obvious and frequent adoption of a typical Centromere pattern in the majority of dividing cells.
Figure 11:

Serial dilution of a Centromere pattern to extinction:
It is clear that that diluting a Centromere pattern to extinction does not replicate the staining of sample 1022.

Fig 11a: Centromere at 1 in 8 dilution x 400 on HEp2000
Figure 11b: - Same serum as Fig 9a at 1 in 64 dilution:

The staining is very weak and barely visible by eye at x 400 but digital imaging always enhances the apparent intensity: The speckled staining of interphase and metaphase/anaphase/telophase cells extinguish at same rate as interphase cells, in contrast to sample 1022, where the staining of dividing cells was very difficult to see for most participants. The granules certainly become finer, less heterogeneous, and less “blobby” with dilution. They still retain the characteristic Centromere cell-cycle-related distribution however.

Sample 1022: An exhausting but informative serum, with plenty of food for thought.

I hope this commentary has been helpful and will stimulate useful reflection on reporting practices and assay configuration for participants.

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