Report

30th ENGL MEETING

JRC Ispra

1-2 October 2019

The European Commission’s science and knowledge service

Joint Research Centre
7) Welcome and approval of the Agenda
The Director of JRC Directorate F ‘Health, Consumers and Reference Materials’ welcomed the participants and highlighted the historic importance of the network.
The Chair enquired whether the Agenda needed modifications. The Agenda (annex 1) was approved without amendments.

8) Approval Report 29th ENGL Plenary
The Secretary requested verifying if personal data was included in the ENGL report. The report was adopted without modifications.

9) Dynamic Action List (DAL) of 29th ENGL plenary
The Secretary reviewed the open points of the action list. He informed that the request of opening a forum on research topics in the ENGLnet will be included in the agenda of the following SC meeting.

10) Outcome of the 36th and 37th ENGL SC meetings (February and June 2019)
The Secretary summarised the main points discussed in the last two Steering Committee (SC) meetings. In the first meeting, the SC decided to postpone the preparation of a report on genome editing in animals and in the second meeting, it established a Working Group (WG) for amending the Methods Performance Criteria (MPR) document. The members also agreed launching a survey on the EURL GMFF web applications.

The Chair considered the report prepared by the ENGL on the detection of food and feed plant products obtained by new mutagenesis techniques as a cornerstone for the subject. He provided an update on the appropriateness checks of CRMs made available in the frame of Regulation (EC) No 1829/2003. He reminded that a survey launched by the EURL GMFF upon request of DG SANTE had identified problems in the quality of and information provided for some CRMs. As a result, DG SANTE had organised two meetings with the applicants and extended the mandate of the EURL GMFF, which has now also to verify the appropriateness of the CRMs according to the requirements laid down in Regulation (EU) No 503/2013. The workflow and criteria have been published on the EURL GMFF webpage and were communicated to the applicants, EFSA and DG SANTE. In the process of GM authorisation EFSA will finalise the dossier only after the successful assessment of the detection method and the reference material by the EURL GMFF. Thanks to the reported observations within the ENGL network and the following survey this aspect has now been added to the regulatory control.

The Chair requested to report problems on CRMs to the EURL GMFF, which has now the mandate to intervene. He also reminded that in the renewal process both the detection method and the CRMs will be verified and that the detection method had to fulfil the MPR ENGL criteria applied at the moment of renewal. A JRC representative informed that several AOCS certificates have been already updated with the required information.

11) Discussion of Progress reports of ENGL WGs:
WG Update of Methods
The WG chair informed that the guideline on update of methods for renewal of authorisation was submitted to ENGL members in 2018; it has been approved by the SC in June 2019 and further revised. The final version will be sent for approval to the SC and then for consultation to EuropaBio and DG SANTE.

WG ENGL Procedures
The WG chair informed that the new document on WG ENGL procedures has been published in ENGLnet. These new procedures are considered as the internal rules as specified in the Consortium Agreement. He notified that the rules for forming an ad-hoc WG, when immediate action is requested, have not been yet defined. The chair asked to maintain the WG on ENGL procedures in standby so that it could be activated when new requests or procedures are needed.

WG multiplex PCR methods
The WG chair reported that a kick-off meeting has taken place in March 2018 and that the WG had formed different subgroups. The speaker presented a flowchart for the verification and validation of the multiplex methods. The document is now in its fourth draft version. It aims at providing practical guidance on experimental set-up, verification and single laboratory validation of multiplex PCR methods. This version will
be discussed on a web-meeting at the 15th-16th of October to resolve the open questions and missing contributions. A physical meeting with all WG members will be schedule in early 2020 to finalise the document. The WG chair asked if the document should also cover multiplex digital PCR since guidance on the technology has been already provided in another ENGL document.

The Secretary remarked that in the mentioned document the chapter on multiplex digital PCR was not covering practical aspects. He suggested including also the latter for digital PCR in the guidance on multiplex assays. The WG chair suggested inviting members of the WG to the workshop on digital PCR planned in November 2019 at the JRC in Geel.

**WG good practice/quality of DNA sequencing data**

The WG chair reported that the WG has been divided into subgroups addressing different subtasks. A videoconference will be organised with the coordinators of the subgroups. A physical meeting will be organised in January 2020, location and date to be decided.

**WG DNA extraction**

The WG chair informed that different aspects of DNA extraction are covered in six subtasks. They will be discussed in a physical meeting on 25-26 November 2019. The group is considering organising a training course on DNA extraction for the following year.

A participant from the United Kingdom reminded that information on DNA extraction collected in a workshop organised in 2017 was later organised and made available in a database on ENGLnet. The Chair commented that during the NRL session of the meeting, DNA extraction was considered a relevant topic and encouraged the WG in suggesting best practice approaches.

**WG Selection of Methods for Validation**

The WG chair reminded that two methods were selected for validation: one for detection of a potato reference gene, the other consisting of a pentaplex PCR assay. These two methods were undergoing further testing and optimisation. She also reminded that the WG requested to visualise the EU authorisation status of different GM events on the JRC GMO-Matrix web interface. The members performed a gap analysis to define the priorities for 2019 and concluded that all species and GM events are covered by the current screening and identification strategies. The members also concluded that digital PCR methods although not covering an analytical gap could provide a useful analytical tool and could be considered for validation.

A list of recommended reference genes for taxon-specific methods will be prepared and finalised by JRC members. A member from Czech Republic screened the literature to identify possible targets for detection of animal species. A short list was prepared considering only targets present at single/low copy number and having a real-time PCR method validated in-house.

The speaker announced that the list could be retrieved on the presentation slides made available on the ENGLnet.

**New WG on Method Performance Requirements**

The WG has been established and the EURL GMFF presented the mandate. It was reminded that the ENGL document on Method Performance Requirements (MPR) provides the parameters for acceptance of a method submitted by the applicant and those that had to be fulfilled after validation. The new mandate specifies three main topics:

1) Verification of the current criteria and their applicability to digital PCR-based methods
2) Verification of the current criteria and their applicability to detection and quantification methods for genome edited products
3) Applicability to methods for detection of GM animals (in a second phase).

As objective, the final draft should be ready for the next ENGL meeting (end September 2020). As a proposed schedule, the WG will have a web meeting in November and a physical meeting early in 2020.

12) Update from DG SANTE
DG SANTE provided an update on the following topics:

**Seed testing**
In October 2018, Member States requested further convergence in Regulatory Committee 2001/18. A technical group, the WG 2001/18/EC received the mandate to explore the possibilities. These were compiled in a room document covering different aspects from risk based sampling plans to sample preparation and proficiency testing. The room document should be finalised at WG level by the end of 2019 or in the beginning of 2020 for subsequent endorsement in Regulatory Committee 2001/18.

**CRMs**
DG SANTE remarked that the Chair had already covered the topic and that the applicants had been very cooperative. In June 2019, a workflow had been agreed for all involved parties. GM events will be granted authorisation only after the EURL GMFF has finalised the assessments of the CRMs and the analytical method and EFSA has published its positive opinion. To avoid delays at the renewal phase of already authorised GM events, applicants were encouraged to verify the appropriateness of the CRMs prior to the renewal application.

**CJEU ruling**
According to the ruling only classical mutagenesis is exempted and products generated by New Breeding Techniques (NBT) are falling under the EU legislation on GMOs. Labelling of products derived from classical mutagenesis could be implemented at national level, but only when not hampering the functioning of the internal market. DG SANTE informed that no legislative proposal is foreseen in the work program of the current College. The Council requested the Commission to submit a study clarifying issues on NBT (on going). This initiative was launched in September.

**Expression of results for stacked GM events**
In April 2019, the ENGL requested a clarification on the expression of results for stacked GM events. As a result, DG SANTE suggested reporting the results per single event per each species. The rationale for that decision is that in absence of information related to product traceability it would be difficult to judge compliance.

**GM presence in additives/enzymes**
The presence of non-authorised GM events in feed additives has been detected in multiple cases. Since the previous week, food enzymes containing living GM cells have been also reported to the Rapid Alert System for Food and Feed (RASFF). The issue has been discussed in several meetings of the Steering Committee (SC) PAFF (mainly in the animal feed section). DG SANTE clarified that any product containing unauthorised GMO is non-compliant with EU legislation. Many of those products were originating from China.

**Russian Federation**
The Commission received a letter from the Russian Federation (RF) in May 2019 claiming that multiple unregistered and undeclared GMO products of EU origin had been found on the market. DG SANTE had a discussion in the SC PAFF in June 2019 to gather information from MS. In July 2019, the Commission requested detailed explanations from the RF. So far, the Commission has not received further clarifications.

**UK leave**
DG SANTE did not receive information on MSs using UK-based laboratories for official control analysis. Competent Authorities (CA) should prepare to appoint non-UK based laboratories as NRL in case the UK leave is finalised.

**Follow-up discussion**
The Chair explained that the JRC was also involved as EURL in the authorisation of Food Additives (FA) and that it was maintaining a repository of the related samples provided by the applicants. He informed that the EURL FA had started verifying potential microorganism contamination in all samples of officially authorised products. He further remarked that only the vitamin B2 feed additive has been tested for GM contamination. A representative from Poland expressed his concern for the presence of Living Modified Organisms in market products. As the information was very recent, DG SANTE expected that non-compliant products would be removed from the market within the following days and that the CA from affected MSs would investigate the origin of the contaminated additives.
Other participants commented that enforcement could not be fulfilled without a detection method and that serious measures should be taken against these health-risk products.

The Chair reminded that the task was under the competence of the CA and that a point in the Agenda was dedicated to the issue. He fully supported the relevance of the work.

13) Challenging the log transformation of PT data (W. Broothaerts, JRC)
The speaker explained that logarithmic transformation of GMO proficiency testing data had been done from the first Proficiency Test (PT) rounds performed by the EURL GMFF up to 2018. This followed the recommendation of Thompson et al. from 2006 based on the analysis of PT datasets from a UK PT scheme, which appeared to be log-normal distributed rather than normally distributed.

However, by log transformation sometimes a multimodal distribution could be hidden. The assumption of log-normality of the GMO PT datasets was challenged by the JRC. The data from a total of 18 PT rounds performed between 2010 and 2018 was analysed, comprising 56 datasets with more than 3000 individual reported results. Statistical outliers were removed, then a series of statistical tests were applied (Kolmogorov-Smirnov, Skewness & Kurtosis, Shapiro-Wilk (S-W)) to evaluate the distribution of the datasets on the raw domain, and if needed, after log transformation. The most powerful test for ‘Goodness-of-fit’ for normality (S-W) showed that for the majority of the datasets (41 out of 56) the results were normally distributed on the raw scale. In six cases the W test value (from the S-W test) was statistically not significant on the raw scale, but even worse after log transformation of the data. The latter approach in that case was therefore not useful for improving the distributions before performance assessment.

Four other datasets were statistically also not normally distributed for the raw data, but had an improved normality after log transformation (although the W test value was not statistically significant). Further investigation showed that all these four datasets were derived from measurements that included laboratories that had used the adh1 (70 bp) gene as taxon-specific reference target. Removing the data obtained with adh1 from these 4 datasets resulted in a statistically significant W value on the raw scale, i.e. the deviation from normality was due to the use of a biased measurement method. Finally, the remaining five datasets had a W test value that was significant on the log domain only. Examination of the corresponding distributions (on the raw scale) showed all of them to have one or more secondary peaks. Such additional peaks are somewhat masked with a log transformation of the data, but they actually indicate the occurrence of technical or experimental problems (adh1, double copy CruA) that should not be hidden. He concluded that for 91% of the PT data distributions, the deviation from normality was statistically not significant and that technical/experimental issues could explain the deviation from normality in the other cases. Log transformation of the data is therefore no longer justified.

The speaker explained that the evaluation of the laboratories’ performance has been done on the raw scale in 2019. He clarified the relationship between the z scores calculated on the raw and log domains. Laboratories with an underestimated result ($x_i < x_{pt}$) will be less penalised by the new approach, while overestimated results will be more penalised.

The Chair clarified that log transformation is not applied to PCR data during ring-trial validation.

14) Development of NRL performance according to EURL GMFF PT data (W. Broothaerts, JRC)
The speaker explained that the EURL GMFF had a unique access to a large dataset reported in proficiency testing rounds between 2010 and 2018. These data could be used to analyse the long-term performance of laboratories, which would be an indicator for the competence development of the official labs and the EURL GMFF support provided to the NRLs.

To this aim, the JRC re-analysed 2667 reported results for 56 measurements on 36 test items provided in 18 PT rounds (2010-2018) by 62 NRLs, totalling 80 independent datasets. The RSD% value was used as an indicator of the spread of the reported results. It was observed that the RSD% value decreased with time, that the variation reported in copy number ratios was higher than when results were reported in mass fractions (m/m%) and finally that the RSD% value on average was larger for results on oilseed rape than maize and the latter larger than for soybean events.

The JRC also recalculated an assigned value based on the NRL results (new robust means) and estimated the deviation between the laboratory data and the assigned value. Highly deviating results were observed mainly
in the 2010-2013 period, and more for the challenging test items than for the simpler matrices composed of ground maize or soybean. There was a trend towards a lower % difference (%D) with increasing $x_{ct}$ for the period 2010-2013, but this trend disappeared in the following years (2014-2018). Laboratories’ performance was calculated and scored by using %D, as satisfactory, questionable or unsatisfactory (similar as Z scores). Importantly, the number of laboratories that obtained always satisfactory or questionable results increased from 41% to 74% in the last two years. These results indicate an increased harmonisation in the performance of the laboratories and an improved performance with time.

The Chair remarked that the data was very valuable and had confirmed the positive impact of the ENGL and EURL GMFF on the GMO control activities.

A representative from UK noted that the data had shown a decreased variability of the mean values and that it would be interesting to also plot the values of the measurement uncertainties.

The Chair informed that the EURL GMFF will provide the code numbers of the participant laboratories so that they could verify their performance in the result tables. He announced that a scientific paper on this study was in preparation.

15) New ENGL WG on GM microorganisms (GMM)

A JRC representative informed that the ENGL Steering Committee decided to create a new WG on detection of GMM in food and feed. The main task of the WG will be to provide a report addressing the challenges and possibilities for detecting GMM in food and feed, including GMM obtained by new mutagenesis techniques. The applicable regulatory framework should only include Commission Regulation (EC) No 1829/2003 and Directive No 2001/18/EC. The document should provide acceptance criteria for the sensitivity and selectivity of detection methods that are not defined in the existing EFSA guideline. The members of this WG still needed to be identified.

The Chair specified that contributions for writing the report were not limited to the ENGL network and extended the invitation to experts from Member States.

A participant from Belgium asked if quantification of GMM should be expressed in absolute values and underlined that detection methods may need to fulfil different requirements when used for risk assessment or for control by enforcement laboratories.

It was confirmed that the quantification results should be expressed as number of GMM products per mg of sample; for risk assessment purposes the method should detect the largest transgenic sequence possibly present in the sample, while for official control purposes the method should detect a short target sequence of recombinant DNA.

The Chair explained that the scope of the report is to describe whether it is possible to detect and quantify up to a certain level the presence of GMM in a sample. The analytical target will not be limited to viable cells, in which case a microbiology approach could be taken. When he requested if there are volunteers in the ENGL interested in joining the WG; participants from Germany, Belgium, France, Poland and Czech Republic declared their interest in participating to the WG activities.

The Chair reminded that input from external members would also be desirable and asked sending the proposals directly to the Secretariat.

16) Genome-edited organisms listed in the EUginius database (T. Prins, WFSR, NL)

The speaker announced the possibility of retrieving information on genome-edited organisms from the EUginius web application. He informed that in the previous year institutions from Poland, Italy and Austria have become partners of the EUropean GMO INItiative for a Unified Database System. He explained that the scope of the EUginius database is to provide reliable information on EU and non-EU authorised GMOs.

The database contains a molecular characterisation of GM events and available genetic elements, describes traits and provides detection methods. Specificity results obtained from the analysis of CRMs can be used for data interpretation in a verification matrix to know if a method can detect a certain GMO. The EUginius web interface provides an analysis tool listing the events, species and elements that can be detected by the
methods and covers GM plants, animals and microorganisms. In particular, it includes 712 GMOs (15 in the pipeline) of which 318 are single events and 394 stacks and 243 state-of-the-art detection methods. The database also stores descriptions and general information including the producing company on 10 genome-edited (GE) organisms. Sometimes not the precise sequence but only the modified trait is known.

The Chair enquired whether it would be possible to visualise for each content the date of the last revision. The speaker remarked that information on the last update is not provided and that the database contains de novo sequences or sequences collected from other sources but that the quality is not guaranteed. Information on the number of accesses to the web interface was not known by the speaker.

17) Results of surveys on the GMOMETHODS database and EURL GMFF IT applications (L. Bonfini, JRC)
A survey on the GMOMETHODS and JRC-Matrix web applications was launched by the EURL GMFF at the beginning of July 2019. It had been designed to verify the use of the two applications by ENGL members, their level of satisfaction and to determine the most important information and options to be provided on the web interfaces.

A JRC speaker presented the results of the survey representing the input of 32% of the invited laboratories. More than half of the respondents declared using the GMOMETHODS database always, very often or often while an additional 23% declared using it only when a new method was published or needed. Overall, more than 73% of the respondents declared to be satisfied with the database. A large majority of the respondents requested to additionally provide information on the number of reference gene copies, recommended screening methods and CRM conversion factors.

Similar results were observed for the JRC GMO-Matrix application. About 83% of the respondents declared using the web tool. More than 70% of them requested providing information on the EU authorisation status of the GM events displayed on the web interface or on EU reference methods detecting custom NGS sequences. Almost of all them (97%) requested providing EU screening approaches for identification of EU authorised GM events.

Finally, the respondents suggested for both applications improvements in the design of the web interface, the queries options or the display of results.

18) Discussion on specific topics:
18.1) Validation aspects of NGS (Moderator: N. Roosens)
The speaker explained that NGS entails a complex workflow including different steps where the experimental approach needs to be adapted to the particular purpose and type of sample. If NGS is applied to GMO analysis, the quality criteria need to be flexible enough to be used for all different purposes, yet sufficiently detailed to produce reliable consistent results. She examined each single step of the workflow (DNA extraction, library synthesis and sequencing, primary data analyses, secondary and tertiary data analyses) and identified possible quality criteria.

The speaker asked if ENGL members were using NGS for their analysis. At least eight laboratories confirmed using NGS (mostly Illumina MiSeq) for pathogen detection and species identification, others outsource sequencing or consider the technology not yet to be applicable to GMO analysis. One laboratory informed having NGS accreditation for microbial whole genome sequencing (WGS) while another requested accreditation for species identification.

The moderator asked if NGS could be considered a useful tool. Some participants proposed using NGS for detection of GMM, unauthorised GMOs or genome edited products; others observed the difficulty in applying the approach to a complex mixture. The moderator noted that NGS would not be the best technology for plasmid characterisation in GMM.

The Chair highlighted the influence of data treatment on the quality of the results and the inability of most laboratories in analysing bioinformatics data. Also ISO working groups in different fields are preparing standards on NGS workflows (e.g. ISO TC34 SC16 WG8 on species detection using NGS; ISO TC276
Biotechnology: standard for general requirements for NGS; ISO TC34 SC9 WG25: WGS for foodborne pathogens, including part on validation) but did not cover yet the bioinformatics level.

The moderator remarked that benchmarking data set and reference databases are needed for comparing results from different pipelines. She underlined the challenges in validating the single steps of the workflow which should be specific for the purpose.

A participant from Germany questioned the idea of having a single NGS instrument for every laboratory and proposed outsourcing the sequencing and data analysis work to external companies. The moderator explained that it is very important to invest in the technology to develop a workflow suited to the analytical needs of the laboratory, control the entire process and obtain accreditation.

Some participants expressed caution in defining validation criteria and parameters before acquiring sufficient experience.

19) Overview on GMO-related activities in India, (Gurinderjit Randhawa, New Delhi, India)

The speaker reminded that her institute, the ICAR-National Bureau of Plant Genetic Resources, has been associated with the ENGL for 10 years. She explained that in 2017 her country was ranking in the fifth position for GM crop cultivation, specifically for GM cotton (four GM cotton events have been authorised since 2002, but the event MON15985 (Bollgard II) covers 95% of the GM cotton grown in India). GM events covering 18 crops, namely brinjal, cotton, corn, cabbage, castor, were undergoing field trials in 8 states.

She explained that since 2012 every package containing genetically modified food should be labelled as GM. In 2018, a draft mandatory regulation has been published requiring labelling for products containing more than 5% of GM ingredient. The labelling threshold is not yet implemented.

She explained that her institute performs molecular profiling of major crops and manages a National Gene Bank database conserving more about half million accessions of 1,762 crop species. For research purposes, import of a maximum seed quantity is permitted. When the material is clear, a procedure for molecular testing of imported transgenic material is followed.

The laboratory follows a stepwise DNA-based GMO testing approach employing for screening a multiplex (6-plex) approach for six target genes, a decaplex for detection of approved GM cotton and GM cotton under field trials and a GMO screening matrix with a decision support system for 141 GM events with 106 genetic element targets covering 21 crops. The laboratory has also developed a crop-specific GMO matrix combined with multiplex PCR for GMO screening in cotton and maize and a real-time PCR-based multi-target system for rapid screening of 21 GM events and 47 targets.

The laboratory developed a commercial duplex real-time PCR kit targeting P-35S and T-nos and published LAMP-based GMO screening for marker elements such as i.e. P-35S, T-nos or commonly employed transgenes. They use their gene bank for testing the adventitious presence of transgenic plants in 50 field trials ex-situ. They participated in international PT rounds (25) and foster partnership for technology transfer.

There are a network of GM testing labs in India and four reference laboratories. They organise biosafety workshops and offer training programs. Thanks to the ENGL and the JRC, they were able to increase their expertise by sharing experiences and participating to international meetings and networks. They would like to continue the collaboration with the ENGL.

The speaker informed that only GM cotton events have been authorised in India while for only a few GM rice events field trials have been authorised in certain areas.

20) Use of High Throughput Sequencing for detection of GMOs and plant species (F. Debode, CRA-W, BE)

The speaker proposed using enrichment technologies for GMO detection to overcome the low representability of the transgenic inserts in the host genome. The enrichment was attained by using capture probes that are complementary to elements present in GM constructs. The DNA was first sheared in fragments of about 400 bp and, after hybridisation with the probes, the bound fragments were collected and sequenced.
The laboratory created a database of target sequences for designing the probes. In the first version of the design they used 458 probes covering 55 kb sequences including mainly promoters, terminators and genes. In the second design they included probes for 20 plant species, which could be enlarged in the future to toxic or allergenic plants. In the third design they modelled probes for other elements such as t75 and bla to avoid background noise. In their tests they confirmed that it was possible to detect even at low % (10% and 1% of GM event GTS 40-3-2) a large panel of elements introduced into GMOs and to re-create partially or completely the contigs of the related GM constructs.

They were able to discriminate in mixed samples GM constructs having a high % similarity such as A2704 soybean and LL62 rice. The approach is not quantitative since the number of reads for the selected genes was not reflecting the GM percentage.

This new approach requires a new update for each run, extensive bioinformatics analyses, management of background noise and of the elements present at low level.

The speaker further explained that they did not use event-specific probes in the enrichment strategy to avoid competition effects. They did not run a significant number of repeatability measurements but obtained the same results when they analysed twice the same sample. Moreover, it is not possible with this approach to distinguish a stack event from a mixture of single events.

**21) CRISPR as new analytical tool for nucleic acid analysis (J. van der Oost, Wageningen, NL)**

The speaker reviewed the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) and associated genes (Cas) in the bacteria genome and their involvement in the antivirus prokaryotic defence system.

He explained that the CRISPR/Cas systems cut the DNA of the virus and insert a copy in a tandem repeat of the bacterial genome. The RNA transcribed from the locus (crRNAs) is incorporated into a CRISPR/Cas complex and is used for recognising and degrading complementary sequences in the genomes of newly infecting viruses. In the absence of a template the CRISPR/Cas system promotes a non-homologous repair of the DNA cleavage site generally inducing a final gene inactivation. In presence of a template the system promotes homology-directed repair and allows accurate engineering of the target genomic sequence.

In recent developments Cas9 and Cas12 have been fused to deaminase domains to obtain a precise engineering transition from CG to TA and from AT to GC. Another novel application is a Cascade & Cas12k with T7-like transposase complex for RNA-guided transposition to introduce any size sequence into the host genome. The speaker informed that his laboratory succeeded in obtaining very precise editing of the human genome with different PAMs and engineered CRISPR/Cas providing activity and specificity levels similar to those of the Talan protein.

The speaker was of the opinion that GMO rules and safety tests should be applied in case of major genetic changes (exchange of genes between unrelated species from bacteria to plant), while genome edited products should be regarded as non-GMOs when resembling natural products (minor changes: insertion/deletions & exchange of genes within species). Moreover, a discussion on safety should be based on sound scientific arguments and on the final products rather than on the technology used. The speaker explained that depending on the position of the PAM motives, mismatches at SNIP sites could be discriminated. He finally added that off-targets effects had been studied in human cells and the specificity of the system has been highly improved.

**22) Mitigating the risk of off-target effects when using CRISPR genome editing (B. Bugarija, Chicago, IL, USA)**

The speaker presented the different strategies for reducing the incidence of off-target effects (OTE) when using the CRISPR/Cas system. OTEs are problematic for clinical applications and may vary with target site and method. In silico tools to predict OTEs are difficult, as they often miss important sites or over-predict.

The group of the speaker tested different conditions and concluded that specificity could be improved by using 18-20 bp long RNA oligonucleotides, preassembled RNP complexes or chemically modified proto-spacers. They performed screenings in bacteria to select high fidelity variants of Cas9 and isolated an Alt-R HiFi Cas9 mutant.
that maintained 99% on-target activity when delivered as RNP (Nature Medicine (2018)).

In vitro assays such as GUIDEseq were also used to identify OTEs but were limited to the cell type. The company developed a multiplexed, amplification-based, target enrichment NGS approach called rhAmpSeq. The primers flanking the editing site are designed with an internal RNA base moiety blocking extension by DNA polymerase after hybridisation to the target DNA sequences. Cleavage of the primers by RNase H2 allows the amplification of the target in the PCR reaction. In a second step, the generated rhPCR amplicons are further amplified with P7 and P5 universal index primers. The approach allows normalising and quantifying each rhAmpSeq library. The speaker explained that by blocking the primers, primer-dimer formation in multiplex reactions is significantly reduced. He ensured that the IDT rhPrimers and universal sequences were free from any user restriction. He added that the company is studying approaches for efficiently delivering the system into plants.

23) Statistical tools for validation and verification of molecular methods (D. Mäde, DE)

The speaker presented a statistical package for verification and single laboratory validation of DNA methods. He explained the statistical model by which the probability that a particular DNA test portion contains at least one copy of the target could be estimated with a probability formula. By resolving the formula, it could be calculated that the best LOD\textsubscript{95%} could not be significantly lower than three DNA copies. A lower number could indicate an incorrect serial dilution.

The speaker programmed free software in R to overcome the limitation of using commercial software with an unknown statistical elaboration. The final package uses an open source code, does not require user registration or programming skills, entails a local installation without connection to an external webserver, is applicable to experiments from other fields (i.e. microbiology) and can be extended on user request.

The package offers a macro where the POD (probability of detection) and related diagrams can be visualised after data input. The POD package can be used also for the calculation of the LOD\textsubscript{50%}. The speaker provided instructions on how to install and use the statistical package. He explained that the major assumption of the package was that a single DNA copy was independently distributed in the replicates. To assess the LOD\textsubscript{95%}, he suggested using measurements in 12 replicates for having an optimal ratio between input and results and performing the test in a solution containing 25 ng/µL of background DNA molecules to saturate adsorption sites at the tube surface.

24) Validation of whole genome sequencing for pathogen surveillance and outbreak detection: a testimony of a possible roadmap towards ISO accreditation of NGS in a public health context (S. De Keersmaecker, BE)

The speaker explained that for pathogen outbreak surveillance and investigation it is very important to detect the pathogen, to discriminate the type and to link the pathogen isolated in the food with the patient. The characterisation, however, is time consuming and expensive.

She remarked that whole genome sequencing (WGS) is potentially able to replace the classical (sub)-typing and characterisation methods, but that the approach needs to be validated to demonstrate that it is fit for purpose within a quality system. Currently there is no official harmonised guideline for validating WGS on pathogenic isolates and there is no data on the performance of the workflow.

The speaker’s group identified four stages in the workflow that needed to be verified and validated: 1) pure culture, 2) DNA extraction, 3) DNA sequencing and 4) bioinformatics (validation of the pipeline for data analysis). They focused on species for which the bioinformatics pipeline was already validated and verified the performance characteristics, namely trueness, precision, robustness and others (carry-over contamination, normalisation).

They replicated sequencing of a reference sample for which the genome sequence was known (NCBI) and compared the sequences obtained at SNP level. With the instrument 99% trueness was achieved and the analysis provided accurate genotyping of bacterial strains. Intra-run and inter-run repeatability were tested with three isolates per species, three replicates of each isolate by the same analyst on the same MiSEQ instrument within one run or overtime.

They did not observe any impact of library preparation or time on the results. They obtained the same results
in the repeatability and reproducibility tests where they observed that the operator preparing the library had no impact and that for proficiency tests the results were depending on the species analysed.

They could not see any impact on the robustness, which was defined, as the degree to which repeated sequence analyses give the same results using different instruments and different protocol conditions. It could be concluded that Illumina NGS sequencing of Nextera XT prepared libraries using MiSEQ reagents generated data that met the mandatory quality control (QC) requirements.

The speaker remarked that the WGS approach for pathogen characterisation could be applied to the GMO field. Other types of library synthesis, sequencing and data analysis pipelines should be employed while maintaining the same validation strategy and performance parameters. Acceptance criteria values could be modified according to the application.

The speaker clarified that pure and not mixed samples were used in the analysis and that, since the approach could be considered as platform dependent, it is important to have methods and material for comparing results to demonstrate that laboratories could reach the same conclusion.

25) NGS applied to GMO detection (D. Scaglione, IGA Technology Services, Italy)

The speaker provided an overview of NGS approaches employed in his laboratory which could also be used for GMO detection. He warned however that those approaches have not been validated on GMO samples and that compliance to performance requirements need to be first evaluated. He remarked that traditional GMOs could be easily detected, while GMOs generated by CRISPR/Cas-mediated genome editing presenting in some cases only a single point mutation need a different analytical strategy.

He described in the first part of the presentation approaches for the detection of traditional GMOs or genome edited organisms generated by homologous recombination (SDN-3). Whole genome sequencing (WGS) approaches such as Pair-end mapping for relatively small inserts (<1kb) or Mate-pairs mapping when the length of the insert could be extended to 3-6 kb, were considered acceptable as tests for pure material but unpractical for mixed samples at 0.1% or 0.01% GM level. Local gene walking strategies for accessing the adjunct point of insertion such as Site Finder involve many steps, require Sanger sequencing and were considered as difficult to interpret.

Hybridisation capture was regarded as laborious and expensive, while Ligation mediated PCR+NGS could be employed for detecting non-authorised GM events but not for quantitative purposes. The Target Locus Amplification (TLA) approach could allow multiplexing while Long-read sequencing with the MINION platform displayed an error rate of 10%, which could be acceptable however for assigning the sequence to a certain locus. Long-read sequencing with Cas9-based enrichment was considered as a promising genome-walking strategy.

The speaker presented approaches also for the detection of SNV and short INDELs for NHEJ (SDN-1) and HR (SDN-2) genome edited organisms. The WGS approach was considered as too expensive since the detection of a GM event present at a 0.15% content level required a 21000 X coverage.

Local (rhAMP-Seq) targeted rhAmp PCR using rhAmp primers blocked by a RNA base moiety was considered as a highly specific technology that allows multiplexing but is vendor specific. The Index hopping technology could hit some cross contamination not existing in the original sample while alignment free methods such as Kmers could be used only on low-error rate sequencers.

The speaker remarked that it is impossible to distinguish for SNVs between a mutation introduced by genome editing and a naturally occurring mutation, but that accessory information such as somatic mutations may be collected on a global scale to differentiate between the two cases. As a conclusion, he remarked that the choice of the instrumentation including the related ‘chemistry’ are crucial for the deployment of NGS in GMO testing and that the reproducibility of the bioinformatics steps of sequence analysis is also important.

Long-read sequencing is a promising technology for the assessment of large insertions while deep analysis of mutations with low-error rate sequencing could provide approaches for comprehensive fingerprinting. Somatic mutations occurring naturally or as a result of the transformation process might be exploited for unique fingerprinting of authorised events.
A participant commented that characterisation of somatic sequences involves WGS. The speaker remarked that sequencing of the somatic variety is required only once and that afterwards markers for known mutations could be used for detecting the GM event.

A participant wondered if the correspondence between SNVs and somatic mutations could be used also after crossing. The speaker commented that some somatic mutations may be lost during crossing but others may be retained.

Another participant commented on the difficulty of pursuing the approach for identification of GE products considering that, for instance, only for the GM event MON810 already 400 different varieties have been registered.

26) Developments in Animal Biotechnology (J. P. van der Berg, Wageningen Food Safety Research, NL)

Recent developments in animal cloning
The speaker informed that somatic cell nuclear transfer (animal cloning) is increasingly being performed by private companies for assisting breeding of “elite” livestock. He remarked that animal cloning is prohibited in the EU but that it is permitted in major meat-exporting countries such as the USA, Argentina, Brasil, Australia, New Zealand and Canada, while no specific regulation exists in China.

EFSA has not found differences in the risk assessment between food products generated from animal clones and their original donors. The procedure is however too expensive to be applied to food production. He informed that potential indicators of animal cloning are the telomere length, epigenetic aberrations and abnormal gene/protein expression or mitochondrial heteroplasmy. Detection methods based on these targets are not sufficiently sensitive and robust yet.

Genetic modifications of livestock
The speaker informed that the genetic traits more commonly modified in GM animals are enhanced growth and disease resistance. Globally a fast growing salmon is the only GM animal that has been approved (Canada and USA). The same company has developed a gene-edited tilapia using CRISPR-Cas-9, which does not contain recombinant DNA and is therefore not classified as GMO in Argentina. Genetic modifications in cattle focus primarily on milk composition and disease resilience.

Some gene-edited organisms are in the pipeline but are not approved yet. A “polled” dairy cattle (no horns) generated by gene editing using Talen is a potential candidate for commercialisation. The US FDA discovered however in the genome a repair template plasmid containing an antibiotic resistance gene. Therefore, all animals had to be sacrificed.

“Slick” beef cattle have been developed using genome-editing in Argentina. The genetic modification improves heat tolerance due to a less dense fur and an increased transpiration.

Detection and traceability
PCR-based assays could be used to detect GM salmon but in case of small genetic alterations the genetic changes cannot be easily distinguished from those occurring naturally. In these cases traceability can only rely on documentation. No food product derived from GM animals has been approved by EFSA, however guidance on GM animal is available from EFSA. The EFSA GMO Panel opinion is limited to a set of new breeding techniques.

27) Final remarks & closure

A participant from Greece wished to share some ideas with all participants. In his opinion, new methodologies could advance the ability of performing official control tests in a more efficient manner and proposed using common microarrays and an expert system for the analysis of the results. He commented that a common approach could ensure harmonisation in the provision of the results by EU official control laboratories. He suggested creating a WG for the task.

The Chair clarified that the ENGL could not establish a detection monopoly or favour methods depending on a unique technology.
The Chair informed that for authorised GM events in the EU the EURL GMFF has the task of supplying control samples (plasmids) to NRLs. The plasmids are produced and distributed as positive controls, but not as calibrants.

As the applicants have to ensure the availability of Certified Reference Materials (CRM) for all GMO events authorised in the EU, the laboratories can use these CRMs for their calibration and quality control needs. He requested addressing issues on the availability of CRMs to DG SANTE or the EURL GMFF.

In line with Regulation (EC) No 1829/2013, the EURL GMFF has also received genomic DNA and food and feed samples from the applicant for the sole purpose of method validation. The Chair remarked that the laboratories involved in the method validation studies of the EURL GMFF can use these samples only for the validation exercise and have to destroy remaining material afterwards. As the EURL GMFF is not monitoring, after the validation exercise, the conditions and quality of the material received from the applicants, it cannot further distribute the remaining material to laboratories.

A participant from Belgium informed that for some GM events withdrawn from the market the production of CRMs had stopped even though the material was still needed. The Chair ensured that the requirements for the availability of the material after withdrawal will be verified with DG SANTE.

A participant from France asked if ENGL members are authorised to use the method provided by the applicant if the related GM event has been withdrawn before authorisation, i.e. for the maize event VCO-01981-5. The Chair remarked that for GM food the situation has to be verified with DG SANTE.

He further clarified that for stacked GM events the CRM and detection methods are available only for each single event. However, the EURL GMFF has already verified whether the method detecting the single event performs equally well on the stack material.

The Chair requested to submit feedback on the organisation of the ENGL plenary and suggestions for inviting experts from other networks. He announced that the table with CRM conversion factors has been published on the EURL GMFF website. He informed that JRC colleagues will try to complete the table for the remaining CRMs as quickly as possible combining the determination of the CF with the assessment of the other appropriateness CRM criteria.

He thanked the participants for their active participation, the WG members for their work in producing the documents, and the speakers for their presentations. He finally closed the meeting.
1 October 2019
Session: ENGL

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<tr>
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<th>Topic</th>
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<td>Welcome and approval of the Agenda</td>
<td>Draft agenda Report</td>
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<td>09:30</td>
<td>Approval Report 29th ENGL Plenary</td>
<td>DAL ENGL29 Report</td>
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<td>09:30</td>
<td>Dynamic Action List (DAL) of 29th ENGL plenary</td>
<td>DAL ENGL29 Reports SC36 and SC37 Report</td>
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<td>10:00</td>
<td>Outcome of the 36th and 37th ENGL SC meetings (February and June 2019)</td>
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<td>09:30</td>
<td><strong>Discussion of Progress reports of ENGL WGs:</strong></td>
<td>Progress reports</td>
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<td>11:00</td>
<td>Update from DG SANTE</td>
<td>Presentation</td>
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<td>11:30</td>
<td>Challenging the log transformation of PT data (JRC)</td>
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<td>12:30</td>
<td>Development of NRL performance according to EURL GMFF PT data (W. Bieiothaerts, JRC)</td>
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<tr>
<td>12:30</td>
<td><strong>Buffet lunch</strong></td>
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<td>14:00</td>
<td>New ENGL WG on GM microorganisms</td>
<td>Presentation</td>
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<td>14:30</td>
<td>Genome-edited organisms listed in the EUgenius database (E. Droganidi, BYL, DE)</td>
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<td>15:00</td>
<td>Results of surveys on the GMOMETHODS database and EURL GMFF IT applications (L. Borfini, JRC)</td>
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<td>181</td>
<td>1600 Discussion on specific topics:</td>
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<td>182</td>
<td>▪ Validation aspects of NGS</td>
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<td>(Moderator: N. Roosens)</td>
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<td>▪ Report on discussion</td>
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<td>17:30</td>
<td><strong>End of day 2</strong></td>
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<tr>
<td>19:30</td>
<td><strong>Social dinner at Hotel Belvedere</strong></td>
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**2 October 2019**

Session: Open Science Day

<p>| Time  | Topic                                                                 | Documents in ENGLnet |
|-------|                                                                      |                      |
| 19    | 0900 Overview on GMO-related activities in India,                      | Presentation        |
|       |     (Gurinderjit Randhawa, New Delhi, India)                           |                      |
| 20    | 09:45 Use of High Throughput Sequencing for detection                  | Presentation        |
|       |     of GMOs and plant species (F. Debode, CRA-W, BE)                   |                      |
| 10:30 | <strong>Coffee Break</strong>                                                      |                      |
| 21    | 11:00 CRISPR as new analytical tool for nucleic acid analysis (J. van | Presentation        |
|       |     der Oost, Wageningen, NL)                                          |                      |
| 22    | 11:45 Mitigating risk of off-target effects when using                 | Presentation        |
|       |     CRISPR genome editing (B. Bugarija, Chicago, IL, USA)             |                      |
| 12:30 | <strong>Buffet lunch</strong>                                                      |                      |
| 23    | 14:00 Statistical tools for validation and verification of             | Presentation        |
|       |     molecular methods (D. Mode, DE)                                    |                      |
| 24    | 14:45 Validation of whole genome sequencing for pathogen surveillance | Presentation        |
|       |     and outbreak detection: a testimony of a possible roadmap          |                      |
|       |     of ISO accreditation of NGS in a public health context             |                      |
|       |     (S. De Koorsmaecker, BE)                                           |                      |
| 15:30 | <strong>Coffee break</strong>                                                      |                      |
| 25    | 16:00 Technologies, analytical tools and challenges for the           | Presentation        |
|       |     sequencing-based detection of old and new genetically modified     |                      |
|       |     organisms (D. Scaglione, IGA Technology Services, Italy)           |                      |
| 26    | 16:45 Developments in Animal Biotechnology (J. P. van                  | Presentation        |
|       |     )                                                                  |                      |</p>
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<tr>
<td>17:20</td>
<td>Final remarks &amp; closure (H. Emons, JRC)</td>
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<td>17:30</td>
<td>End of the meeting</td>
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Meeting documents available at:  