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Human Organ Tissue Identification by Targeted RNA Deep Sequencing to Aid in the Investigation of Shooting and Other Traumatic Injury Incidents

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I. Purpose

A number of criminal cases requiring forensic investigation involve significant trauma to the human body in which internal organ tissue is transferred from the injured party to another individual or an item. Examples include tissue adhering to bullets that have exited the body, tissue present on the clothing of an individual responsible for causing the trauma through his/her close proximity to the victim through the use of a firearm or knife or other implement, tissue present on a suspected murder weapon and tissue present on the walls, ceilings or furnishings of the scene of a suspected homicide or serious assault in which the body of a missing person has been removed. The nature of the transferred tissue would be dependent upon the circumstances of the crime but could include adipose, skeletal muscle, lung, liver, heart, brain, kidney, stomach and intestine. In combination with standard DNA analysis to identify the individual source of the transferred biological material, positive identification and differentiation of the organ tissue from blood or other secreted body fluids could provide important probative information. The problem is that currently the identification of traces of desiccated organ tissue can be problematic and requires the expertise of a cellular pathologist and/or histologist and the use of immunohistochemistry methods. Investigators and/or forensic scientists often have limited, if any, access to such personnel and facilities and, in any case, many such tissues are intractable to such analysis due to limited material

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and/or the fact that the cellular structures are non-canonical in appearance due to dehydration and are difficult to discern due to limited quantity or crushing damage. Thus at present, many case situations involving organ tissue are resolved at the DNA level alone without the investigator being able to ascertain the potentially important contextual information about the organ tissue source of the DNA on the person, weapon or other item.

II. Project design

The purpose of this project was to utilize targeted multiplexed deep sequencing (next generation sequencing, NGS) of mRNA transcripts in order to identify the tissue or organ source of origin from putative tissue fragments in order to aid in the criminal investigation of shooting and other traumatic bodily injury incidents.

The goals were to use next generation sequencing for the definitive identification of eleven human tissues from different organs (brain, lung, liver, heart, kidney, intestine, stomach, skeletal muscle, adipose, trachea and spinal cord). We have developed two prototype biomarker panels: a 48-plex that contains numerous gene targets for each tissue and organ (brain, spinal cord, lung, trachea, liver, skeletal muscle, heart, kidney, adipose, intestine, stomach, skin and spleen) and an additional 54-plex which expands upon the 48-plex assay with the inclusion of biomarkers for fetal brain, placenta and prostate. The 48-plex assay has been validated including testing and evaluating the limit of detection (LOD), sensitivity and specificity. Fresh autopsy tissue, post mortem interval samples and *bona fide* casework samples were tested in order to evaluate its efficacy for potential use in forensic casework. The assay has been transferred to a collaborating international forensic laboratory, the University of Zurich (PI:Dr. Cordula Haas) who has successfully utilized the assay to identify tissue samples. The 54-plex panel validation is currently underway.

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The objectives were to (i) finalize the development of a targeted oligonucleotide panel comprising numerous biomarkers for the identification of eleven human tissues and organs, (ii) test and optimize the assay with studies on tissue samples from commercial sources (i.e LOD, sensitivity and specificity), (iii) evaluate assay performance with fresh autopsy tissue and autopsy tissue and (iv) transfer the developed assay to collaborating laboratories. All objectives have been successfully met and the work performed has resulted in the development of novel targeted multiplexed deep sequencing (next generation sequencing, NGS) of mRNA biomarker transcripts in order to identify the tissue or organ source of origin from putative tissue fragments.

III. Methods

**detailed protocols are provided publications (Appendix A) resulting from this work*

Multiplexed targeted mRNA NGS assay for body fluid identification - Illumina MiSeq platform

NGS libraries of targeted body fluid gene candidates were prepared using the TruSeq® Targeted RNA kit (January 2016 protocol version; Illumina Inc., San Diego, CA). Total RNA input ranged was 50 ng. Pooled libraries were quantitated using the 2200 TapeStation (Agilent Technologies, Santa Clara, CA) and High Sensitivity D1000 Screen Tape according to the manufacturer's protocol. Pooled libraries were diluted and denatured according to the manufacturer's recommended protocol and a 600 µl of sample (6 pM) was pipetted into the MiSeq® V3 150 cycle reagent cartridge for sequencing on the MiSeq instrument. Sequencing was performed using 51 cycles (single read). Local sequencing software on the MiSeq analyzed the data (base calling, demultiplexing and alignment to the provided manifest file using a banded Smith Waterman alignment). A minimum sample total read count (MTR) of 5000 was used (samples below this threshold were excluded). A minimum biomarker read count (MBR) of 500 was used as an individual biomarker threshold (counts per biomarker below 500 were removed).

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A third threshold was then used in which individual biomarker read count values that were less than 0.5% of the total reads for the sample were also removed. Bar graphs of threshold-filtered counts were prepared by sample and by gene to evaluate gene expression and specificity. The percent contribution of reads was next calculated in order to provide the percentage of total sequencing reads for each individual sample that was attributable to tissue-specific markers.

IV. Findings

**detailed results are provided in the publications (Appendix A) resulting from this work*

A. 48-plex tissue identification assay

We initially formulated a prototype 46-plex targeted mRNA NGS assay for tissue identification. The results of assay development and initial performance checks were published (Appendix A). The next goal was to optimize and further develop the prototype assay to not only improve identification of some of the already included tissue types but to identify novel biomarkers for additional organ tissue types. Two additional targeted mRNA NGS assays for tissue identification were developed both using the Illumina MiSeq platform, a 48-plex and a 54-plex assay.

The 48-plex assay contains biomarkers for the identification of the following organs and tissues: brain (4 biomarkers), spinal cord-neural (3 biomarkers), lung (3 biomarkers), trachea (3 biomarkers), liver (5 biomarkers), skeletal muscle (4 biomarkers), heart muscle (1 biomarker), heart (3 biomarkers), kidney (4 biomarkers), adipose (4 biomarkers), intestine (3 biomarkers), stomach (4 biomarkers), skin (3 biomarkers) and spleen (4 biomarkers). This assay utilizes ~50 ng of total input RNA, although the limit of detection of numerous organ tissue biomarkers is well below this input level. Once biomarker selection was completed, validation experiments were performed. To evaluate the specificity of the assay, numerous donors of both target and non-target

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tissues were tested. For the target tissues, the percent contribution of total reads attributable to the target biomarker class were as follows: brain - 78 – 96% brain biomarkers (with a majority of the remaining reads attributable to spinal cord-neural biomarkers; spinal cord – 100% brain-spinal cord-neural biomarkers); lung 92 – 100% lung biomarkers; trachea – 98 – 99% trachea biomarkers; liver – 99-100% liver biomarkers; skeletal muscle – 97 – 100% skeletal muscle biomarkers; heart – 84 – 100% heart biomarkers; kidney – 99 – 100% kidney biomarkers; adipose – 45 – 94 % adipose biomarkers (with the remaining read counts attributable to skeletal muscle biomarkers (in 3 of the 5 donors) and stomach (in 1 of 5 donors)); small intestine – 97 – 100% intestine biomarkers; stomach – 97-100% stomach biomarkers; skin – 94 – 97% skin biomarkers; and spleen – 80 – 96% spleen biomarkers. The results of this specificity testing demonstrate the high degree of specificity of the assay for the target tissues. Numerous non-target tissue samples as well as forensically relevant body fluid samples (blood, semen, saliva, vaginal secretions and menstrual blood) were tested with many falling below MTR thresholds and therefore not analyzable. For those tissues above MTR thresholds, a majority of expression appeared to originate from skeletal muscle biomarkers. It is possible that trace amounts of *bona fide* skeletal muscle tissue were actually present in these non-skeletal muscle samples and therefore may (or may not) represent low level background basal transcription.

The ability of the assay to identify multiple tissues within an individual sample was also demonstrated through the evaluation of 2- to 5-tissue admixtures. For the 2-tissue admixtures, lung and heart samples were mixed in varying ratios (25ng total input): 20/5ng, 15/10ng, 12.5/12.5ng, 10/15ng, 5/20ng. This range permitted each of the two tissue types to be the major contributor to the mixture. Lung and heart tissue were identified in each of the mixtures. As the input of each tissue type increased, so did the percent contribution for each biomarker class, and vice versa when

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the input was decreased. Seven 3-tissue admixtures were tested. In four of the seven samples, all three tissue types were correctly identified. In the remaining three mixtures only two of the three tissues were identified. Four 4-tissue admixtures were tested. In two of the four samples, all four tissue types were correctly identified. In the remaining two samples, either 2 or 3 of the tissue types were identified. Three 5-tissue admixtures were tested. All five of the tissues were not detected in any of the mixtures, but four of the five tissues were detected in one of the samples and three of the five tissues were identified in two of the samples. With these complex mixtures, the input total RNA was low for each of the individual samples (e.g. ~10 ng for the 5 tissue admixtures) which is below the optimal input level. However the above demonstrated the ability to detect multiple tissues within individual admixed samples.

The samples used in the testing described above consisted of commercially available total RNA samples. We next evaluated the 48-plex assay with actual organ tissue samples. Target tissue from 3 different corpses was collected post-mortem. The percent contribution of total reads attributable to biomarkers for the target tissue ranged from 93-100% for most samples. In addition to these corpse tissue samples, samples from two suicide cases were available for testing: 1) material recovered from train tracks and 2) material recovered from a hooded jacket. For the train track sample, skeletal muscle was identified which would be expected as part of the material recovered after a person is hit by a train. For the hooded jacket sample, brain was identified which is consistent with a case in which the cause of death was a self-inflicted gunshot wound to the head. Additionally, the liver biomarkers have been evaluated with a series of post mortem liver tissue samples, with post mortem intervals (PMIs) ranging from ~3.5 hours – 37 days. While not tested with the full 48-plex assay, all liver biomarkers present in the 48-plex assay were included in the evaluation of these samples. While each biomarker was not present in every sample, the

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presence of liver tissue was correctly identified in all samples that were above the MTR threshold, including the 37-day sample. The results of the cadaver and *bona fide* casework samples therefore demonstrate the usefulness of such an assay in the identification of the tissue source of origin of samples in traumatic injury investigations.

A manuscript describing the results of this validation is currently being prepared and will be submitted for publication soon.

B. 54-plex tissue identification assay

During the development of the 48-plex assay, we identified biomarkers for the identification of additional tissues including fetal brain, placenta and prostate. The 54-plex assay now contains 2 biomarkers for fetal brain, 2 biomarkers for placenta and 2 biomarkers for prostate. Figure 1 below shows an example of the expression levels of one of the new fetal brain biomarkers. Its presence is not detected in either of the other two adult brain samples (the next two samples after the fetal brain sample) but shows high expression in the fetal brain sample (grey bar).

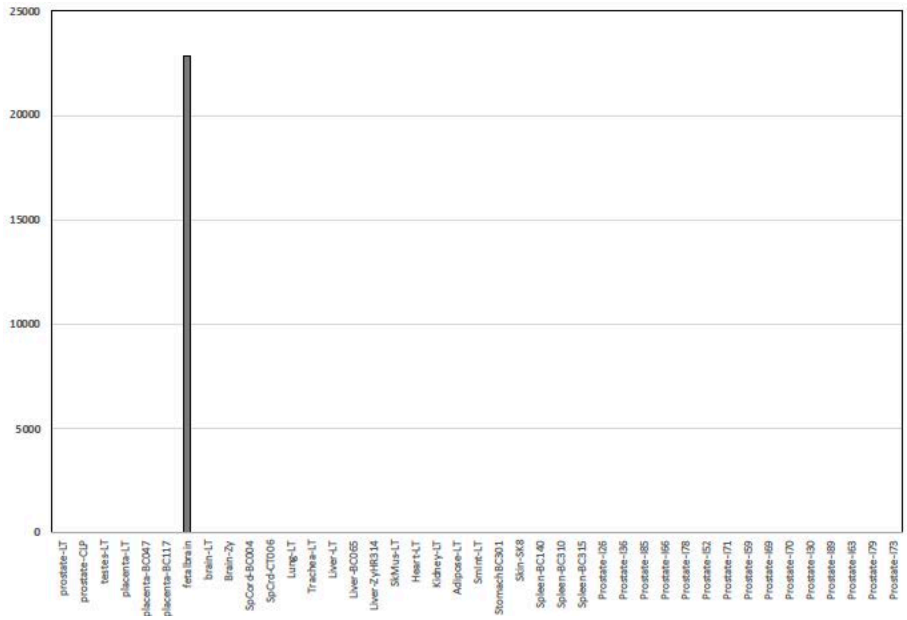

Figure 1. Expression (threshold corrected read counts) of a new fetal brain biomarker.

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Similarly, expression of the placenta biomarkers is only observed in placental tissue samples and in combination with the fetal brain biomarkers could prove useful in cases involving pregnant victims. Additionally, expression of the prostate biomarkers is observed mainly in prostate samples. These biomarkers have also been detected in prostate tissue samples with PMIs ranging from 24 hour – 10 days. The prostate is an organ that is less susceptible to rapid atrophy compared to other tissues in cadavers over time and may be useful subsequently for post-mortem interval (PMI) estimation studies.

While the 54-plex assay is now finalized, additional samples are needed for testing, particularly for the fetal brain biomarkers, before a full validation can be performed. Currently only one fetal brain sample has been tested as this is a difficult tissue type to obtain. For both assays we are also currently utilizing the data to train a multivariate statistical model that predicts the tissue type based on the mRNA profiling results. By considering co-expression of markers the model can recognize distinct expression patterns in each tissue.

C. Technology transfer

Technology transfer of developed assays to operational forensic science casework laboratories and other research collaborators was an integral part of the work. The laboratories can address specific needs of operational laboratories, with any feedback ultimately incorporated into the assay design and performance if possible. During the course of the this project, we were able to successfully transition and implement this assay to the Institute of Legal Medicine, University of Zurich (Switzerland) thus demonstrating the feasibility of use of this technology in operational laboratories.

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To transfer the developed 48plex assay to Zurich, initially the assay and associated protocols for library preparation and sequencing were provided. Commercial tissue samples of known tissue source were used in initial testing to ensure that the proper consensus results were obtained upon implementation by their lab. The results of the initial sequencing runs were provided by Zurich to our laboratory for review. Read counts and percent contributions from the samples tested were analyzed and compared to our own data. There were no technical or analysis issues encountered demonstrating the ease in which this assay can be incorporated into other laboratories' workflows. Upon successful implementation of the assay in the Zurich laboratory, they were able to continue utilizing the assay with additional specificity samples as well as *bona fide* tissue samples and forensic case samples.

V. Implications for criminal justice policy and practice in the United States

An NGS-based molecular organ tissue assay that can definitively identify internal organ tissue and that could be used by any laboratory with forensic NGS capabilities will facilitate the investigation and prosecution of cases in which potentially important contextual information about the organ tissue source of the DNA is present on a person, weapon or other item. Many of the cases expected to be impacted include shootings or stabbings whereby the bullet or knife trajectory through the body or the mere presence of particular internal organ tissue indicating proximity to, or involvement in, a significant trauma-producing event might be demonstrated. The relative ease-of-use of the assay by forensic molecular biologists will in time, once labs are up and running with NGS technology, ‘democratize’ the ability to routinely identify organ tissue when necessary instead of having to rely on specialized (and not always readily available) cellular pathology services.

It is possible in the future that the organ tissue targets described will be incorporated together

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with body fluid specific biomarkers into a combined comprehensive tissue identification assay to identify both externally secreted body fluids and internal organ tissues. Such an assay would require approximately 100 or fewer co-analyzed targets, a number that is easily accomplished with current multiplex NGS technology. This combined assay would simply become another modular component of the forensic scientist's NGS armamentarium to be employed, whenever necessary, along with DNA typing. Commercial vendors are already making plans to incorporate RNA based body fluid identification into their NGS products and this effort could be easily expanded to include organ tissue markers.

Appendix A. List of Publications

[1] Hanson E. and Ballantyne J. Human Organ Tissue Identification by Targeted RNA Deep Sequencing to Aid the Investigation of Traumatic Injury. Genes 2017, 8, 319. Doi:10.3390/genes8110319

[2] Hanson E., Salzmann A., Dorum G., Fliss B., Hess S., Haas C. and Ballantyne J. mRNA MPS Tissue Identification Assay to Aid in the Investigation of Traumatic Injuries. Forensic Sci Suppl Series (2019). In Press. https://doi.org/10.1016/j.fsigss.2019.09.012

**an additional manuscript describing the validation of the 48-plex assay is currently underway*
***once full validation of the 54-plex assay is complete, an additional manuscript will be prepared describing the results of this validation*

Appendix B. Acknowledgments

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