CCQM Cell Analysis Working Group
Online

29 April 2021

Meeting Report

WG Participants: J Campbell (Chair, NML@LGC, UK), B Fu (Vice-Chair, NIM, China), N Faruqui (Rapporteur ,NPL, UK) , S Zou (NRC, Canada) , J Choi (KRISS, Korea), J.Y.Lee (KRISS),), J Cavalcante (INMETRO, Brazil), F.Leve (INMETRO), J.Martins (INMETRO, D.Cavalcanti (INMETRO), E.Barrias (INMETRO), G.Pinheiro (INMETRO), C.Lopes, A.Leon (INM Columbia), J.Leguizamon (INM). R.Morato (INM), F Rojas (ISP, Chile), C Divieto (INRIM, Italy), F Pennecchi (INRIM, Italy), L Revel (INRIM, Italy), S Vessillier (NIBSC, UK), D Rajagopal (NIBSC, UK), S Fuji (NMIJ, Japan), A Kummrow (PTB, Germany),L Wang (NIST, US) S Sarker (NIST), L Pierce (NIST), N Lin (NIST), L Tian(NIST), I Kepiro (NPL), M Ryadnov (NPL), M.Vonsky (VNIIM, Russia), A Runov (VNIIM),

1. Introductions

J Campbell welcomed delegates to the meeting and proceeded to go through the different elements of the agenda for the three online meetings that has been organised for the Spring 21 meetings. He highlighted that the three discussions for the 29th April are the current pilot studies under consideration. He also wanted to discuss about some workshop ideas that he had made to the plenary meeting for viral quantification. He further highlighted that the 6th May discussion would be focus on the particle number quantification proposal from NMIJ alongside a further two new proposals, one was by M.Ryadnov regarding the transfection of virus-like particle and from M.Vonsky regarding mycoplasma quantification. Both of these proposals are also scheduled for discussion at the joint CAWG/NAWG meeting on the 18th of May.

2. Current pilot study

2.1 Quantification of membrane intact *E.coli* in drinking water (P205)

B Fu (NMI-C) provided an update to the proposal and study materials, and went into details of the study design. He presented the two methods that will be used in the study, membrane filtration and flow cytometry to enumerate membrane intact *E.coli* in drinking water. He proceeded to the updates from the discussion in the last meeting and said that they have prepared a new sample of *E.coli* which is a non-pathogenic strain. NIM China presented data verifying that the strain did not contain virulent genes by gel electrophoresis of PCR product**.** B.Fu proceeded to talk about the homogeneity analysis of the new sample with the results indicating that the inhomogeneity of the material was insignificant. A detailed updated protocol was presented with an acknowledgement given to Nancy Lin (NIST,USA), especially for help with the flow cytometry method.

The chair asked for confirmation of the deadline for the feedback to the study leads. B Fu said that he will share the document with the members and will hope to get the feedback by the end of May from them. The Chair agreed that it was a good idea to do so as it might not be possible to so today. He also stated that there was a concern about the safety aspect of the material and wanted to know from the members if they had any comments on that. He added that members can also comment on it when they provide their feedback on the protocol. B Fu added that the virulent genes assessment was done,

and the certificate was shared with NIST colleagues who reported it to be suitable for import purposes. N.Faruqui asked if the strain was a GMO and B Fu confirmed that it was not. J.Cavalcante asked about the stability time of the material under storage. B.Fu replied that it can be stored for more than year. The new material was prepared last year, and it is stable so after the sample is received and if it is stored at -80C freezer then it can be stable for 1 year. G.Pinheiro had a query about figure 5 related to flow cytometry method and wanted to know if there was any update as he compared it to the one that was present in the last document. He suggested that we should do staining at each dilution point, then 3 technical replicates were also stained. In the calculation of result, there are three independent standard procedures for each dilution. So, considering this he thinks we should do three independent stainings. He wanted clarification of which one was correct - Whether we should do three staining of the standard dilutions or just one staining for the three technical replicates. B Fu replied that for each sample, we get three replicate dilution, for each one we need to do one staining. For each staining, we do three-time flow cytometry analysis. B Fu said that he will confirm and modify the description for the highlighted query. The chair highlighted that we would have a week of getting the feedback regarding the protocol to B Fu and we should expedite it as soon as possible. He also suggested that we should organise another meeting post the feedback period.

Action:
Lead to modify the description of the staining procedure in light of discussion on the 29th April.
Chair to circulate the updated protocol to all members after the online meeting.
Participants to expedite feedback on the updated protocol.
Chair to organise another meeting post the feedback period in a few weeks' time.

2.2 Quantification of fixed peripheral blood mononuclear cells in suspension (P217)

D Rajagopal provided the update to the project. She highlighted that all participants of the project who have provided results will present summaries. There were ten NMI participants in the study and nine of them will be presenting the results today. The enumeration methods ranged from manual to automated, volumetric to bead based flow cytometry evaluation were performed. She proceeded to provide a detailed description of the evaluation conducted at NIBSC, followed by individual participants results which was presented by representative from each measurement institutes. S Zou from NRC, A Kummrow from PTB, B Fu from NIM-C, C Divieto from INRIM, J Choi from KRISS, F Leve from INMETRO and L Wang & S Sarkar from NIST presented the results obtained for the study. Finally, D Rajagopal summarized the results obtained from all institutes using different methods employed.
S Vessillier thanked Deepa for the compilation and summary of the results and she said that the main point they wanted to discuss was about the statistical analysis of the data whether it would follow the same analysis as done in P165 or in any different way. She also added that the use of the graphical analysis of NIST would be great. L Wang added that in their analysis they see some debris and after discussion with Sandrine and Deepa, they have included everything in their analysis but it would be useful to test whether some centralised analysis could be done and Sumona's automatic analysis could be used for synchronized analysis. She also highlighted the analysis done by PTB could be another way to analyse the data. A Kummrow added that he was not sure if they could help a lot from the pipetting side, as there are different types of pipettes. It depends on what type of pipettes people are using and if they are using different types then results will be different. Gravimetric control will be easily reproducible, but this was not the approach taken here. So, pipetting will be part of the uncertainty of the measurements, but this was not the major problem. The major source of uncertainty is coming from the counting of cells in flow cytometry. Staining can help in counting to determine what is a cell and what is not. All other sources of error is small in comparison to this. J Campbell highlighted that the main concern is the different ways in which people have interpreted the protocol and Lilli's point of having a centralised method of analysis is good or take into consideration several NMIs. S Sarkar added that they would like to use the tool that they have developed to do a centralised analysis. The

tool does not cover the uncertainty analysis per se. It provides other metrics, like CV, R^2, proportionality index, measure of bias coming from proportionality etc. It would be helpful to analyse any type of data whether it is generated by cytometry or microscopy methods. She recommended that they could provide the data template and if the participants could enter their data into the template as that would really ease the analysis process for them. There is also room for more metadata for e.g. add time for flow data if it is available. Also considering the number of particles as that would help to establish the higher uncertainty associated at lower dilutions. These were some of the thoughts that she shared with the members regarding the analysis. S Vessillier also reiterated that it is better that the participants entered the data themselves into the template some of the data was difficult to decipher when trying to consolidate data for the analysis (check Excel presentation of the data). S Vessillier added that it would be helpful to use the NIST developed template and compare it to the analysis that they will do at NIBSC, taking the approach like the one adopted in P165 study. D Rajagopal also added that it would be great if NIST shared the template and guidance for the analysis method that they have developed. S Sarkar confirmed that they would provide it via email in a week or so. S Vessillier added that they were still waiting for two participants to return the data. D Rajagopal added that for the manual counting method whether they should follow one approach and A Kummrow added that he is not sure whether they would or not as PTB, INRIM AND NIM-C have microscopic images but he was not sure if all participants had it or not. He highlighted that the problem that he saw was in the lower concentrations. He said that maybe the differences were due to type of chamber used for the analysis. S Sarkar mentioned that they were running four parallel methods and only one of the method showed some challenge in lower dilution level and other ones were fine so she could say that in their case it was not the sample handling per se which was contributing to the error, but there is something about the assay itself that is struggling at lower dilutions and there will be some interesting results to look at in the study. The Chair summarised that the next steps was to get all the participants to submit their result and have a centralised analysis portal to be decided and certain elements of this would be coming from NIST. A Kummrow highlighted that the fluorescent beads measurement would be conducted at PTB and he wanted the participants to send it to PTB prior to the summer break as it could get delayed if it was done during the summer break period.

Action:
NIST to circulate via email the analysis app and template.
Participants to provide PTB with their Trucount bead samples to summer break

2.3 Proliferative stem cell number per unit area (P197)

The update for the project was provided in combination between three institutes, NPL, INRIM and NIST. N Faruqui gave an update on the cell model and with the previous discussion with the members it was highlighted to use a more clinically relevant cell model and MSC (ATCC cell line) was selected based on the previous discussions in the group. These cells are widely used in clinical studies for various disease therapy. The sample preparation using this cell line was discussed by I Kepiro and she showed the cells were labelled using a nuclear and proliferation stain and how the entire well of the chambers used was covered. She also highlighted the various standards that would be considered in formulating the imaging protocol for the study. This was underway in collaboration and active participation from INRIM and NIST. The sample packing and transportation was also discussed and efficacy of it was demonstrated as the samples had been sent to NIST and INRIM.

C Divieto then proceeded to present the analysis that they had conducted. The area measurement of the grids in the chambers using laser profilometry was performed using SI traceable sensors. They also looked at the prepared samples and initially looked at the cleanliness of the bottom of slide and asked NPL to provide a cleaning protocol for the chambers as from their experience of P123, it is important

to have a suitable protocol to minimise any issue of the breakage etc of the samples. They also imaged the sample at two different magnifications, and they found that the cell distribution was quite homogeneous, but she stated that they will perform homogeneity and stability studies of the sample which would require time to perform. She also added that some of the issues in counting highlighted by I Kepiro for e.g. cells which lie on the line of the grid etc. She said that they will define and measure the area inside the grid line to get a better area measurement. She also introduced the concept of using the fluorescent beads to consider the different nuclei in different stages of proliferation with different expression of fluorescence intensities. She pointed to the use of fluorescent beads to get a standard for the fluorescence intensity. She concluded by saying that they will contribute in the development of the protocol and homogeneity and stability study of the samples.

S Sarkar (NIST) provided an update. She spoke about Spherotek fluorescent beads that has been used for flow experiments which could potentially be useful in this study to bench mark the fluorescence intensity of the labelled cells for imaging purposes. They have been assigned reference values and the same rainbow bead could be used for both channels that is used in the current study. She highlighted that it was important to look at the phase image alongside the fluorescence ones because it could give information about the morphology of the cells and can explain the confounding fluorescence images. The also used the fluorescent beads at the same acquisition settings for the sample and it shows that the beads that they have tested are in the same range as the sample intensities and this was a critical parameter to verify if these beads were to be used in the study. They proposed that the empty wells in the chambers could be used for the beads and they could be used together with the sample to benchmark the fluorescence intensity. The beads were used without any mounting media and it would be required to be tested further. The stability etc of the beads would need to be tested and if it works then it could provide a means of testing the various imaging systems/regimes that will be used by various participants.

A Kummrow said that years ago he had used these beads and commented that the proportionality is good but some beads at higher magnification does not have homogeneous fluorescence. The other problem was the higher dynamic range. When the beads are put on the slide then they cluster but maybe with the use of mounting media they could be dispersed. S Sarkar said that the analysis of these beads would require some expertise from the imaging people at NIST and expertise of L Wang and A Kummrow. There is a potential of using these beads in the study and using it for thresholding purposes to get a positive or negative proliferative cell. A Kummrow also highlighted the wide debate within the standards community regarding MSCs. ISO decided to use the term mesenchymal stromal cells, although the abbreviation does not change. He said that it was like a religion some believe, and some do not! He also highlighted that for this study it does not matter what type of cell is used but it may have consequences for publication. He said particularly that we need to be careful with the chosen term. The Chair also reiterated that we need to be careful too. He also mentioned about how we word it if it is proliferative or proliferated. He said that we will need to carefully consider this aspect. N Faruqui added that we will continue to work with INRIM and NIST especially with the use of beads for benchmarking purposes. We will be using these beads at NPL as well and try different mounting media to analyse the stability of these beads. We will continue to have a small group meeting and report the results in Oct with the aim of sample distribution after that.

Action:
Chair to organise a meeting before October meeting to touch base on the progress of the project.

3. Future meetings

3.1 2021 online meeting: 6th May & 18th May

9 Any other business

There was no other business.

10 Close

The CAWG meeting concluded at approximately 14:55 (GMT) on 29th April 2021.

Annex 1: List of actions for CAWG 12th meeting 04/05 2021

ID	Action	Comment
CAWG/2021-1/1	P205: Lead to update the protocol with regards to the comment received at the meeting (29th April) Chair to circulate the updated protocol to all members after the online meeting. Chair to organise another meeting post the feedback period in a few weeks' time.	Complete Complete Complete. 25th May. 1pm Paris time.
CAWG/2021-1/2	P217: NIST to circulate a data template to facilitate a centralised analysis of the data. Participants to provide PTB with their Trucount bead samples prior to summer break Statistical analysis will be conducted at NIBSC following receipt of results	
CAWG/2021-1/3	P197: Chair to organise a meeting before the October meeting to touch base on the progress of the project.	