DEQAS REVIEW

2016/2017
Introduction

DEQAS is the largest specialist external quality assessment (proficiency testing) scheme for the vitamin D metabolites 25-hydroxyvitamin D (25-OHD) and 1,25-dihydroxyvitamin D (1,25(OH)₂D). A pilot scheme for 24,25(OH)₂D was launched in 2015. The scheme is equally suited to hospital laboratories, research institutions and kit manufacturers. In January 2017, 918 participants returned results for 25-OHD and 169 for 1,25(OH)₂D. Nine laboratories returned results for 24,25(OH)₂D.

DEQAS has close links to the US National Institute for Standards and Technology (NIST) and the Vitamin D Standardization Program (VDSP) of the NIH Office of Dietary Supplements (ODS). In April 2013 DEQAS became an accuracy-based scheme for 25-OHD₃ and 25-OHD₂ with values assigned to all samples by the Reference Measurement Procedure of NIST. A similar procedure is used to assign values for 3-epi-25-OHD₃. Participants can now assess the accuracy of their results by comparing them to an internationally recognized reference method.

DEQAS is accepted by the College of American Pathologists (CAP) as a proficiency testing scheme for 25-OHD; CAP accredited laboratories in North America can use DEQAS as their primary proficiency testing scheme for 25-OHD.

The primary purpose of DEQAS is to assess the accuracy of results produced by its participants but it also investigates particular aspects of 25-OHD and 1,25(OH)₂D methods; these have included linearity, specificity and the effect of anticoagulants. Occasionally, samples with abnormal levels of other constituents (eg. high lipid content, haemoglobin) are distributed to assess methods’ resistance to matrix effects. Because DEQAS has over 1000 participants in 56 countries using 30 25-OHD methods or variants of methods (January 2017), the statistics are very robust and much more representative than studies done in a single laboratory or among small groups of collaborators.

Another important service is the provision of advice and/or additional samples to participants and manufacturers wishing to introduce or develop new methods and troubleshoot existing methods. Participants are not normally charged for this. In some circumstances DEQAS samples can offer an inexpensive alternative to NIST SRMs.

DEQAS has a panel of Advisors, which includes acknowledged experts in the field of vitamin D, proficiency testing schemes and biostatistics. All are available to provide participants with help and advice should they require it. Initial contact should be made by e-mailing administrator@deqas.org.

DEQAS NEVER ASSESSES PARTICIPANT PERFORMANCE ON SPIKED SAMPLES. Increasing the concentration of vitamin D metabolites by spiking serum can give erroneous results [1,2].

DEQAS ALWAYS USES SERUM HARVESTED FROM BLOOD COLLECTED ACCORDING TO NCCLS GUIDELINES [3]. Blood collected in local clinics or donor matrix sensitive non-extraction 25-OHD methods.[4].
ACB Meeting

In July 2016 DEQAS organized a meeting on vitamin D on behalf of the Southern Region of the ACB (UK Association for Clinical Biochemistry and Laboratory Medicine). The meeting was held at Imperial College, London and abstracts of the talks are available on the DEQAS website (www.deqas.org) - document library.

Scheme Design

Five samples are distributed quarterly at ambient temperature, by first class post in the UK and by airmail to laboratories within Europe. Express mail (Royal Mail ‘international signed for’) is used for countries outside Europe with the exception of the USA and Canada; samples for laboratories within these countries are sent by overnight courier to an agent in Atlanta GA who forwards them to the laboratories by US Postal Service Priority Mail.

Some overseas laboratories who have experienced delays in receiving their samples have opted to pay for delivery by a courier service eg. FedEx, which we are happy to arrange on the client’s account.

Source of serum used by DEQAS

Until relatively recently DEQAS has used serum from blood collected from polycythaemic or haemochromatosis patients undergoing therapeutic venesection at a clinic in Charing Cross Hospital where DEQAS is based. However, in 2012/13 the clinic was moved to Hammersmith Hospital which is several miles from Charing Cross Hospital.

In July 2013, NIST reported that all DEQAS samples prepared from blood donated in the Hammersmith clinic contained a substance which apparently co-eluted with 3-epi-25-OHD₃ and produced an M/S peak overlapping that of the 3-epi-25-OHD₃. This had not been seen in serum from Charing Cross donations and the most likely explanation was that something, possibly a plasticiser, was leaching from the collection bags. Information from the manufacturer of the bags (Fenwal) revealed that they contained the plasticiser Di(2-ethylhexyl) phthlate (DEHP). The bags used in the Hammersmith and Charing Cross clinics were from the same manufacturer but those purchased by Hammersmith were sterilised by gamma irradiation whereas the Charing Cross bags were steam sterilized.

It was later confirmed that high concentrations of DEHP were present in serum from the Hammersmith clinic (also found in sera used by another PT scheme). DEHP which has no structural similarities to vitamin D metabolites is unlikely to be the interferent itself but its presence indicates that substances are leached from the plastic bags unless the blood is removed immediately (as was the case with Charing Cross donations)

To investigate this, it was decided to compare serum 25-OHD results on blood donations collected simultaneously in plastic bags and plain glass tubes from the same subject. In a comparison of serum 25-OHD results using 4 methods (DiaSorin Liaison, IDS iSYS, Abbott Architect and the Siemens Advia Centaur) only the Siemens assay showed higher results from blood collected in plastic bags [4]. Other methods, as yet untested, might also be affected and it was decided to purchase ‘plasticiser free’ serum from a commercial supplier (Solomon Park) in the US. Serum from this supplier (also used in the CAP accuracy based survey) is harvested from blood collected according to the NCCLS C37-A guidelines, which minimizes the possibility of leached substances appearing in the sera [3].
This has two advantages:

1. The serum is known to have minimal content of leached materials and its use removes any lingering doubts about leached substances in DEQAS samples interfering in methods for vitamin D metabolites.

2. DEQAS will be able to specify what range of values are required. This will enable us to send out more samples with higher levels of 25-OHD and more samples containing 25-OHD$_2$.

### 25-Hydroxyvitamin D scheme

DEQAS Collaboration With NIST (US National Institute of Standards and Technology)

From **April 2013**, every DEQAS sample will have had target values assigned by the NIST Reference Measurement Procedure (RMP) for 25-OHD. In addition to ‘Total 25-OHD’ (25-OHD$_3$ + 25-OHD$_2$) participants are given the NIST values for the individual metabolites **25-OHD$_3$**, **25-OHD$_2$** and **3-epi - 25-OHD$_3$**. This will be of particular interest to those laboratories using HPLC/UV and LC-MS/MS methods.

Funding for the provision of target values is currently provided by the NIH Office of Dietary Supplements (until July 2018).

The NIST assigned value has replaced the All-Laboratory Trimmed Mean (ALTM) previously used as the target value for performance assessment although the ALTM will continue to be reported.

**College of American Pathologists (CAP) Acceptance of the DEQAS 25-OHD scheme**

CAP accredited laboratories wishing to use DEQAS as an alternate PT provider for 25-hydroxyvitamin D should contact DEQAS at administrator@deqas.org quoting their CAP identification code (LAP number).

As part of their agreement with CAP, DEQAS is obliged to submit the performance scores for laboratories enrolled in the DEQAS programme after each distribution: April (event 1), July (event 2), October (event 3) and January (event 4).

**Exclusion of Sample 5 from Performance Assessment (25-OHD only)**

Occasionally, DEQAS may include an ‘experimental sample’ as part of a special investigation. Only the fifth sample of a distribution is used for investigative purposes and results will be excluded from performance assessment, *whether used for this purpose or not*. Despite not using this sample for performance assessment, all the usual statistics will be published.

**Sample stability**

Sample stability is an important issue for DEQAS as samples are sent worldwide at ambient temperature. Solutions of vitamin D and its metabolites are known to be light sensitive and relatively unstable. However, in experiments conducted before the regular dispatch of samples, both 25-OHD and 1,25(OH)$_2$D were shown to be very
stable in serum, probably as a result of the tight binding to vitamin D binding protein
and the relative opacity of aqueous solutions to UV radiation. However the stability
studies were performed using a chromatographic method and we cannot guarantee
that matrix changes (eg. a rise in serum pH) which inevitably occur at ambient
temperature might not affect the performance of less rigorous methods.

Details of the Stability Study are given on www.deqas.org (document library).

Vitamin D Standardisation Program (VDSP)

DEQAS is closely associated with the VDSP which was inaugurated in 2010 by the
US Office of Dietary Supplements (ODS) [5].

The objectives of the VDSP are to:

• Standardize the laboratory measurement of 25(OH)D to the NIST Reference
  Measurement Procedure (RMP) in national health surveys worldwide.
• Promote standardized 25(OH)D measurement in:
  • Commercially developed laboratory procedures and
  • Clinical and research laboratory procedures.
• Study differences in 25(OH)D data found among standardized national
  health surveys worldwide.
• Conduct an international research program devoted to improving the
  laboratory measurement of 25(OH)D.
• Conduct commutability study of Proficiency Testing samples, including
  DEQAS.

Commutability of DEQAS samples

This section appeared in the previous DEQAS Review

Introduction

Proficiency Testing samples are said to be ‘commutable’ in an assay when they
behave identically to ‘normal’ patient samples.
A commutability study was organized by the VDSP in 2012 and a further study was
undertaken in 2016 the results of which should be available in late 2017.

Method

The principles of commutability are explained in Fig. 2
Briefly, participating laboratories were sent a panel of 50 single donor samples
prepared from blood collected according to the C37-A guidelines, together with
samples from DEQAS (collected in plastic bags), CAP and NIST accuracy based
surveys. All samples were assigned 25-OHD concentrations by the NIST and Ghent
Reference Measurement Procedures. Results from the 50 samples obtained from
each participating laboratory were plotted against the RMP values and a linear
regression line constructed. PT samples were said to be commutable if the results fell
within the 95% confidence limits of the regression line.
Fig.2. Assessment of Commutability (courtesy of Dr. Christopher Sempos). The chart shows real data obtained from a widely used automated immunoassay.

Results

Results of all DEQAS samples fell within the 95% prediction interval of the regression line and were deemed to be commutable on the analytical platforms used. A second commutability study was conducted in 2016 and the results should be available in late 2017.
Methods used by DEQAS participants

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<td>IncStar RIA (until January 1999)</td>
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<td>Nichols Advantage (discontinued in April 2006)</td>
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<td>Abbott Architect</td>
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<td>Jan 16</td>
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<td>Organtec Alegria 25-OH Vitamin D</td>
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<td>April 17</td>
<td>IDS iSYS New</td>
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Table 1. Method timeline for 25-OHD: year methods first appeared in DEQAS and number of results submitted in July 2017 and April 2016

The changing pattern of method usage over the past 12 years is illustrated in Fig. 3. A marked increase in fully automated assays in 2008 was followed by a sharp decline in manual methods. There has also been a steady increase in LC-MS/MS methods since 2008. Overall, the total number of participants in the 25-OHD scheme has
declined in recent years. Reasons for this are uncertain but some will be due to laboratory closures/amalgamations or rationalizing of specialist assays. To more easily fulfill accreditation requirements, some laboratories (mistakenly in our view) may choose schemes where performance is judged against peer-group means rather than the ‘true’ values of an accuracy-based scheme such as DEQAS.

Figure 3. Number of participants submitting 25-OHD results (2005 – 2016)

Performance

Method Accuracy

Long-term assay performance of 25-OHD methods is given in figures 4 – 7 which shows trends in bias and imprecision since NIST target values became available in October 2012. Individual values represent the mean % bias (figures 4 and 5) and CVs (figures 6 and 7) of the samples in each distribution after excluding those containing 25-OHD$_2$. Bias of individual samples is calculated from the submitted result ($X$) and the NIST assigned value for the sample (TV): $\%\text{ Bias} = \{(X - TV)/TV\} \times 100$. The shaded areas represent limits of acceptable performance suggested by Stöckl et al [6] and adopted by the VDSP. For comparison purposes, samples containing 25-OHD$_2$ have been omitted as the Abbott Architect is stated by the manufacturers to recover only about 80% of 25-OHD$_2$. Samples containing 25(OH)D$_2$ do not currently form part of the annual performance assessment, a policy which is under review. In general the results are erratic but have shown a recent improvement. In April 2017 five of the 6 fully automated methods had a bias within the VDSP limits. At 6.7% and 9.3% respectively, the bias of the HPLC and LC-MS/MS assays were outside the VDSP limits in April 2017.
Figure 4. Method bias of Total 25-OHD results submitted to DEQAS. Mean % Bias from NIST assigned values for the major automated ligand binding assays (October 2012 to April 2017).

Figure 5. Mean % Bias from NIST assigned values for HPLC and LC-MS/MS methods (October 2012 to April 2017).
25-OHD Assay Imprecision

The variability (CV%) of results among laboratories using the same fully automated method is shown in figure 6; in April 2017, 2 methods (Abbott Architect and DiaSorin Liaison) had a mean CV (5.4% and 8.1% respectively) below the VDSP threshold (10%). CVs for the HPLC and LC-MS/MS assays in April 2017 (figure 7) were 13.9% and 9.4% respectively (figure 7).

Figure 6. Mean CV% for the major automated ligand binding assays (October 2012 to April 2017)
Figure 7. Mean CV% for HPLC and LC-MS/MS methods (October 2012 to April 2017).

24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃)

There has been a recent upsurge of interest in measuring 24,25(OH)₂D₃; a succinct account of the clinical reasons for measuring this metabolite was presented by Professor Glenville Jones in a talk given at the ACB meeting in 2016. An abstract of Professor Jones’s talk can be found in the document library on the DEQAS website (www.deqas.org).

Measurement of 24,25(OH)₂D is confined to laboratories using a suitable LC-MS/MS method and DEQAS has invited those participants to measure this metabolite on the 25-OHD samples. This is a pilot study and the results are presented in the quarterly report. Some recent results are given below (table 2). The results highlight the inter-laboratory variability of 24,25(OH)₂D measurements which, in part, might reflect different approaches to assay standardization.

A Reference Measurement Procedure has been developed [7] and Standard Reference Materials (SRMs) with assigned values of 24,25(OH)₂D₃ are available from NIST.
24,25-dihydroxyvitamin D results for samples 511 - 515

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Comment:
The statistics were calculated on trimmed data for samples 511, 512, 513 and 515 and untrimmed data for sample 514. Clearly, with such a small number of results the summary statistics are very unreliable.

This data is for information purposes only.

Table 2. Measurement of 24,25(OH)₂D on samples distributed in April 2017. Table taken from the final report.

**Concentration Dependent Bias in 25-OHD Assays**

Most, if not all, ligand binding assays show a high cross reactivity with 24,25(OH)D₃ (100% or more in some assays) and 3-epi-25-OHD₃ interferes in some routine chromatographic assays. Since both metabolites are strongly correlated with 25-OHD (figures 8 and 9), and concentrations of 24,25(OH)₂D₂ can be as high as 10% of that of 25,OHD, bias of ligand binding assays might be expected to be higher in samples with high concentrations of 25-OHD. The 3-epimer does not cross react in immunoassays but, since the mass is the same as 25-OHD, LC-MS/MS methods which do not resolve the 3-epimer from 25-OHD are likely to give to give spuriously high results in samples with high levels of 25-OHD.
Figure 8. Relationship between 24,25(OH)$_2$D$_3$ and 25-OHD$_3$ in a series of DEQAS samples.

Comment

The tight correlation of 24,25(OH)$_2$D and 3-epi-25(OH)D$_3$ with 25-OHD is illustrated by this series of DEQAS samples (figures 8 and 9). From these results it appears that 24-hydroxylase is switched off when 25-OHD levels fall to about 15 nmol/L. This makes physiological sense – conservation of the substrate for 1,25(OH)$_2$D production in D deficient subjects.

Figure 9. Relationship between 3-epi-25OHD$_3$ and 25-OHD$_3$ in a series of DEQAS samples.
Is there evidence of a concentration – related bias in 25-OHD Assays?

Because the concentration of 24,25(OH)₂D increases as the concentration of 25-OHD increases, the high cross reactivity could theoretically cause a spurious increase in 25-OHD results at higher levels. This was looked at in the most commonly used methods.

Plots of % Bias vs NIST target values (samples 496 – 505).

Figure 10. Abbott Architect

Figure 11. Beckman Dxi
Figure 12. DiaSorin Liaison

Figure 13. Roche Total

Figure 14. IDSiSYS
Figure 15. Siemens Advia Centaur

Figure 16. HPLC
Comments.

Only the Abbott Architect showed evidence of a concentration–dependent *increase* in bias. The manufacturer’s package insert gives a cross reactivity for 24,25(OH)$_2$D$_3$ of 101.9 – 189.2% over a concentration range of 50–100 nmol/L. More surprising is the Siemens Advia Centaur which shows a marked *decrease* in bias as 25-OHD concentrations increase. There seems to be no reference to the cross reactivity of 24,25(OH)$_2$D in the manufacturer’s package insert. This is apparently the only immunoassay using a monoclonal antibody, which may be significant.
Sample to Sample Variability of Bias

**Figure 18.** Method-related bias of results submitted to DEQAS. Mean % Bias from the NIST target value for samples 511 to 515 distributed in April 2016; mean results (nmol/L) are given in parenthesis (legend). Figure taken from the April 2016 Final Report.

**Comments**

Sample 495 (symbol x) contained endogenous 25-OHD₂ 35.3% of the Total 25-OHD (97.6 nmol/L). Of the ligand binding assays, both Abbott methods, the DiaSorin Liaison, the IDS EIA and IDS iSYS showed a greater negative bias for this sample than for the other samples in the April 2016 distribution. This was also apparent in the HPLC/UV methods. This suggests (but does not prove) that these methods under-recover 25-OHD₂ despite most manufacturers claiming co-specificity for 25-OHD₂ and 25-OHD₃. In the manufacturers’ package inserts, cross reactivity of 25-OHD₂ is stated to be 109%, 105% and 100% in the IDS EIA, iSYS and DiaSorin Liaison methods respectively. Abbott state that the Architect’s cross reactivity of 25-OHD₂ is 86.5% (62.4 nmol/L) and 82.4% (163.2 nmol/L). IDS and DiaSorin use spiked samples to determine cross reactivity although no experimental details are given. Abbott determine the cross reactivity of endogenous 25-OHD₂ in samples containing minimal 25-OHD₃ (below the LOQ).

**Conclusion**

Despite the general increase in the accuracy of 25-OHD methods, undoubtedly helped by improved standardization, there remains a very large sample to sample variability in bias, particularly in the ligand binding assays. This presumably reflects the presence of other constituents in the sample that interfere in the assay (matrix effects) which in the HPLC/UV and LC-MS/MS methods are removed in the extraction and chromatography steps. Short of introducing an extraction step, it is difficult to see how these matrix problems can be overcome. Automated ligand binding assays for 25-OHD may have reached a performance ceiling.
The number of participants in the 1,25-dihydroxyvitamin D EQA scheme has increased gradually over a number of years with a marked increase in 2015 following the earlier introduction of the fully automated immunoassay for the DiaSorin Liaison XL in 2014. Participant numbers appear to have levelled off in 2016 with a modest decline in 2017.

Table 3. 1,25-DIHYDROXYVITAMIN D METHOD TIMELINE

<table>
<thead>
<tr>
<th>From</th>
<th>Method</th>
<th>Returns</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 1997-1998</td>
<td>HPLC + RIA</td>
<td></td>
</tr>
</tbody>
</table>
|           | IDS RIA                    | 1  
|           | In-house Receptor assay    | 0  
|           | In-house RIA               | 0  
|           | Incstar Receptor assay     | 0  
|           | DiaSorin RIA (formerly Incstar) | 20
|           | Nicholls Receptor assay    | 0  
| October 2004 | DIAsource CT assay        | 1  
| April 2005   | IDS EIA                    | 17 
| October 2007 | LC-MS/MS                  | 11 
| July 2009    | AMP RIA                    | 0  
| April 2010   | Immunodiagnostik ELISA     | 1  
| April 2012   | IDS iSYS                   | 31 
| January 2014 | Cusabio ELISA             | 1  
| April 2014   | DiaSorin Liaison XL        | 2  
| October 2016 | IDS iSYS New              | 0  

Fig 19. Number of DEQAS participants registered for 1,25-dihydroxyvitamin D for distributions between January 2011 and July 2017 and results submitted.
The most notable changes in $1,25(OH)_2D$ methodology over the last 5 years have been the introduction of the automated immunoassays which have seen a rapid increase in use. Consequent on this has been the decline in use of the long established manual radioimmunoassays and enzyme immunoassays; the DiaSorin RIA was withdrawn in 2017. Although there are still only a small number users, there has been a steady increase in participants using LC-MS/MS.

Figure 20 shows the shift in use away from the manual radioimmunoassays, and enzyme immunoassays, to the automated assays on the DiaSorin Liaison XL and the IDS iSYS analysers. The automated analyser method groups comprise 76% of the results returned for the July 2017 distribution, with the DiaSorin Liaison XL being the dominant method with 59% of the submitted results.

**Method Bias**

The bias of individual samples from the ALTM for the major methods over the last six sample distributions (30 samples) is shown in figure 21. Over this period there is significant sample to sample variability; however, in April 2017 three method groups (DiaSorin Liaison XL, IDS iSYS New and LC-MS/MS) had biases within +/-10% of the ALTM.
Fig 21. The % bias from the ALTM of each of the major 1,25 (OH)\(_2\)D immunoassay methods for the 35 samples distributed between January 2015 and April 2017.

Inter-laboratory imprecision

Inter-laboratory imprecision for 1,25(OH)\(_2\)D assays continues to be high, perhaps reflecting the low levels of this metabolite and more complex methodology required (separation from VDBP and other interfering substances). However, the mean CV% for the automated immunoassays; DiaSorin Liaison XL and IDS iSYS New, show a considerable improvement over the other methods.

Fig 22. Mean inter-laboratory imprecision (CV%) of 1,25(OH)\(_2\)D results for distribution cycles since April 2015.
Performance Assessment

The bias limit for acceptable performance for 1,25(OH)2D has remained the same since it was introduced for the 2009-2010 distribution cycle. To achieve acceptable performance over the distribution cycle, participants are required to return results for all 4 distributions and to have 80% of their results within 30% of the Target Value. For the 2016 - 2017 distribution cycle, 129 of the 195 eligible laboratories were awarded a proficiency certificate (66%). 46 participants (24%) failed to return any results on one or more occasions and were therefore unable to achieve acceptable performance.

Due to the considerable method-related variability, the use of the ALTM as a target value for 1,25-dihydroxyvitamin D proved controversial and since October 2013 performance has been judged against the Method Mean. As can be seen in Figure 21 there is still a large disparity of results given by the different methods and the ALTM would be heavily influenced by the dominance of the DiaSorin Liaison XL method group.

Conclusion

Despite the limited clinical need for the measurement of serum 1,25(OH)2D, there has been a small but steady increase in the number of participants submitting results for this analyte. This is largely due to the availability of the assay on the DiaSorin Liaison analyzer.

From an analytical standpoint there is clearly a need for a Reference Measurement Procedure (RMP) and/or standard reference materials (SRMs).

Summary

1. DEQAS is a specialist external quality assessment (EQA) scheme for the vitamin D metabolites 25-OHD₃, 25-OHD₂, 1,25(OH)₂D and 24,25(OH)₂D.
2. DEQAS distributes 5 unadulterated human serum samples quarterly for all metabolites. Serum used for performance assessment is never spiked with additional material.
3. Participants in the UK and Republic of Ireland can enroll in a supplementary scheme in which an additional 3 samples are distributed quarterly (in between the main distributions).
4. DEQAS is an accuracy based scheme for 25-OHD. Results are assessed by comparison with those of an internationally recognized Reference Measurement Procedure.
5. DEQAS has successfully worked with the VDSP to improve the standardization of 25-OHD assays.
6. The influence of sample matrix is believed to be largely responsible for the persistent differences in sample bias seen in non-extraction automated ligand binding assays. LC-MS/MS methods show much less sample to sample variability in bias.
7. Performance of 24,25(OH)₂D and 1,25(OH)₂D assays remains poor.

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Organiser  Administrator  Consultant Clinical Scientist

October 2017
References


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