CCQM Cell Analysis Working Group
Online

6th May 2021

Meeting Report

Participants: J Campbell (Chair, NML@LGC, UK), 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), 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), H Goenaga Infante (NML@LGC), D.Bartczak (NML@LGC), J.Norieaux (LNE, France), P. Fiscaro (LNE), T.Sutthinun (NIMT), A. Röthke (PTB), K.Tong (GLHK), YT Tsoi (GLHK), K.Tse (GLHK), A.Gadelha, M.Winchester (NIST), K.Murphy (NIST), A.Gadelha (INMETRO), A Montoro Bustos (NIST), H.Sakurai (NMIJ), J.Abella (INM Columbia), R.Carvalho Silva, J.Serna Saiz (INM), M.Johnson (NIST), A.Gorbushina (BAM), Y.Kuruma (NMIJ), D.Ahumada (INM)

1. Introductions

J Campbell welcomed delegates to the meeting and proceeded to go through the different elements of the agenda for the day. He highlighted that the three discussion today was on the new proposals followed by an administrative discussion regarding the working group.

2. New Proposals

2.1 Quantification of virus-like particles. Cross – WG activity (NPL)

E.deSantis presented a proposal and highlighted the need and challenge related to cell and gene therapies and vaccine development. She highlighted different types of viral vectors which were currently in use. She went on to highlight measurement challenge including number of particles (count), Cell particle uptake (count and mass fraction), amount of functional cargo at the intracellular target (copy number and mass fraction) and the need for a reference material to bench mark the different stages of these complex processes. There was also a need for traceable methods and correlated measurements to scan the entire processes. Based on these challenges, the study was proposed to look at number/ concentration measurements of virus like particle. Given the various challenges, a cross working group study was proposed to dissect the different elements of the process and utilise the strength of each working group to carry out the study by looking at different stages of the process and the quantification requirements. The proposed study would utilise a synthetic virus like particle, developed at NPL using a bottom up approach. This bioengineered system has advantages; biocompatibility with control of encapsulation properties. She presented some examples of the systems that has been developed at NPL, e.g. with of predictive sizes, charges etc. She also presented the standardisation drive for these peptides where the size distribution of these encapsulation systems was studied under the remit of VAMAS. The size range of the particles under examination was within the range of AAVs which is a viral vector commonly used for gene delivery. She shared the pre-validation and pre characterisation studies conducted under an ongoing VAMAS project. These systems also have biological function as one of the systems could be encapsulated with

a plasmId DNA and the successful transfection of this was monitored by examining the effect of expression of GFP inside a cell.

Following the presentation, the Chair started the discussion by asking if NPL was proposing a stand alone study (CAWG) or joint studies (ie. with NAWG). E DeSantis answered that it depends how the working group felt but we could have joint and or standalone studies. Initially if the group felt that it would be required to have a preliminary study to then drive a joint one. M Ryadnov added that in the first instance it would be good to agree within CAWG and NAWG on the technical details, but both stand alone, and joint studies were feasible. The current strategy of CAWG is to count the cells and related entities, and we need to agree if we want to do fluorescent counting using microscopy or flow cytometry. With respect to NAWG, we need to decide on the type a target sequence to integrate. They would use PCR analysis for their study, and we need to decide with them if they want to use mRNA or SiRNA in their design. For the CAWG we will be using the same material, which can be used for both Working groups. J Campbell added that this is also of relevance to PAWG and it would be a good springboard for us to approach them. M Ryadnov added that yes it would be good too, as they normally use mass spectroscopy for their analysis, and it can be used to get the mass fraction of the monomer. They can also derive K_D values which can be a useful parameter, but PAWG is not there yet. There is a subgroup under PAWG, and it can be proposed there but at this time it is perhaps not realistic. J Campbell added that he has spoken to the chair of NAWG and there is a lot of interest in that group and he said that he will reach out to the chair of PAWG and he believes that they would also express interest. He added that he has additionally proposed a workshop related to this theme in the plenary, with support from the NAWG chair. Historically, he said that they have had this discussion as to where this activity will sit whether it a CAWG or NAWG and he said that he was willing to trigger this discussion and hence proposed a workshop. J Campbell asked members of the group for comment regarding the proposal and since there were no additional comments, M Ryadnov added that the group will perhaps need time to digest and we will need a feedback from the group. J Campbell said that we do have a joint session on 18th May and if the slide deck was distributed to the members along with the minutes of the meeting then members will have time to think about the proposal. He also requested that the presentation be given again at the joint CAWG/NAWG meeting. L Wang from NIST said that it is a topic of interest for them and she added that it was difficult one too. A lot of capabilities would need to be taken into consideration and she said that they would support the study if CAWG would want to do it. She also requested NPL share the slides as it would help in their internal discussion and how they can support and join the study. J Campbell suggested that the slides could be shared which could give time for members to think about the project.

Action:
NPL to share the slides of the presentation to the working group members.
NPL to deliver the presentation for further comment at the joint meeting of CAWG/NAWG
Chair to contact the PAWG to gauge interest

2.2 Particle number concentration measurement for cellular analysis (NMIJ)

Y Kuruma presented the update to the study proposal. He had complied the result of the questionnaire which was received from all the members interested in participating and wanted to discuss the draft protocol for the study. He stated that the proposed study has four main purposes; to confirm NMI measurement capability of number/ concentration of red blood cells using stable artificial particles, although number/concentration of RBC is dependent on the dilution procedure for measurement using particle analysers and the primary dilution factor is out of the scope of this study, second purpose is to investigate differentiation between different measurement principles, third purpose is to state the applicability of using of artificial material for cellular analysis and the fourth purpose is to

establish study protocol for a future key comparison and a basis for CMC registrations for cell/particle number concentrations. He went into the details of the study material which he stated would be an aqueous suspension of monodisperse, spherical, non-coated particle which will have a nominal diameter of 5 µm. It also contains trace surfactant and preservatives. The raw PSL suspension will be purchased from a commercial source and diluted around 50 times with water with no additional surfactant added. Concentration of the particles will be in the range $0.5x10^6$ - $1.5x10^6$ particles/g supplied in a 50mL bottle with a screw cap. 2 -3 bottles will be sent to each laboratory. NMIJ can produce 40 bottles (maximum) which limits participants to around fifteen. NMIJ have conducted preliminary homogeneity and stability testing of the material. They concluded that the candidate sample will be suitable for the proposed study. Y Kuruma elaborated the sample handling requirements of the material and also some details of the analysis process. He then stated that the number/g was a desirable unit in comparison to number/mL. NMIJ will only provide a nominal value of density and participants will be required to measure it themselves. A preliminary schedule of the study was given (finalisation of protocol, study number assignment , material prep May, Call for participation, September, distribution of material October, submission of results March 2022). He proceeded to give details on how the study material will be distributed and the format in which the participants were required to send the results to NMIJ. He also added that the reference value will be calculated from the average of all the results by eliminating the outliers. He then discussed the results from the questionnaire that NMIJ had obtained from interested participants (12 labs) including the instrumentation available to the study. There was a question about the homogeneity of the diluted material, and he said that NMIJ will conduct it on the stock material and the participants can conduct it on the diluted material themselves. There were questions regarding the custom clearance requirements for the material and he said that they will provide the required details. Fluorescence properties of the material were also queried, and he confirmed that they are not fluorescent particles and no fluorescence molecules will be attached. Next query was regarding the addition of the preservative/surfactant and he confirmed that the stock material contains it but no additional ones are added to the material the sample preparation stage. He concluded by stating that he wanted to decide in the meeting today the specification requirements for the study material.

A Kummrow started the discussion by saying that in the presentation the particles were compared to red blood cells, but it can also be compared to white blood cells and in fact it fits in better. He added that some companies send PTB particles for calibration compare it to white blood cells especially the concentration range if raised by a factor of two or so higher. He commented that the perspective should not be limited to red blood cells only. He added that if you look in cell counting most people (clinical) will be looking at number in mL and this is what they want but it in this study he feels that it will be better if we focused on number per g and not on mL. Participants should report in per g but if they find it impossible to calculate the density of the material then they can do so in per mL. He added that it is not trivial to measure density , but not a major obstacle. He also added that there are questions regarding the dilutions of the material. If they are controlled gravimetrically then there is no problem in reporting it in per g. If volumetric instruments are used then it has to be reported per mL (and cannot be in g).

Another point was on how to create the result of the complete study and it is proposed to calculate the simple arithmetic mean by the removal of the outliers. This is a delicate business, (LGC have used Der-Simonian-Laird mean) and we should ask S Ellison from LGC or another statistician to do the analysis of the study. It will automatically take care of the outliers. He also highlighted that outlier testing is difficult and we should ask a statistician to do as its not so simple to do. J Campbell added that we should be reporting the outliers and A Kummrow agreed and said that they should also contribute to the result and they have less weight. It is important to relate it to different techniques and how they can produce different results. There could be a technical reason for the outliers and why a particular technique produces different results. He said that he has made a list and he will send it to

the Japanese colleagues directly along with some minor points in the protocol. He added that PTB is providing service in these kinds of measurements and would very much like an interlaboratory study. It sounds a bit strange to use CAWG for particle counting but manufacturers of flow cytometers have interest in these kinds of measurements, and they use it for internal calibration. PTB will send these comments in an email.

J Campbell added that this study fits with our reference method development strategy. He further added that the major discussion in the last meeting had centred around the size of the particles and he wanted to ask the attending members regarding this. He added that there was some discussion regarding a smaller sized material but that would push the study back with regards to time. A Kummrow added the size of the particle is the right size esp. if people are looking at the flow cytometer method. Light scattering can be an issue especially with some methods. J Campbell added that using techniques that IAWG use will give us a traceability aspect. J Campbell wanted to know if we needed to have a formal voting for the study and he then asked the members about the participation in the study; PTB, NIBSC, NPL indicated interest. LGC had a question regarding the size of the material. She added that they have developed a reference methodology directly traceable to the SI unit of Kg, it allows them to measure the number/ concentration measurement, but they are only able to do it with sizes up to 2.5 micron. She said that she understands the rationale behind the 5 µm. She added that they are very interested to participate in the study but would not be able to use the reference methodology with the current proposed size. They would need to discuss internally whether they can still go ahead with the study if the size is fixed at 5 µm. They can take other approaches that can be used but of course the reference method cannot be used. J Campbell added that this is the first one and there could be other iteration of the study that would consider using a lower size of the material. D Bartczak added that they would require more time to discuss internally and will then get back to the WG regarding the participation. Following on J Campbell went through the NMIs regarding the participation, C Divieto confirmed that INRIM would be participating using microscopy and had two observation, we should indicate the claim of the study and clearly state the measurand for CMC claim for later on. It should be clearly stated in the protocol when it is sent in the final version of the protocol to all participants. S Zou from NRC stated that they have very limited technologies to access now which could be used in the study and she didn't know now, but later may be able to give a conclusive answer. L Wang added that her colleagues oversee particle measurements and also added that in terms of the unit they measure per volume so in that sense they would need to know the density in order to convert particle/g and whether the manufacturer can provide the density with or without uncertainty. They will be using optical counter and flow cytometer for the measurement. NIST provides measurement in this size range. M.Johnson (NIST) added along with her colleague they do single particle ICPMS measurements and the size is larger than what the instrumentation is used for, but they will be making some considerations. J Cavalcante affirmed that they would be interested in participation but will need to speak to her colleagues internally. J Choi from KRISS expressed his interest and added that they would use flow cytometer and optical microscopy in the study.

The Chair concluded that from the NMI roll call there was a lot of interest in the study and some have expressed that they would need to discuss internally and he encouraged them to get back to NMIJ once that happens within each NMIs. S Fuji from NMIJ finally added that they will incorporate the feedbacks about the sample from the members and modify the protocol with the material specification. He finally asked if 5 µm sample size was fine and J Campbell added that it was the relevant size for biology and NMIs will accommodate this with their technologies. A Kummrow further added that the pilot study proposed now is 5 µm but in the future for key comparison you plan to use a smaller size and S Fuji confirmed it. A Kummrow said that using smaller sizes would allow to use other methods. J.Campbell wanted some clarification with regards to flow method regarding the number of particles, ie, flow rates if we needed to have it or leave it to participants. A Kummrow added that based on the protocol and dependent on the technique, the participants can dilute the sample as

different techniques would have different requirements. J Campbell also wanted to know when NMIJ would be able to modify the distribute the updated protocol and y Kuruma answered that they would need to discuss internally. J Campbell added that he will contact NMIJ separately to initiate a study number (CCQM-F-02).

Action:
NMIJ to modify the protocol on the basis of feedback from the WG.
Chair to contact NMIJ for the generation of study number
IAWG working group members to feedback to NMIJ regarding instrumentation suitability for the method.
PTB to provide some formal comment to NMIJ in an email.
Study analysis to coordinated with Statistician input.

2.3 Mycoplasma quantification (VNIM)

M Vonsky presented the new study proposal which was initially presented to NAWG but it could have an interest in CAWG as well. He elaborated on the different mycoplasma species that are common contaminants of many biological systems. They can also cause disease to animals and humans. PCR based detection system is currently used and most biological production systems should avoid the presence of these organisms. There is a regulatory requirement for the detection of these microorganisms esp. in biopharmaceuticals etc. There are a variety of species of mycoplasma and he proposed a species which is non-pathogenic to humans but is present in various matrices and is in the top five of the cell culture contaminants. He went on to describe the culture condition of these microorganisms and different structures of mycoplasmas. He commented that in the previous study we were discussing about µm sizes whereas these are much smaller at 500 nm. There are various cell culture methods to detect the presence of mycoplasma. CFU count is the best method for the viable cell count of these organisms and he detailed the methodology used for these counts. The other method is to get the genome copy number. He went on to describe the study materials that could be used in the study. It could be a liquid culture material, frozen culture or lyophilised culture. It survives all these conditions very well. It would be very interesting to study the eukaryotic contamination by these microorganisms but as a first step it might be too complicated.

J Cavalcante wanted to know about the biosafety level of these mycoplasma and M Vonsky replied that it is safe but he wanted to check before confirming the level. He added that they did not require any special permissions to work with it. She confirmed that they would be interested to participate in the study. J Campbell added that it is a very important measurement as it's the first question that people ask with regards to biobanking etc about the mycoplasma infection. He wanted to ask about any requirement for flow cytometry use and if anybody has done this kind of study and if we need to have a pre-pilot study. M Vonsky, added may be, and that it is very easy to grow this mycoplasma and they can provide with this culture. He also wanted to know if the pilot can be based on the PG8 strain from ATCC. J Campbell added that it makes sense to do it on the largest target mycoplasma type. C Divieto asked if we can use microscopy on slides which is already prepared, or the participants would need to grow it in their lab. M Vonsky clarified if the suggestion is to copy the design of P123 or fix it on the agar media. C Divieto elaborated that this is to just avoid any contamination in labs that use eukaryotic cells. M Vonsky said that of course eukaryotic cell culture laboratories would have to avoid growing these cultures and the suggested idea was good and he would like to discuss further. N Lin added that NIST would be interested in this study. They work on other types of bacteria and have the measurement capabilities to do cell number and genome copy analysis. J Campbell added that this will be another study to discuss in the joint session with NAWG and will need to consider in the session which will have the maximum impact for stakeholders. M Vonsky added that they have been asked

from biotechnology industry about the reference material in this area and this is an important point for them and their stakeholders. J Campbell suggested to M Vonsky to distribute the slides to the members so that they can further consider the proposal. He said that there are lot of considerations that needs to be thought about in the proposal. He suggested that maybe a small working group within the group that would help to look at the technical challenges of this study. He wanted to nominate few people like M Vonsky, J Cavalcante, C Divieto and N Lin to work out the technical details for the proposal. J Cavalcante added that they would also need to discuss internally and there could be other technologies from other groups which could be useful for the current study. J Campbell suggested to M Vonsky to present this study again in the joint session for a wider discussion. He said that it would be good to know what capability the CAWG can bring to the study and how much standalone measurement that CAWG members could do.

Action:
VMIM to distribute the slides to members.
VNIIM to check the biosafety level of material and feedback to the WG

3. **Administrative**

3.1: Roles

Chair started off by the volunteer positions in the group. He mentioned that C Divieto was our KCWG member and it is an opportunity to make a formal proposal to her if she would continue with this position for this period and C Divieto confirmed that she will continue. Chair then asked if N Faruqui will continue with the rapporteur role and she also confirmed to continue.
He showed that the BIPM website has been updated which gives us an opportunity to consolidate our own website. He wanted to know if there was a volunteer in the group who help him with it. He wanted them to get in touch.

3.2: Plenary discussion

He went on show the slides that he presented in the plenary. He summarised from the questionnaire that was circulated to the group and the compilation of the stakeholders was done from that exercise. How the group is split and the technologies that are related to the group. He added that the attendance to the group meetings have increased, especially due to the online set up. He presented the various study's and their status and wanted to know if there was anything incorrect there. There are two studies in progress and three studies in planning and another T cell study which is in planning without a study number and it would be good to see flow cytometry based studies for eukaryotic cell quantification would evolve. He reinforced the cell counting metrology angle for eukaryotic and prokaryotic cells and how further studies could increase in complexity. He said that he made the case to BIPM that we will be putting forward a key comparison in the next decade –possibly three key comparisons in the next decade. He said that counting was not the only thing that we can do and at some point, we need to discuss about viability and function studes. We started to uncover counting, differential counting and there is a huge task to do both for reference method development and characterisation of biological function. He highlighted that it is important to keep the strategy document as a live document and these kinds of discussions are best when done face to face so hopefully in 2022 we need to reassess and have a session when we can meet to face to face to work on its development. He added that he gave some priorities for the working group for e.g. establishing reference methodology for cell counting, total differential cell count by relevant cell properties, to dovetail our studies with measurement expertise and priorities of other working groups and also

alignment with CIPM challenges together with continue to liaison with JCTLM and ISO committees, perhaps establishing a taskforce in working group to examine complex challenges.

He added that he proposed a workshop to CCQM based on virus like particles, which will be an online workshop and will be arranged later in the year. He continued to go through slides about the tentative project ideas and challenges. One of the questions is the requirement of accuracy in the area and people make do with lower accuracy and whether it is required in the field so he said that he felt that if we will get higher accuracy in our measurements then people will need it and the regulatory framework will change accordingly and this is a huge task; to improve accuracy and precision in our measurements. He asked if we needed to parcel some challenges in the area and create a task force to think about it. He added that he didn't need an answer today but its something for the members to think about going forward. He added that he has proposed a workshop on virus like particles and it will be discussed further in the joint session on 18th May but he wanted to know if members wanted to propose more ideas for workshop for the next year. He highlighted the CCQM strategy document available, which has been prepared by R Wielgosz and the SPWG. He added that he has prepared a plenary report that hopefully gives a fair reflection of where we are with our studies. He elaborated on the areas where our group can have an impact on for e.g. digital pathology, environment etc.
He elaborated on the new BIPM website which will have a CAWG area and he will post the documents on that website. Members should be able to create their passwords etc for accessing the website.
He summarised that we have had two online spring meetings and we are due to have a joint meeting with NAWG on 18th and another meeting to be organised soon for P205 study. He again added that P197 would need to have another meeting and that could be either joined with P205 or another separate one that can be organised.
S Sarkar added her thoughts that the work programmes is coming together in terms of complexities over time. She suggested maybe some time needs to be spent on the theoretical kind of goals could be for reference materials and reference methods and if we could outline what could constitute those things without actually build those or decide on what they are. Outlining the key principles which would guide the development of those things as it is a whole new world in terms of stable, reference materials etc. Chair added that we need to think along these lines and start to build up complex measurands. S Sarkar added that part of this thinking would be when we think about what the design of a synthetic material will look like which will represent a cell better, if we have a list of criteria that we need to have to better capitulate the properties and then it can help in circumventing the issue of transportation, stability etc. J Campbell said that we have started to think about reference specifications for complex measurands and its something that we have thought about more in eukaryotic cells than prokaryotic ones. S Sarkar said that its something that she meant and added about reference methods and Chair said that he will add it to the list. Chair added that we need to have an interim meeting for a subset of the group before the Oct meeting to discuss some of these ideas. L Wang added that it should not be a small meeting but should be open to all. A brainstorming session for a bigger group to collate all the ideas. Chair agreed that it will help to map it all. He added that we need to work as team to liaise with other standard organisation like JCTLM, ISO etc and to stay abreast with the developments in each of these committees. A Kummrow said that one option would be to have a half an hour in the meeting to update the group on the ISO committee that the members are part of. The ISO committees are not focused on cells, nucleic acids but relevant to the group can be reported here. The chair suggested to have an exclusive P205 meeting (on the 25th of May) and then in the late summer, early autumn organise a meeting before Oct to take a critical look at what we have provided in the strategy document. We can have a dedicated meeting so that members can come prepared to input their ideas and we can keep the impetus going for the strategy of the group and also other committees relevant activities can also be fed in that meeting.

Action:
Chair to post the plenary report on the website and meeting reports and talks on the website.
Call for volunteer to help with the CAWG website.
Chair to organise a meeting in September 2021 to have a focus discussion on the group's strategy.

4. Future meetings

4.1 2021 online meeting: 18th May (Joint with NAWG). 25th May (P205 protocol feedback)

5 Any other business

There was no other business.

6 Close

Chair thanked all the participants and presenters. The CAWG meeting concluded at approximately 14:12 (GMT) on 6th May 2021.

Annex 1: List of actions for CAWG 2019-10

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 update progress of the project.	
CAWG/2021-1/4	New Proposal I: Transfection with virus-like particles. Cross – WG activity (NPL) NPL to share the slides of the presentation to the working group members.	

	NPL to deliver the presentation for further comment at the joint meeting of CAWG/NAWG Chair to contact the PAWG to gauge interest	
CAWG/2021-1/5	New Proposal II: Particle number concentration measurement for cellular analysis (NMIJ) NMIJ to modify the protocol on the basis of feedback from the WG. Chair to contact NMIJ for the generation of study number IAWG working group members to feedback to NMIJ regarding instrumentation suitability for the method. PTB to provide some formal comment to NMIJ in an email. Study analysis to be coordinated with Statistician input. Chair to approach NMIs (LGC?)	Form-02 provided to NMIJ 20/05/21
CAWG/2021-1/6	New Proposal III: Mycoplasma quantification (VNIM) VNIIM to distribute the slides to the members. VNIIM to confirm biosafety level of material with members	
CAWG/2021-1/7	Administrative: Volunteer to help with the CAWG website. Chair to post the plenary report / talks / strategy / minutes on the website. Chair to organise a meeting in September 2021 to have a focus discussion on the group's strategy.	