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Body fluid identification using a targeted mRNA massively parallel sequencing approach – results of a EUROFORGEN / EDNAP collaborative exercise

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Highlights

- Targeted RNA sequencing assay for body fluid identification tested within 17 EUROFORGEN / EDNAP laboratories
- Identification of stains containing blood, semen, saliva, vaginal secretion, menstrual blood and skin
- 2 assays containing 33 and 29 biomarkers for Illumina MiSeq/FGx and Ion Torrent PGM/S5, respectively
- Statistical analysis of results using partial least squares analysis (PLS)

Abstract

In a previous study we presented an assay for targeted mRNA sequencing for the identification of human body fluids, optimised for the Illumina MiSeq/FGx MPS platform. This assay, together with an additional in-house designed assay for the Ion Torrent PGM/S5 platform, formed the basis for a collaborative exercise within 17 EUROFORGEN and EDNAP laboratories, in order to test the efficacy of targeted mRNA sequencing to identify body fluids. The task was to analyse the supplied dried body fluid stains and, optionally, participants' own *bona fide* or mock casework samples of human origin, according to specified protocols. The provided primer pools for the Illumina MiSeq/FGx and the Ion Torrent PGM/S5 platforms included 33 and 29 body fluid specific target sequences, respectively, to identify blood, saliva, semen, vaginal secretion, menstrual blood and skin. The results demonstrated moderate to high count values in the body fluid or tissue of interest with little to no counts in non-target body fluids. There was some inter-laboratory variability in read counts, but overall the results of the laboratories were comparable in that highly expressed markers showed high read counts and

less expressed markers lower counts. We performed a partial least squares analysis (PLS) on the data, where blood, menstrual blood, saliva and semen markers and samples clustered well. The results of this collaborative mRNA massively parallel sequencing (MPS) exercise support targeted mRNA sequencing as a reliable body fluid identification method that could be added to the repertory of forensic MPS panels.

Key words:

Forensic science, body fluid identification, mRNA profiling, massively parallel sequencing

1. Introuction

Messenger RNA (mRNA) profiling has emerged in the last years as a new method for body fluid identification [1-6]. It is based on the premise that each single tissue type is comprised of cells that have a unique transcriptome or gene expression (i.e. mRNA) profile. A number of markers have been identified for the forensically most relevant body fluids and tissues: blood, saliva, semen, vaginal secretion, menstrual blood and skin [7-18]. Conventional mRNA profiling includes the following steps: RNA extraction, reverse transcription, tissue-specific PCR amplification and separation/detection with capillary electrophoresis (CE). The main advantages of mRNA profiling compared to conventional protein-based methods are a significant improvement in specificity, additional body fluids and tissues that can be tested (e.g. menstrual blood, vaginal secretion, nasal secretion, skin), the ability of multiplexing numerous mRNA markers for the identification of one or several body fluids and the possibility of simultaneously

isolating RNA and DNA from the same piece of stain [19, 20]. The co-extraction of RNA and DNA allows for positive identification of the tissue/fluid source of origin by mRNA profiling as well as a simultaneous identification of the body fluid / tissue donor by STR profiling [21].

The European DNA Profiling Group (EDNAP – http://www.isfg.org/EDNAP) performed six collaborative exercises during the last few years, in order to evaluate the robustness and reproducibility of CE-based mRNA profiling for blood, saliva, semen, menstrual blood, vaginal secretion and skin identification [22-26]. The results of these collaborative exercises support mRNA profiling as a reliable body fluid identification method that can easily be combined with current STR typing technology.

The introduction of massively parallel sequencing (MPS) also revolutionised transcriptomics. With targeted mRNA sequencing approaches it is possible to multiplex numerous markers avoiding time and sample consumption from multiple separate analyses. In addition, mRNA sequencing provides a larger dynamic range for quantitative analyses and the possibility to identify sequence variation, compared to the endpoint PCR/CE-based methods. Furthermore, MPS is specifically suitable for degraded samples, since mRNA is usually fragmented prior to the reverse transcription step in MPS protocols. Within the EUROFORGEN Network of Excellence (https://www.euroforgen.eu) we established a targeted mRNA massively parallel sequencing approach for body fluid/tissue identification [27]. This included the evaluation of a suitable library preparation protocol and sequencing platform, as well as the selection of the most appropriate markers, based on the experience with the PCR/CE-system. The assay was developed to analyse six different body fluids / tissues, i.e. blood,

semen, saliva, vaginal secretion, menstrual blood and skin. In a proof-of-concept-paper the assay we developed and ran on the Illumina MiSeq/FGx platform was demonstrated to identify body fluids with good sensitivity in single source and mixed stains [27].

Based on this knowledge, a EUROFORGEN / EDNAP collaborative mRNA MPS exercise was organised by the Zurich Institute of Forensic Medicine, Switzerland. In addition to the Illumina MiSeq/FGx specific assay, we developed a separate assay for the Ion Torrent PGM/S5 platform (Ion AmpliSeq™ RNA custom panel, Thermo Fisher Scientific, Waltham, MA), enabling laboratories running this alternative MPS system to participate in the collaborative exercise. We started with a PGM/S5 panel including the same 33 biomarkers that were used for the MiSeq/FGx specific assay. Then, in an iterative process, we added new markers and removed poorly performing markers (panels 2 and 3 included 61 and 37 biomarkers, respectively). The final PGM/S5 assay included 29 biomarkers in total. In this exercise, either of the two multiplexes was provided to each participating laboratory. The exercise included the analysis of 16 and 8 mock casework samples for MiSeq/FGx and PGM/S5 users respectively, and optionally additional *bona fide* or mock casework samples from the participating laboratories. Here, we present data from the 17 participating laboratories.

2. Materials and methods

2.1 Samples and materials

The organising laboratory (Zurich Institute of Forensic Medicine) sent 8 stains to laboratories using PGM/S5 (n=9) and 16 stains to laboratories using MiSeq/FGx (n=10). Two laboratories performed the experiment on both platforms. Samples

were collected from healthy volunteers with their informed consent. The sampling was approved by the local ethics commission (KEK), declaration of no objection (No. 24-2015). Blood samples were obtained by venipuncture (S-Monovette® EDTA, Sarstedt, Nümbrecht, D) and 50 µL were spotted on sterile cotton or cellulose swabs. Semen and saliva samples were collected in sterile cups and microtubes; respectively and 50 µL each were spotted onto sterile cotton swabs. Vaginal secretion and menstrual blood samples were taken using sterile cotton swabs from the vagina and 1/4 of a swab was sent to the laboratories. Skin samples were collected by rubbing the forearm with a pre-wetted (90% ethanol), sterile cotton swab. Mixed samples were produced with different ratios of two body fluids. Varying volumes of saliva and semen were spotted on sterile cotton swabs containing blood (25 µL), vaginal secretion (1/4 swab), menstrual blood (1/4 swab) or skin cells (whole swab). This resulted in a final volume of 50 µL of two different body fluids per whole swab or 12.5 µL per 1/4 swab (Table S1). All swabs were dried at room temperature for at least 12 hours.

Laboratories using MiSeq/FGx were asked to analyse 8 additional stains containing either human blood, semen, saliva, vaginal secretion, menstrual blood or skin or any mixtures thereof, but not more than 2 body fluids/tissues per stain (Table S2).

MiSeq/FGx laboratories used a TruSeq Targeted RNA Expression panel (Illumina Inc., San Diego, CA) to amplify the 33 body fluid specific transcripts [27] (Table 1). PGM/S5 laboratories used an Ion AmpliSeq™ RNA custom panel (Thermo Fisher Scientific) detecting 29 body fluid specific transcripts (Table 1). Primer pools for both MPS platforms were provided by the organising laboratory.

2.2 RNA-extraction and quantification

Laboratories could use any RNA extraction method of their choice. The organising laboratory recommended a manual organic method [1] and a silica column based method (RNeasy® Mini Kit, Qiagen, Venlo, NL). For laboratories using a PGM/S5, it was highly recommended to use the organic extraction method which results in higher RNA yields. We suggested using the TURBO DNA-free™ kit (Thermo Fisher Scientific) for DNase treatment.

To define the desired input amount of RNA, laboratories were advised to quantify the RNA extracts using fluorescence or electrophoresis based quantification methods. The following methods were recommended: 1) Quant-iT RiboGreen RNA kit / Fluorescence microplate reader (high- and low-range protocol options) (Thermo Fisher Scientific); 2) QuantiFluor RNA System / QuantiFluor-ST Fluorometer (Promega, Madison, WI); 3) Quant-iT RNA assay kit / Qubit Fluorometer (Thermo Fisher Scientific); 4) Bioanalyzer (normally bad RNA quality / low RIN numbers with these kind of samples). In case the laboratories had no means of quantifying the RNA extracts, the organising laboratory proposed a specific input volume for each stain (Table S3).

2.3 Library preparation and sequencing

The MiSeq/FGx library preparation was performed according to the manufacturer's protocol (#15034665 v01, January 2016) using 50 ng RNA in 3 µL and following the "degraded RNA" protocol. Targets were amplified using 34 cycles. The final library, diluted to 6 pM, was sequenced using 51 cycles single-read and the MiSeq Reagent Kit v3 (Illumina Inc.). The PGM/S5 library preparation was performed according to the manufacturer's protocol

(MAN0007450, Revision A.0) using 50 ng RNA and 30 amplification cycles. Pooling and diluting of Ion AmpliSeq™ RNA libraries was recommended as follows: for an Ion 314 chip, pooling to 25 pM, resulting in an end concentration of 4 pM; for Ion 316/318 chips, pooling to 100 pM, resulting in an end concentration of 16 pM; for Ion 520/530 chips (S5 sequencing system), pooling to 100 pM, resulting in an end concentration of 6.25 pM. 500 flows were used for sequencing.

2.4 Data analysis

Following sequencing, the organising laboratory collected the sequencing raw data (fastq/bam files) for further analyses. MiSeq/FGx fastq files were used to run the TruSeq Targeted RNA application on BaseSpace cloud (Illumina Inc.), resulting in a matrix displaying read counts per amplicon in each sample. PGM/S5 bam files were used as input files for the Ion AmpliSeq™ RNA plugin on the Torrent Server (Torrent Suite Software v4.6, Thermo Fisher Scientific), resulting in an amplicon coverage file.

2.5 Statistical analyses

To visualise major trends in the data, we performed a partial least squares (PLS) analysis. PLS is a decomposition technique similar to principal component analysis (PCA). It searches for a small set of orthogonal components, or latent variables, that explain as much as possible of the covariance between a set of predictor variables and a dependent variable [29]. In this case the predictor variables correspond to the mRNA markers, and the dependent variable is the body fluid category of each sample. An advantage of using PLS compared to

evaluating each mRNA marker separately, is that PLS considers the co-expression of the markers. Information about mRNA markers that are not expressed may be just as valuable as information about markers that are expressed, if this is a repeated pattern for samples of that body fluid.

The PLS analysis requires a normalisation of the data to adjust for different input amounts. A centered log-ratio (CLR) transformation [28], commonly used for read count data, divides each read count by the read counts sum for the sample, and takes the log-ratio of these relative reads. To avoid zero reads, one pseudo count was added to each read count.

R scripts presented here are available from the authors upon request.

3. Results

3.1 Samples, materials and methods

Seventeen laboratories participated in the exercise, 10 using the MiSeq/FGx platform and 9 using the PGM/S5 platform (2 laboratories did the experiments on both platforms). We provided the protocols and primer pools for PGM/S5 and MiSeq/FGx to assure conditions were as similar as possible for all laboratories.

To keep costs reasonable, the smallest possible modular number of samples was analysed, which comprised 24 samples for the MiSeq/FGx laboratories (half of a TruSeq Targeted RNA Expression Panel Kit) and 8 samples for the PGM/S5 laboratories (Ion AmpliSeq™ RNA Library Kit). The samples and primers were sent at ambient temperature by courier mail. Fifteen laboratories received the parcel within 4 days, only one parcel was held back by customs and was in transit for 16 days. Two laboratories did not store the primers immediately at -20°C and kept them at room temperature for 1-3 weeks. The laboratories were asked to fill

in a questionnaire on what methods they used, their quantification results, and other relevant information about the experiments conducted (Table S3). Table 2 shows the RNA extraction and quantification methods used by the participating laboratories. As predicted, the manual organic extraction method resulted in much higher RNA yields than the inorganic kit-based methods.

3.2 Data analysis

The final data analysis was performed by the organising laboratory. We identified issues with counting reads of some markers using the Ion AmpliSeq™ RNA plugin (Figure S1). Usually, only end-to-end reads are considered for the total read count of each marker. Figure S1a shows how the amplicons/reads along the target sequence are expected to look like when the markers are analysed end-to-end. Most of the markers were analysed in this manner. The target sequence of marker SEMG2 was only partly covered and the end-to-end reads did not represent the actual coverage (Figure S1b). For SEMG2, all reads were counted although shorter than the target region. For marker MUC7 a lot of short reads were aligned to a region adjacent to the actual target region and were wrongly counted as target reads (Figure S1c). To remove the non-target short reads, end-to-end analysis of marker MUC7 was performed. The analysis of MiSeq/FGx data did not show any of these issues.

The results for both marker sets (MiSeq/FGx and PGM/S5) demonstrated moderate to high count values in the body fluid or tissue of interest with little or no counts in non-target body fluids. Two laboratories (6 and 7) analysed the 8 samples with the MiSeq/FGx and the PGM/S5 marker set (the same RNA extracts were used to produce the respective libraries). Representative

MiSeq/FGx and PGM/S5 results from 6 samples (one sample per body fluid) of laboratory 6 are shown in Figure 1. Overall the MiSeq/FGx and PGM/S5 results were similar in that markers of the target body fluid were highly expressed, some markers showed higher reads with the MiSeq/FGx or PGM/S5 assay. This is most likely a result of different primer design, PCR efficiency and existing isoforms. Non-specific reads were covered on average 1650x for MiSeq/FGx and 1945x for PGM/S5.

In Figure 2 the read counts of the laboratories are compared (MiSeq/FGx laboratories 1-7, PGM/S5 laboratories 6, 7, 10-16). Three MiSeq/FGx laboratories (laboratories 8, 9 and 17) had no or only a few specific reads and/or some non-specific reads. Interestingly, these included the two laboratories not using the RT enzyme that was recommended in the library preparation protocol. Results from laboratories 8, 9 and 17 were therefore omitted from further analyses. All results of the PGM/S5 laboratories could be included in downstream analyses. There was some inter-laboratory variability in read counts, but overall the results of the laboratories were consistent, in that highly expressed markers showed high and less expressed markers lower read counts. For PGM/S5 the detection of the low-level RNA stains (blood and semen) worked only for the laboratories that used a manual organic extraction method, where the required RNA input of 50 ng was achieved.

As shown in Figures 1 and 2, variability could also be observed among markers within a body fluid / tissue. Results from the MiSeq/FGx assay showed highest reads from ALAS2 and AMICA1 for blood samples, from PRM1 and PRM2 for semen samples and from HTN3 for saliva samples. Corresponding results from samples analysed with the PGM/S5 assay were difficult to interpret for the low-

level RNA stains blood and semen. For saliva samples, STATH and MUC7 were highly expressed. HTN3 was – in contrast to the MiSeq/FGx assay – the least expressed marker, probably due to unfavourable primer- or marker design. FAM83D and MMP10 were the most prominent markers in both panels for vaginal secretion and menstrual blood, respectively. Skin markers were observed only sporadically and only with the MiSeq/FGx assay.

3.3 Body fluid inference

Raw read counts were used to calculate mean percent contributions of all six body fluid categories for the analysed stains (Table 3). Samples with < 5000 total reads were excluded. All blood, semen, vaginal secretion, menstrual blood and skin samples showed expected contributions and were correctly identified with both the MiSeq/FGx and the PGM/S5 assay. For saliva samples, moderate contributions from vaginal secretion markers could be observed and the identification was therefore inconclusive. All four mixed samples were correctly identified.

3.4 Statistical analyses

We performed a partial least squares (PLS) analysis on both the MiSeq/FGx and the PGM/S5 data to be able to visualise and compare any major trends in the two data sets. The plots in Figure 3 show the correlation between the markers and the first four PLS components, and how the body fluid specific markers correlate with the respective body fluid categories in the MiSeq/FGx and PGM/S5 data. The further the markers are away from the origin, the more influential they are on the components. The saliva markers form a clear cluster in the MiSeq/FGx data, and

they are also closely correlated with the saliva samples. The same can be observed for the semen markers and semen samples. Components 3 and 4 seem to be more influenced by the vaginal secretion markers as they are further away from the origin compared to components 1 and 2. For the PGM/S5 data the saliva markers are slightly more scattered for components 1 and 2 compared to the MiSeq/FGx data, while the semen samples are not as strongly correlated with the semen markers. The skin and vaginal markers are closer to the origin, and hence are less influential on these components. The menstrual blood samples are strongly correlated with both the blood markers and menstrual blood markers. The blood samples are not strongly correlated with the blood markers in the plot of components 1 and 2, indicating the blood markers do not explain the variance of the blood samples. For both the MiSeq/FGx and the PGM data, the blood and menstrual blood markers are difficult to separate with the first two components, while the two marker types are negatively correlated in the plot for the third and fourth component. The first two components together explain 46% of the variance in the MiSeq/FGx data and 56% in the PGM/S5 data, while the third and fourth component together explain 20 % and 24 %, respectively.

Figure 4 shows score plots for the first four PLS components for the MiSeq data, including the single source samples used for the model and the predicted scores for the four mixture samples (semen-vaginal secretion, blood-saliva, semen-menstrual blood, saliva-skin). There are seven replicates of each sample since there were seven laboratories. The left plot (components 1 and 2) shows distinct groupings of semen and saliva samples. In the right plot (components 3 and 4) especially the vaginal samples are distinctly grouped. The blood-saliva mixtures cluster more towards the saliva samples for components 1 and 2, while for

components 3 and 4 they are somewhat more correlated with the blood samples. The saliva-skin samples show similar tendencies, clustering with saliva samples in the left plot and more with skin samples in the right plot, however the replicates look more spread. The semen-vaginal secretion samples are positioned more midway between the semen and vaginal samples in the left plot, while they cluster with the vaginal secretion samples in the right plot. The semen-menstrual blood samples cluster somewhere between semen and menstrual blood for components 1 and 2, and are closer to the menstrual blood samples for components 3 and 4.

The same score plots were produced for the first four PLS components for the PGM/S5 data, including the single source samples used for the model and the predicted scores for the mixture sample, semen-vaginal secretion (Figure 5). There are nine replicates of each sample since there were nine laboratories. The plots show less clear groupings than the MiSeq/FGx data (Figure 4). Saliva is most easy to separate from the rest for components 1 and 2 (left), while the remaining samples are more overlapping. Blood and menstrual blood samples are especially difficult to separate. For component 3 and 4, blood and menstrual blood can be separated, and the vaginal secretion samples show more separation from the rest. The semen-vaginal mixture samples cluster more with semen samples in the left plot, and more towards the vaginal secretion samples in the right plot.

Figure 6 shows the average read counts obtained by the 7 MiSeq/FGx laboratories in the MiSeq/FGX data mixture samples. The mixtures semen-vaginal secretion, blood-saliva, semen-menstrual blood and saliva-skin are presented in separate rows. The known body fluids present in each mixture show

high read counts. The standard error on each bar reflects the quite large variations observed among laboratories. Figure 7 shows the average read counts obtained by the 9 IonTorrent laboratories in the PGM/S5 data mixture sample, the same vaginal-semen mixture as in the MiSeq/FGx sample set. Mainly semen and vaginal markers show high reads, even higher than for the corresponding sample in the MiSeq/FGx data.

Table S2 lists the additional stains which were analysed by the MiSeq/FGx laboratories. Figure 8 shows the corresponding score plots for the first four PLS components for the MiSeq/FGx data. The provided samples were included in the model, while the laboratories' own samples were predicted. The laboratories' own samples differ a little from the provided samples; some of them were mock casework samples with lower amounts of body fluids being analysed and substrates other than cotton swabs being used (e.g. Kleenex, FTA® card and panty liner). The additional blood and saliva samples cluster well within the pristine blood and saliva samples but are nevertheless distinct even within each of the pristine sample groupings. Prediction of semen samples appear to be better for components 1 and 2 than for 3 and 4. The same can be observed for vaginal secretion samples. The additional menstrual blood samples are rather randomly spread for all 4 components at the edge of the menstrual blood cluster. Skin samples show a similar pattern for components 1 and 2 as well as for 3 and 4. For mixed stains, blood-saliva samples tend to cluster more with saliva samples in both plots. Semen-vaginal secretion samples are positioned between semen and vaginal secretion samples. Blood-vaginal secretion samples cluster more with vaginal secretion samples in both plots. In addition, there are some samples with body fluids not included in the model, namely nasal secretion and

urine. All three samples are positioned randomly and not within specific groups, with even the two urine samples being far apart from each other in both score plots.

4. Discussion

In a previous study we presented an assay for targeted mRNA sequencing for the identification of human body fluids, optimised for the MiSeq/FGx MPS platform [27]. To encourage other laboratories not having access to a MiSeq/FGx platform to participate, an *ad hoc* in-house PGM/S5 assay was developed for this study. The purpose of this study was to implement and evaluate the MPS application in an extended group. We organised a collaborative exercise within 17 EUROFORGEN and EDNAP laboratories to test the platform-specific assays. The samples and primers were in transit / kept at room temperature for up to 3 weeks. There was no indication that the temporary non-ideal storage of the primer pools at ambient temperature had a detrimental effect on primer performance.

The 17 laboratories analysed 8 (PGM/S5 system) or 24 samples (MiSeq/FGx system). The conditions of the prepared stains that were provided by the organising laboratory were ideal, i.e. they were relatively fresh (1-2 months from preparation to shipment), prepared and stored under laboratory conditions (i.e. room temperature, stored in the dark), and in forensic terms relatively large amounts of body fluids were used (e.g. 50 µL of blood, saliva, semen). However, casework samples are usually compromised and/or old and therefore can be insufficient in terms of RNA quality and quantity as was observed with some of the additional samples the laboratories analysed. In further experiments, mock

and real casework samples will be included to assess more systematically the performance of the application to cope with old, degraded and compromised samples.

Most of the laboratories had previous experience with mRNA analyses from former collaborative exercises [22-26, 30] and some prior knowledge of MiSeq/FGx / PGM/S5 library preparation procedures. The laboratories applied their preferred RNA extraction methods, but all further steps were performed according to our provided protocols. The library preparation protocols of the assays are straightforward, although the PGM/S5 workflow (except for protocols including the Ion Chef™ robot) is more time-consuming and requires more hands-on experience (e.g. chip loading) than the MiSeq/FGx workflow.

The manual organic RNA extraction that was recommended by the organising laboratory is a simple and cheap method providing higher RNA yields than silica-column based kit extractions (based on our own experience). This was confirmed as laboratories using the organic RNA extraction recovered considerably higher RNA concentrations and correspondingly higher read counts. However, if DNA has to be analysed in parallel from a casework sample, different extraction methods allowing RNA and DNA to be recovered from the same sample have to be applied.

The RNA quantification methods NanoDrop™ and Qubit™ are not very specific and sensitive. For Qubit™ the lower detection limit was reached especially for blood, semen and skin. NanoDrop™, which is based on a spectrophotometric measurement, rather overestimated the actual RNA quantities since residual DNA and other contaminants, also absorbing light at 280 nm, are quantified as well. One laboratory using NanoDrop™ did take this into account and used the

proposed input volumes for library preparation instead of relying on NanoDrop™ results. Quantus™ and Quant-iT™ Ribogreen were more sensitive compared to Qubit™ and were able to quantify even the low input samples.

Both RNA extraction and quantification are important steps for forensic RNA analyses, especially for sequencing applications where sufficient input amounts are pivotal for optimal assay performance. Nevertheless, for some body fluids (e.g. blood, semen and skin), despite the ideal and high input RNA stains and the use of a manual organic RNA extraction method, the desired input amount of 50 ng could not be reached. Therefore, extraction methods with maximal yields as well as specific and sensitive quantification methods are essential.

The results of three laboratories had to be excluded from final evaluation, because of no or non-specific reads. Two of the laboratories did not use the RT enzyme which is part of the library preparation protocol. For the reverse transcription reaction several brands of RT enzymes might work. However, some incompatibilities of buffers or other reagents from these RT reactions may have had a negative influence on subsequent steps of library preparation. For the third laboratory we could not find an explanation for the poor results.

The results from 7 MiSeq/FGx and 9 PGM/S5 laboratories were used for the final evaluation. Both marker sets (MiSeq/FGx and PGM/S5) demonstrated moderate to high read counts in the body fluid or tissue of interest with little to no counts in non-target body fluids. The PGM/S5 workflow seemed to be less reliable with low input samples, e.g. blood, semen or skin, in that only manually extracted samples resulting in higher RNA yields showed a meaningful result. Although 27 markers are shared in both workflows, the choice of the respective amplicons and the corresponding primer design are different. This can lead to inconsistent results

when the same RNA is analysed with the two different panels (Figure 1). There was some inter-laboratory variability in read counts, especially for skin and vaginal secretion samples, but overall the results of the laboratories were consistent, highly expressed markers showed higher and less expressed markers lower read counts. Due to the relatedness of some body fluids that are detected with the presented assays, some cross reaction reads for some markers were not surprising. For example, markers for vaginal secretion could sporadically be observed also in saliva samples since both body fluids are produced by mucous, epithelial cells. To visualise the complex data, we performed a PLS analysis, where blood, menstrual blood, saliva, semen and vaginal secretion markers and samples clustered nicely, although better for MiSeq/FGx than for PGM/S5 data. This reflects the issues with low RNA inputs that could be observed for the PGM/S5 workflow, resulting in few or no specific reads. Skin samples in particular are difficult in several regards. Usually, RNA yields are very low for contact stains. In addition, there was mainly one skin marker (LCE1C) that seemed to be sensitive enough to be detected in skin samples.

The score plots of the mixed samples showed that they do not cluster exactly in between the two involved body fluids but rather with one of them, depending on the type of mixture. This pattern could also be observed using average read counts, where one of the two body fluids was dominant, leading to a higher coverage than for the other body fluid / tissue. The dominant body fluid in mixtures (e.g. vaginal secretion in semen-vaginal secretion mixtures or menstrual blood in semen-menstrual blood mixtures) was usually the body fluid that also had higher RNA yields in single stains. It is therefore important that for mixtures containing body fluids with usually low RNA amounts, e.g. from blood, semen,

skin, the latter are not missed due to overrepresentation of the dominant body fluid. Future experiments will particularly need to analyse mixed samples comprised of variable ratios of these body fluids.

The additional samples that were tested by laboratories using MiSeq/FGx better represented real casework or low input samples. They generally clustered with other additional stains of the same body fluid, but sometimes separately within the clusters of the pristine, provided stains of the respective body fluid. This observation suggests that ideal stains do not reflect the variability of real, casework stains. Further analyses should therefore include more casework-like samples. In addition, body fluid samples that were not included in the panels (urine, nasal secretion) clustered completely randomly, which implies that the chosen markers are not suitable, but were also not designed to detect these types of body fluids.

The presented prototype assays and the corresponding results from the participating laboratories will serve as a basis for improvements regarding marker selection, library preparation and sequencing. Our results suggest that the analysis of mRNA by targeting body fluid / tissue specific amplicons is a promising tool for body fluid identification. Future developments of the current assay will employ coding region SNPs (cSNPs) within the tissue specific genes that vary considerably within individuals. In this way we will be able to assign a body fluid to a specific individual in mixed donor stains. This will be the task for a second collaborative exercise within the same laboratories.

Acknowledgements

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Figure captions

Figure 1: Comparison of results generated on the MiSeq/FGx and PGM/S5. The RNA from one sample per body fluid (analysed by laboratory 6) was run on both platforms. The reads of the markers (black bars = MiSeq/FGx, grey bars = PGM/S5) are displayed as percentage of total reads per sample. Only markers which are included in both panels (n=27) are displayed. For menstrual blood samples, reads from blood and vaginal secretion are also expected.

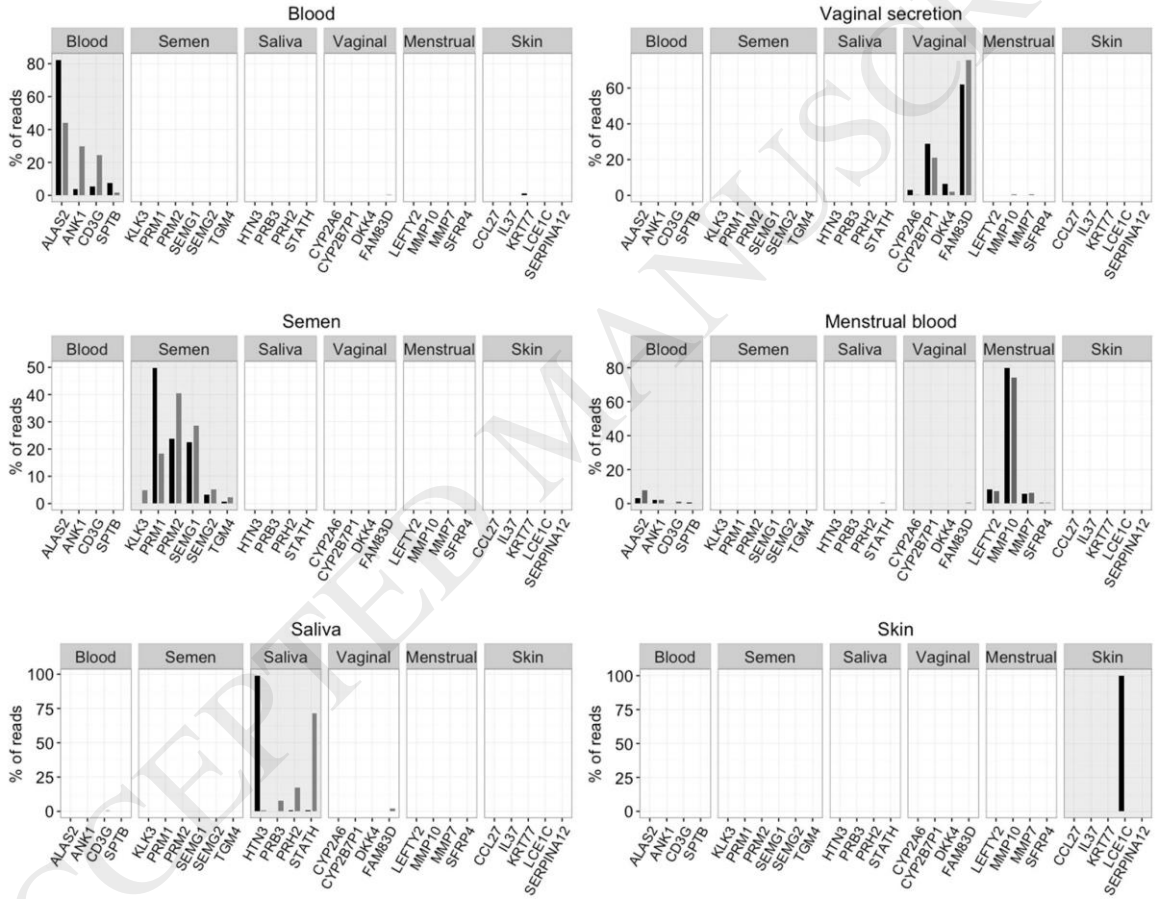


Figure 2: Interlab comparison for MiSeq/FGx and PGM/S5. The first row shows the number of reads per blood marker from all blood samples in relation to the read counts sum of all blood markers, from MiSeq/FGx laboratories (left) and PGM/S5 laboratories (right). The following rows show the same for semen, saliva, vaginal secretion, menstrual blood and skin. Non-specific reads are not accounted for in this figure. Not all markers in MiSeq/FGx are represented in PGM/S5 and *vice versa* (see Table 1).

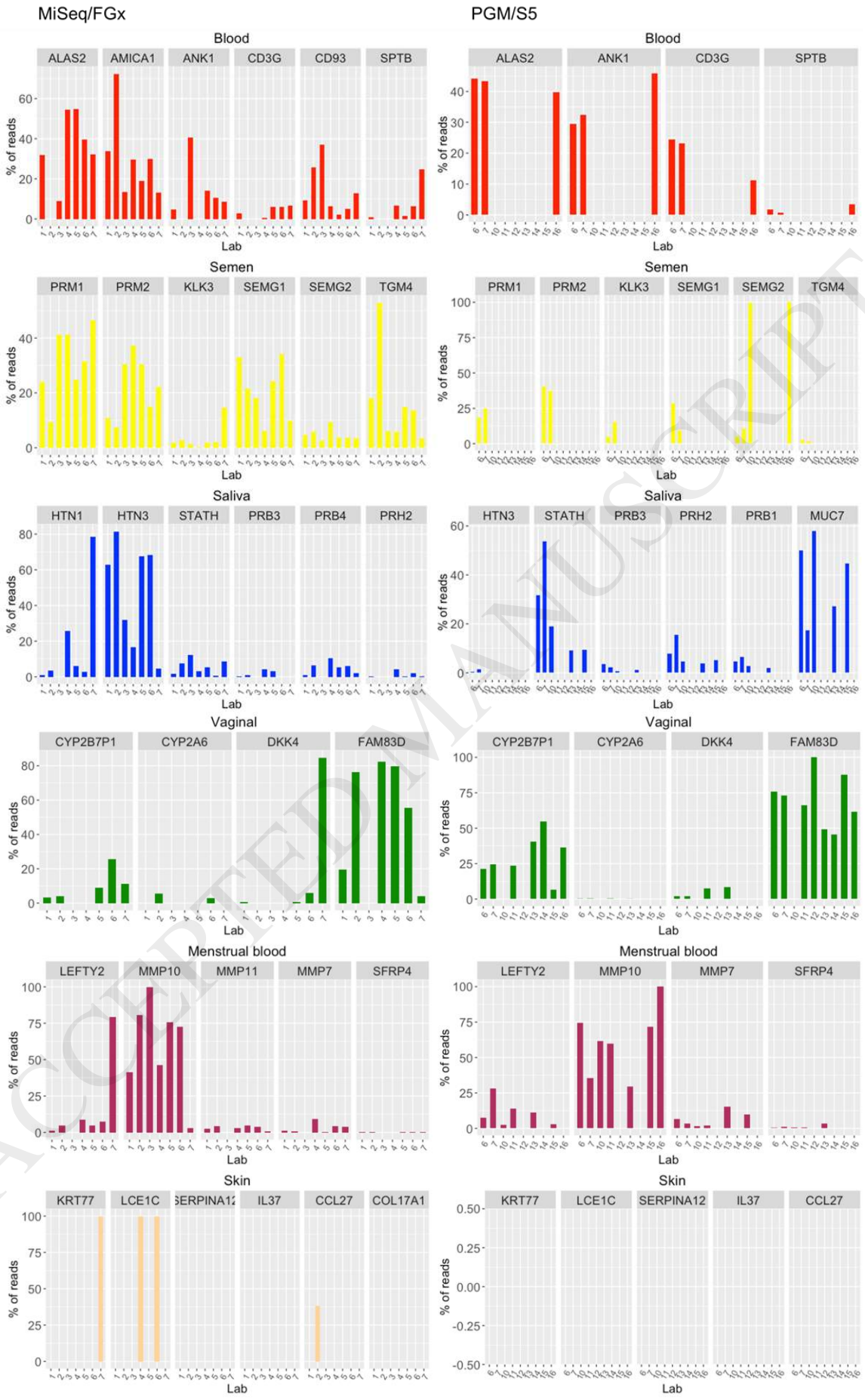


Figure 3: PLS correlation loading plots for the first four components for the MiSeq/FGx and PGM/S5 data show correlation between the markers and the components, and how different body fluid categories (black text) correlate with the markers. Markers further away from the origin are more influential on the components. The markers are colour coded as red=blood, yellow=semen, blue=saliva, green=vaginal secretion, purple=menstrual blood, nude=skin.

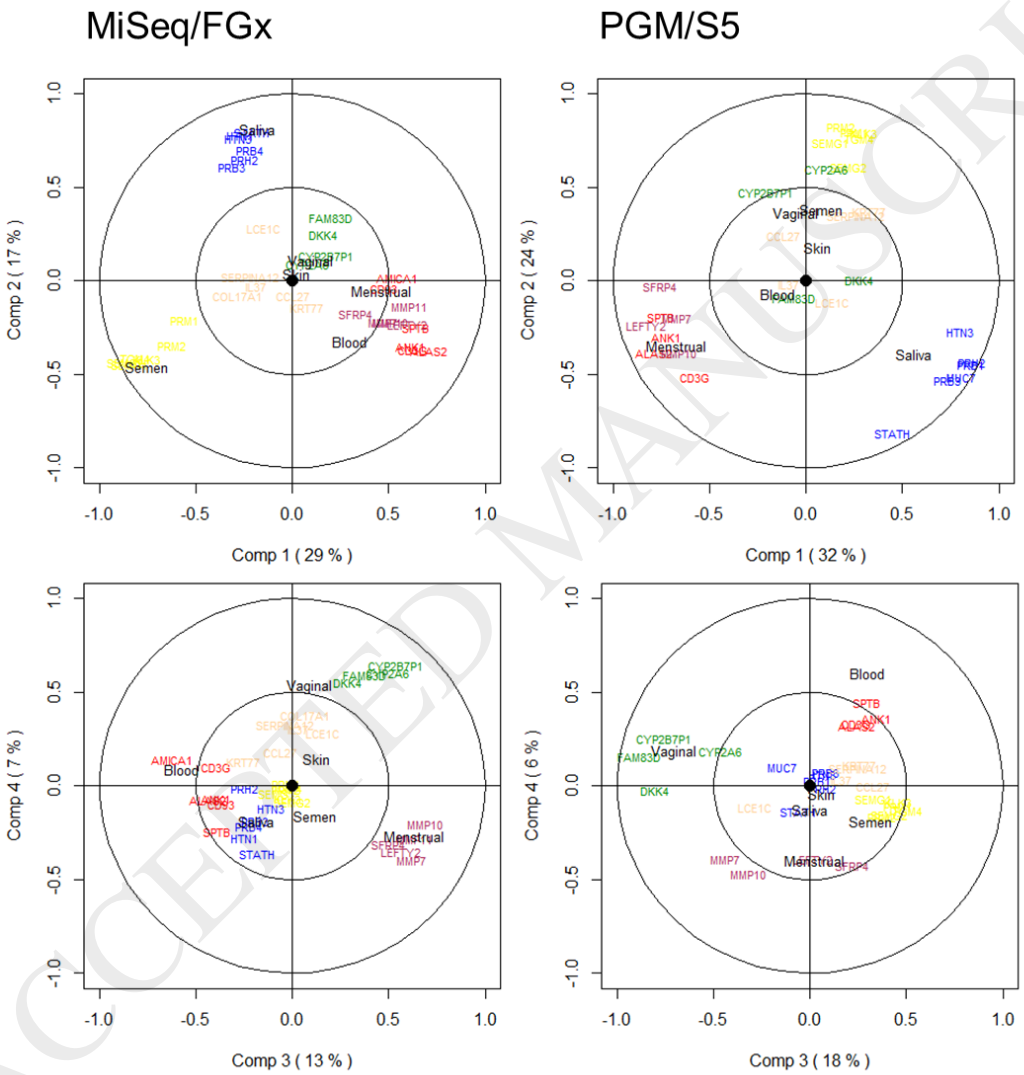


Figure 4: Score plots for the first four PLS components for the MiSeq/FGx data. The single colour circles are the single source samples included in the model. The two-coloured circles are the predicted scores for the four mixture samples, where the colours represent their mixture composition.

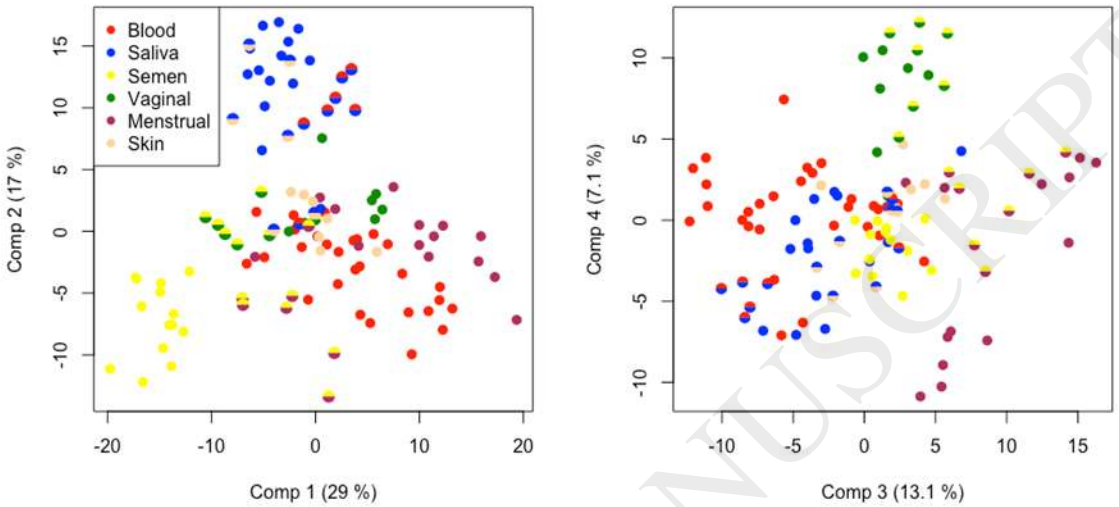


Figure 5: Score plots for the first four PLS components for the PGM/S5 data. The single colour circles are the single source samples included in the model. The two-coloured circles are the predicted scores for the mixture sample, semen-vaginal secretion.

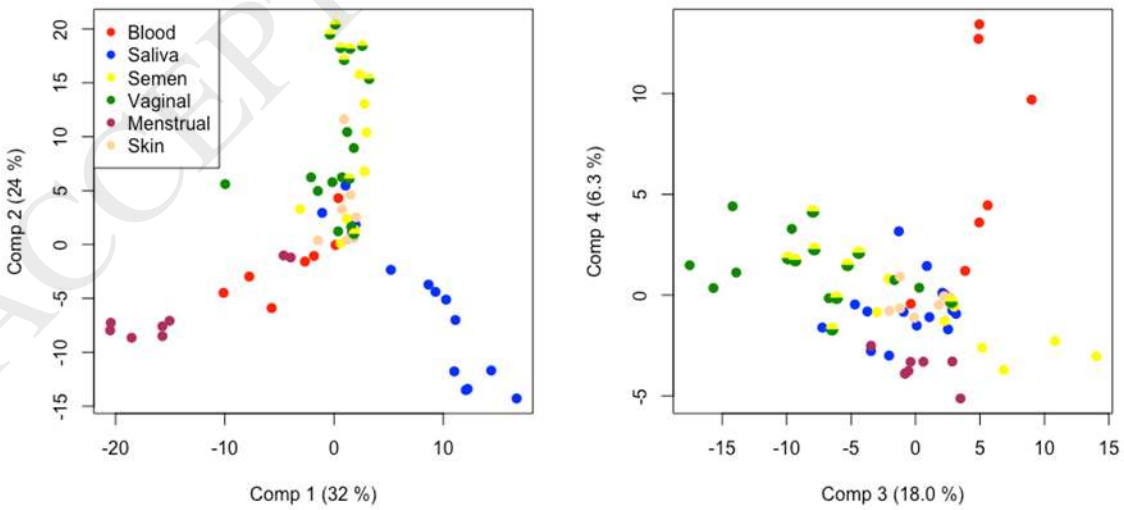


Figure 6: Average read counts for the MiSeq/FGx data mixture samples. Each row represents a mixture of two body fluids / tissues, specified on the right side. The standard error on each bar shows the variation per marker among laboratories.

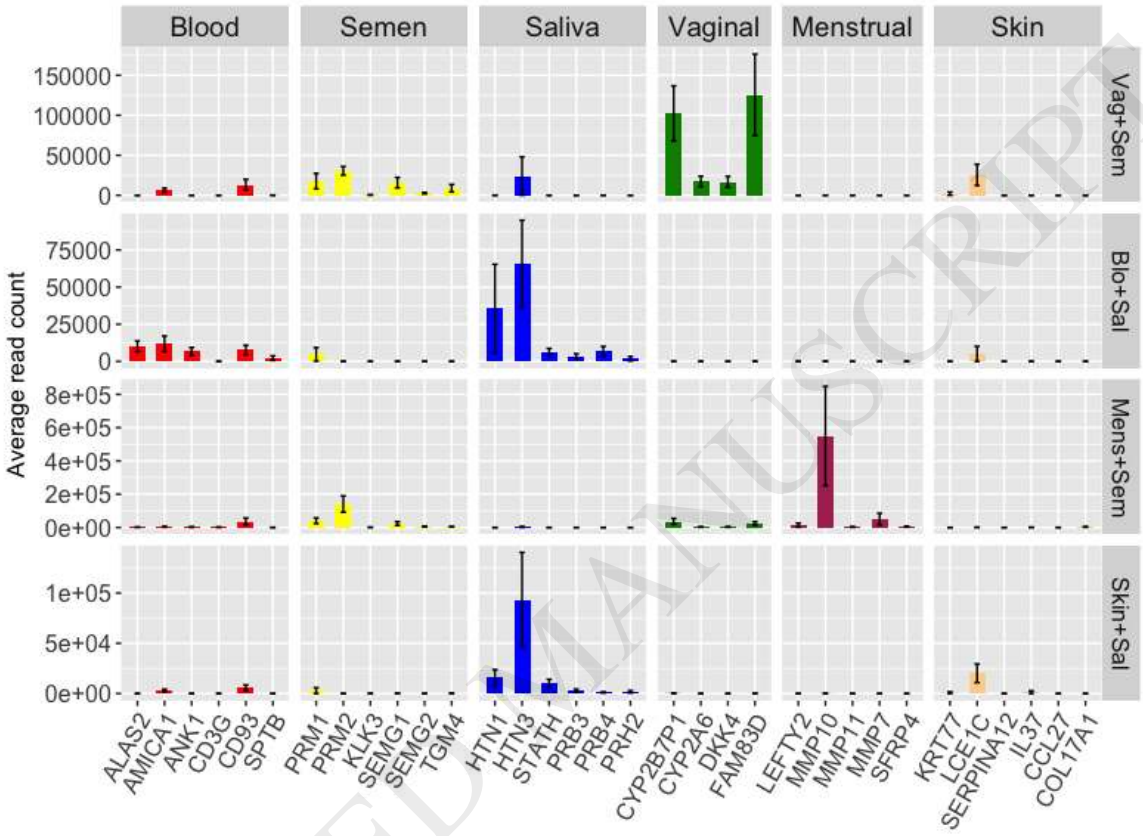


Figure 7: Average read counts for the PGM/S5 data mixture sample. The standard error on each bar shows the variation per marker among laboratories.

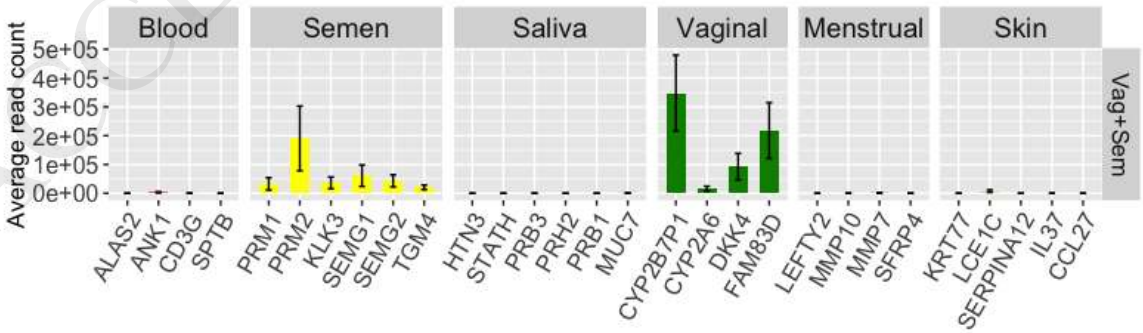
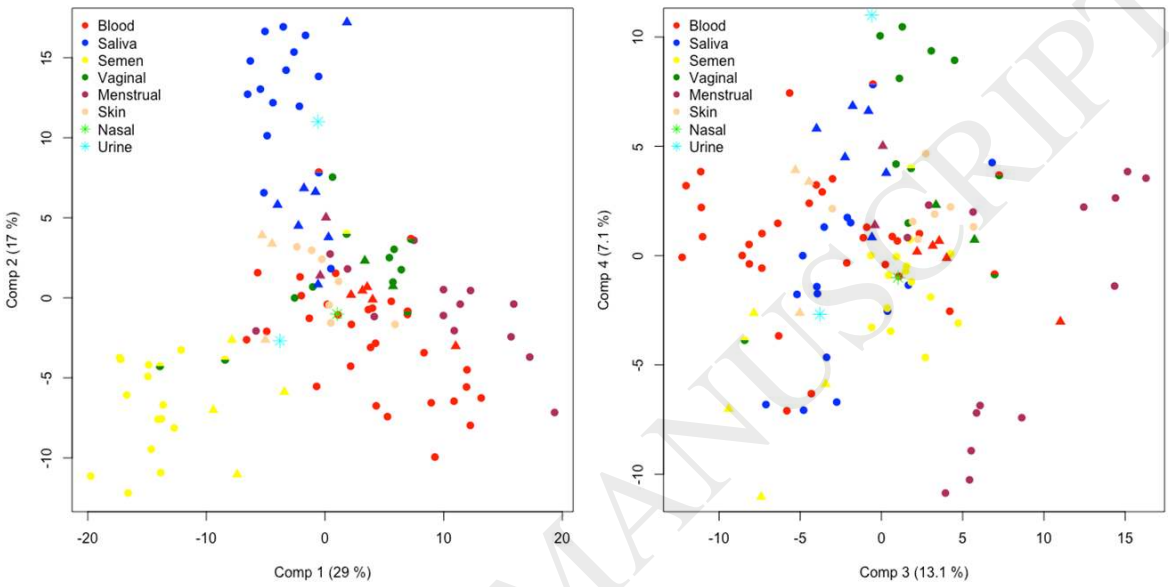


Figure 8: Score plots for the first four PLS components for the MiSeq/FGx data, with predicted scores for the laboratories' own samples. The circles represent samples included in the model, while the triangles represent the laboratories' own samples (cp. Table S2).



Tables

Table 1: Markers included in the TruSeq Targeted RNA Expression panel (MiSeq/FGx) detecting 33 body fluid specific transcripts and in the Ion AmpliSeq™ RNA custom panel (PGM/S5) detecting 29 body fluid specific transcripts.

Body fluid/tissue	Gene	MiSeq/FGx 33plex	PGM/S5 29plex
Blood	ALAS2		
	ANK1		
	SPTB		
	CD3G		
	CD93		
	AMICA1		
Semen	PRM1		
	PRM2		
	TGM4		
	SEMG1		
	SEMG2		
	KLK3		
Saliva	HTN3		
	HTN1		
	STATH		
	PRB3		
	PRB4		
	PRH2		
	PRB1		
	MUC7		
Vaginal	CYP2B7P1		
	DKK4		
	FAM83D		
	CYP2A6		
Menstrual	MMP10		
	LEFTY2		
	MMP7		
	MMP11		
	SFRP4		
Skin	LCE1C		
	CCL27		
	IL37		

	SERPINA12		
	KRT77		
	COL17A1		

Table 2: RNA extraction and quantification methods used by the participating laboratories. Results of RNA quantification are listed for the provided stains 11-28. Results from manual extractions are highlighted in green. nd = not detected.

[ng/µL]	extraction method	quantification method	11	12	13	14	15	16	17	18	19	20	21	22/23	24	25	26	27/28
Lab_1	Kit	Qubit	nd	nd	nd	nd	16.5	24.6	4	nd	23.2	71	29.4	nd	128	6.1	31.4	nd
Lab_2	Kit	Qubit	nd	nd	nd	nd	2.63	3.05	nd	nd	2.3	nd	4.76	nd	20.5	nd	11.5	nd
Lab_3	Kit	NanoDrop	3.8	4	2.2	2.1	30.1	130.4	15.4	3.9	62.2	86.9	39.6	2.7	127.2	10.9	30.1	7.2
Lab_4	Kit	Qubit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lab_5	Kit	Qubit	<0.5	0.81	1.04	0.76	4.44	15.2	2.17	1.22	9.19	44.6	9.63	0.5	>60	0.69	51	0.87
Lab_6	manual	Quantus	29.35	34.6	43.3	57.25	26.9	190	24	61	259.5	234.5	69.6	11	377.5	74.85	212.5	36.9
Lab_7	manual	Quant-iT RiboGreen	23	25.2	64.3	79.9	18	119	33.7	68.2	334.8	471.3	44.7	nd	525	50	392.4	16.6
Lab_8	Kit	no quant	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lab_9	Kit	Qubit	nd	nd	nd	nd	10	10	nd	nd	3.3	9.7	2.7	nd	32	nd	21.5	nd
Lab_10	Kit	no quant		-			-	-	-		-		-	-	-			
Lab_11	Kit	no quant		-			-	-	-		-		-	-	-			
Lab_12	Kit	Qubit/NanoDrop*		4.8			5.5	8	3.8*		11.6		4.4	4.7*	9.6			
Lab_13	Kit	Qubit		nd			nd	56	nd		12.9		9.02	nd	54.8			
Lab_14	manual	Qubit		16.2			8.2	15.6	5.6		65		39.3	2.4	129			
Lab_15	manual	NanoDrop		117.7			223	143.7	107.7		436		107.7	66.25	477.4			
Lab_16	manual	Qubit		nd			2	10.2	2.1		43.6		8.12	nd	33.6			
Lab_17	Kit	no quant	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3: Body fluid inference displaying mean percent contributions for the six body fluid categories (BL = blood, SE = semen, SA = saliva, VS = vaginal secretion, MB = menstrual blood, SK = skin), resulting conclusions and actual components of stains (16 stains from 7 labs using MiSeq/FGx and 8 stains from 9 labs using PGM/S5). Samples with <5000 total reads were excluded (column "used data"). SD = standard deviation.

		Mean Percent Contribution (SD)							
stain	**used data**	**BL**	**SE**	**SA**	**VS**	**MB**	**SK**	**conclusion**	**actual**
11	**7/7**	92 (8)	2 (3)	3 (7)	3 (4)	0 (1)	0 (0)	BL	BL
12	**7/7**	98 (4)	0 (1)	1 (2)	0 (0)	0 (0)	1 (1)	BL	BL
12 PGM/S5	**3/9**	100 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	BL	BL
13	**4/7**	90 (10)	0 (0)	2 (2)	6 (10)	2 (1)	0 (0)	BL	BL
14	**4/7**	95 (4)	2 (2)	3 (5)	0 (0)	0 (0)	1 (1)	BL	BL
15	**7/7**	8 (8)	2 (3)	87 (12)	1 (2)	0 (0)	2 (5)	SA	SA
15 PGM/S5	**3/9**	0 (0)	0 (0)	86 (19)	14 (19)	0 (0)	0 (0)	SA (+VS?)	SA
16	**7/7**	5 (4)	2 (4)	69 (31)	18 (23)	5 (13)	2 (4)	SA (+VS?)	SA
16 PGM/S5	**4/9**	0 (1)	0 (0)	72 (20)	27 (21)	0 (0)	0 (0)	SA (+VS?)	SA
17	**7/7**	0 (1)	98 (4)	1 (3)	0 (0)	0 (0)	0 (1)	SE	SE
17 PGM/S5	**4/9**	0 (0)	100 (0)	0 (0)	0 (0)	0 (0)	0 (0)	SE	SE
18	**7/7**	0 (0)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)	SE azoosp	SE azoosp
19	**6/7**	9 (4)	3 (5)	7 (17)	78 (25)	0 (0)	2 (5)	VS	VS
19 PGM/S5	**7/9**	0 (0)	1 (2)	0 (0)	98 (2)	1 (1)	0 (1)	VS	VS
20	**5/7**	1 (1)	5 (7)	3 (5)	25 (10)	64 (20)	3 (4)	MB	MB
21	**6/7**	13 (6)	1 (3)	3 (7)	0 (0)	81 (13)	1 (3)	MB	MB
21 PGM/S5	**6/9**	24 (8)	0 (0)	0 (0)	1 (1)	74 (10)	1 (1)	MB	MB
22/23	**2/7**	1 (0)	1 (0)	0 (0)	0 (0)	1 (1)	97 (2)	SK	SK

22/23 PGM/S5	**0/9**	-	-	-	-	-	-	-	SK
24	**7/7**	7 (7)	26 (13)	2 (6)	57 (18)	0 (0)	7 (5)	VS / SE	VS / SE
24 PGM/S5	**8/9**	0 (0)	32 (17)	0 (0)	67 (18)	0 (0)	1 (1)	VS / SE	VS / SE
25	**5/7**	27 (14)	2 (3)	69 (14)	0 (0)	0 (0)	2 (4)	BL / SA	BL / SA
26	**7/7**	4 (2)	26 (7)	0 (1)	8 (3)	61 (8)	0 (0)	MB / SE	MB / SE
27/28	**6/7**	7 (5)	3 (3)	75 (12)	1 (1)	0 (1)	14 (11)	SK / SA	SK / SA