Advancing forensic RNA profiling

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Chapter 6

Development of a mRNA profiling multiplex for the inference of organ tissues

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Abstract

Forensic characterisation of organ tissue generally occurs through histological and immunological assays of limited sensitivity. Here, we explore an alternative approach and examine a total of 41 candidate mRNA markers for their ability to differentiate between brain, lung, liver, skeletal muscle, heart, kidney and skin. Various selection rounds are applied involving 85 organ tissues (36 excised autopsy specimens and 49 frozen tissue sections, with at least ten specimens for each organ type), 20 commercially available RNAs from different human tissues and at least two specimens of blood, saliva, semen, vaginal mucosa, menstrual secretion or touch samples. Finally, 14 markers are regarded tissue-specific and included in an endpoint RT-PCR multiplex together with one general muscle, one blood and one housekeeping marker. This 17-plex is successfully used to analyse a blind test set of 20 specimens including mixtures, and samples derived from stabbing of organ tissues. With the blind test set samples, it is shown that an earlier described interpretation strategy for RNA cell typing results [1] is also effective for tissue inference. As organ-typing is embedded in a procedure of combined DNA/RNA extraction and analysis, both donor and organ type information is derived from the same sample. Some autopsy specimens presented DNA profiles characteristic for degraded DNA. Nevertheless, the organ-typing multiplex could generate full RNA profiles, which is probably due to small sizes of the amplicons. This assay provides a novel tool for analysis of samples from violent crimes.

Introduction

Evidentiary items collected at the scenes of violent crimes can include trauma-causing objects such as guns, bullets, knives or impact-inflicting implements. These objects can contain cell material from the victim and/or the perpetrator, which can be examined by DNA profiling [2, 3]. The biological material on these trauma-causing objects often represents blood and/or skin cells but in addition tissue fragments from organs may be present due to the perforation of the body [4]. These organ remnants may hold forensically relevant information. For instance, the presence of organ tissue matching the DNA profile of the victim on a knife provides a link to the crime and argues against an accidental finger cut. With shootings, inferring what type of cell material resides on which bullet is useful for crime scene reconstruction. For complex cases involving multiple shooters, specific bullets with particular organ tissues may be linked to the individual firearms thereby indicating from which gun the lethal shot was fired. Furthermore in practical cases of run-over traffic accidents, identification of human brain tissue was found to be important [5].

For the forensic identification of human organ tissues, enzyme-linked immunosorbent assays and histological methods are mostly employed [5–8]. These methods have limited sensitivity, and when little cell material is present, organ-typing analyses are complicated [9]. We reasoned that an alternative approach may lie in the use of mRNA markers specific for various organ tissues. In recent years, mRNA profiling has emerged as a sensitive, human-specific method for the simultaneous inference of several body fluids and skin cells [10–16]. RNA profiling is typically accompanied by a DNA/RNA co-isolation enabling DNA profiling on the exact same sample, which is of criminalistic importance. Whilst RNA molecules are generally considered to be less stable than DNA molecules, successful mRNA profiling on aged and degraded body fluid samples has been achieved upon selection of stable markers and the use of small amplicons [16–18]. It is not known whether forensic RNA analysis for tissues is possible, as the ex vivo stability of their RNA is unclear. Tissues with a low turnover rate and low concentrations of digestive enzymes such as the central nervous system may have the best quality of RNA [19]. Lung and liver, on the other hand, are described to be most prone to degradation resulting in low-quality RNA [20].

Here, we investigated a total of 41 candidate mRNA markers for the ability to identify brain, lung, liver, skeletal muscle, heart, kidney or skin. We focused on these tissues, as these are most likely found on trauma-causing objects. Specific and sensitive markers were selected and combined into an endpoint RT-PCR (reverse transcriptase PCR) multiplex system that allows analysis by capillary electrophoresis (CE) following routines standard in forensic laboratories. Such an assay will be of value in forensic casework.

Materials and methods

Organ samples

This study aimed at mRNA-based inference of human organ types. Organ specimens were collected at medical institutions and the necessary approvals were obtained from the required ethical examination commissions by these medical institutions. Seven different organ tissues (brain, lung, liver, skeletal muscle, heart, kidney and skin) were studied for which 85 specimens were collected. Thirty-six specimens represented autopsy samples and were excised from four individuals who voluntarily donated their body for research to the Academic Medical Centre (AMC, Amsterdam, The Netherlands). Cadavers were fresh frozen upon arrival and thawed (for other purposes) several days before sample excision. This resulted in 12 brain (four frontal lobe, four occipital lobe, four cerebellum), three lung, three liver, four skeletal muscle, three heart, three kidney and eight skin (four finger, four palm) samples. Excised tissues were transferred to a container with RNA*later*® (Ambion® by Life Technologies, Carlsbad, USA), transported to the forensic institute and stored at −80 °C until use. For each excision, new scalpel blades were used to minimize the risk of contamination. For each DNA/RNA co-isolation, tissue cubes of around 5×5×5 mm were used. Forty-nine specimens involved sections prepared from frozen tissues (University Medical Centre Utrecht (UMC) Central Biobank, Utrecht, The Netherlands). This set comprised seven samples of each of the seven organ types. These samples mainly represented tissues remaining after surgery or diagnostic research that were frozen directly after collection or diagnosis. Sections were stored at −80 °C until use. For extraction of nucleic acids, tissue squares of around 2×2 mm were excised from the sections.

To assess specificity on a larger set of human tissues than those targeted by the selected markers, the FirstChoice® Human Total RNA Survey Panel (Ambion® by Life Technologies, Carlsbad, USA) was used which contains pools of total RNA (of at least three donors) for 20 different human tissues in a concentration of 1 µg/µL. These RNAs were diluted to 10 ng/µL to facilitate analysis.

Body fluid samples

To assess the performance of the organ-typing multiplex when body fluids are involved, RNA extracts of donations of blood, saliva, semen, menstrual secretion, vaginal mucosa and skin (touched cotton patches) were used [16]. When testing the final multiplex, two samples provided by different individuals were used for each of these cell types except for semen for which two donations from fertile donors and two specimens from sterile donors were used. Volunteers had given informed consent.

Mock casework samples

For the preparation of a blind test set, small portions of the autopsy samples were cut, transferred to a petri dish and rubbed with dry swabs (Deltalabs, Barcelona, Spain) to collect some of the cell material. Mixed samples were prepared by sampling multiple excised portions with the same swab (using different sides of the swab if possible) and each tissue portion was discarded straight after a sampling. The aim was to have approximately equal amounts of each organ type on the swab. The blind test consisted of 20 swabs representing three single organ samples, one neat blood specimen and 16 mixed samples: eight two-organ mixtures, five three-organ mixtures, two four-organ mixtures and one seven-organ mixture. Each organ was represented four to ten times and neat blood was present once.

To mimic violent crime scene samples, autopsy samples were stabbed using scalpel knives. Scalpels were inserted once and pulled out immediately. To mimic an abdominal stabbing, a stacking of liver, skeletal muscle and skin was prepared. In addition, liver covered with skin, skeletal muscle covered with skin and skin alone were stabbed. Liver and skeletal muscle samplings had a thickness of 6 to 10 mm and skin specimens that were excised from finger, a thickness of 2 to 3 mm (width and length were around 1.5 to 2 cm). Immediately after stabbing, cell material was collected from the scalpels using swabs.

Retrieval of candidate markers

Marker candidates for brain, lung, liver, skeletal muscle, heart or kidney were selected from the gene annotation portal BIOGPS [21]. Candidates were included because of high expression values (in the first selection round above 1,600, later above 900) and expected specificity, which refers to much higher expression values in the target tissue than in other tissues, especially those assessed in this study.

Primer design

For the mRNA markers, cDNA-specific primer sets were designed that either spanned an intron or had one primer covering an exon–exon junction using Ensemble and NCBI Primer-BLAST [22, 23]. For 18S-rRNA and HBB, primers were adopted from literature [11]; for LOR [16] and LCE1C [13], primers were redesigned. In silico BLAST analyses affirmed human specificity of the amplifications. Details for the amplicons and primers of the markers included in the final multiplex are given in Table 1. In the process of developing this final multiplex, other multiplexes were used for which markers and amplicon numbers are depicted in Table 2. The labelled primers underwent HPLC-purification (Applied Biosystems™, Warrington, United Kingdom).

Table 1. Characteristics of markers included in organ-typing multiplex. For each amplicon one of the primers covers an exon-exon junction unless indicated otherwise.

Tissue	Gene	[Primer] µM	Forward primer (5'-3') Reverse primer (5'-3')	Dye	Size (bp)	Biological function
Brain	SNAP25	0.02	TCGGCGGCTCCACCACAGTT TTGGCTCTGGACCTGGGCTTCTC	6FAM	78	Synaptic vesicles: maintenance & signalling Reported expression in spinal cord [24]
	RTN1	0.08	AGGCCACCCTTTCAAGGCCTACTT AGAACTGCAGGCAGTCCGTGT	NED	86	Neuroendocrine cells: maintenance & signalling Reported expression in spinal cord [24]
Lung	SFTPB	0.06	CCATGATTCCCAAGGGTGCGCTA CGCCACCAGAGGTACCACGC	NED	68	Stability of pulmonary tissues by reducing surface tension
	SFTPD[a]	0.10	CCTGCCTGGTCGCGATGGAC CCAGGCATCCCTGCTTGCCC	6FAM	99	Stability of pulmonary tissues by defence against inhaled micro-organisms
Liver	AMBP	0.03	TTGGCTGACCGAGGTGAATGTGTCC[b] ACCAGTTGCCCACCCCCTGAT	VIC	119	Protease inhibitor & lipocalin transport protein secreted in plasma
	VTN	0.05	GTGCAAGCCCCAAGTGACTCG CATAGACCGTGTACTCATCCTCCG	VIC	65	Secreted by hepatocytes dampens early MAC formation
Skeletal muscle	TNNI2	0.13	ATGTCTGAAGTGCAGGAGCTCTGC GTCGTACTTCTCCTCTTCAGCCGC	PET	72	Fast-twitch skeletal muscle protein. Reported expression in thyroid and tongue [24]
	MYH1	0.8	TTCGCATCTCTACGCCAGGGTCCTTA[b] AGGAAAGGAGCAGCCTCCCCAAA	NED	104	Heavy chain myosin, muscle contraction Reported expression in thyroid [24]
Heart	MYL7	0.08	AAGCTCAATGGGACAGACCCCG CACCACCCCTTTGCCGCTGG	VIC	82	Light chain regulatory myosin motor protein
	MYBPC3	0.4	TCATAGAGGCAGAGAAGGCAGAGC CCCTTTGGGACTTGGGGCACT	VIC	130	Myosin-associated protein Cardiac isoform
General muscle	NMRK2	0.4	GGAGGAGAAGTACCCGCAACTACA ACACCGTTGGCCTCCATCTCCT	6FAM	109	Muscle β1 integrin binding protein regulation of terminal myogenesis
Kidney	UMOD	0.12	ATCACACGGAAAGGTGTCCAGGC TTTTTTGGAGCACAGGGCTTTCCGC[b]	6FAM	145	Formation of a water permeable barrier inhibition calcium crystallization in renal fluids
	FXYD2	0.06	CTCCATCCAGGCCCCAGGCA CGGTCTCATAGTCATAGTAGAACGGG	PET	143	Modulates the activity of the Na,K-ATPase Reported expression in pancreatic islet [24]
Skin	LCE1C	0.2	TGTGACCCCGCTCCTGAATCCG CTTGGGAGGGCACTTGGGGGTG	NED	99	Component of the cornified envelope Mouse homolog expressed in stomach [24]
	LOR	0.6	CTTTGGGCTCTCCTTCCT TCTTCACGCAGTCCACTG	PET	84	Component of the cornified envelope In terminally differentiated epidermal cells
House-keeping	18S-rRNA[c]	0.015	CTCAACACGGGAAACCTCAC CGCTCCACCAACTAAGAACG	PET	110	Small ribosomal subunit protein biosynthesis in all cells
Blood	HBB	0.1	GCACGTGGATCCTGAGAAC ATGGGCCAGCACACAGAC	6FAM	61	Component hemoglobin oxygen binding in red blood cells

[a] Primers span an intron.

[b] Underlined nucleotides are 5' tails added to improve multiplex spacing.

[c] Primer pair does not cover an exon-exon junction nor spans an intron.

RNA profiling

DNA/RNA co-isolation was performed as described by Lindenbergh et al. [16]. Tissue debris that had not fully lysed after 2 h of incubation at 56 °C was discarded using a QIA shredder column. RNA extracts were DNase-treated [16], and copy DNA (cDNA) was synthesised [16] using 10 µL RNA extract. Variable amounts (up to 5 µL)

Table 2 Performance of all candidate markers in various assays and selection rounds.

		Selection round[a]					
Tissue	Marker	1[b]	2[c]	3[d]	4[e]	5[f]	Remark
Brain	MBP						
	SNAP25						
	OLFM1						
	RTN1						
	NEFL						
	PLP1						
Lung	SFTPC						
	SFTPB						Among two most sensitive markers in round 1
	BPIFB1						Specific but less sensitive than SFTPB & SFTPD, so not taken to round 2
	SCGB1A1						Specific but less sensitive than SFTPB & SFTPD, so not taken to round 2
	SFTPD						Among two most sensitive markers in round 1
	ALOX5						
Liver	APOA1						
	ALB						
	APOC3						
	AMBP amp1[g]						1 extra T residue added to rv primer in round 2 for multiplex spacing
	AMBP amp2						Amplicon 2 dismissed after round 3 because superfluous
	CYP2E1						
	AHSG						
	VTN						Included in round 3 to have 2nd liver marker
Skeletal muscle	TNNC2						
	MYLPF						
	MYL1						
	TNNI2						
	MYH2						
	ATP2A1						
	NEB						
	MYH1						2 extra Ts to rv primer in round 2 for multiplex spacing; thyroid expr.
	TNNT1						
Heart	ACTC1						
	TNNI3						
	MYL7 amp1[g]						
	MYL7 amp2						Amplicon 2 dismissed after round 3 because of low sensitivity
	TNNT2						
	MYBPC3						Included in round 5 to have 2nd heart marker
General muscle	NMRK2						Initially included in round 3 for heart, found to mark general muscle
Kidney	UMOD						5 extra T residues to rv primer in round 4 for multiplex spacing
	ABP1						
	SLC13A3						
	PDZK1IP1						Not specific when assayed via CE in round 2
	FXYD2						Included in round 3 to have 2nd kidney marker
Skin	LOR						
	LCE1C						
Housekeeping	18S-rRNA						Final primer concentration lower than in [16] to prevent saturation
Blood	HBB						Co-expressed in several tissues
Number of markers		37	14	16	16	17	
Number of amplicons		37	16	18	16	17	

[a]Color coding: good performance | dismissed | not analysed

[b]1: Tissue-oriented small multiplexes & detection on agarose gel; 85 single source samples - see Supplementary Table 1

[c]2: 16-plex & detection by CE; selected set of single source samples; results not shown

[d]3: 18-plex & detection by CE; 85 single source samples (each two cDNA inputs); 20 human tissues (various RNA amounts); six common forensic samples (body fluids and touch DNA, four different donors each, one cDNA input); 20 blind samples including mixtures (each two cDNA inputs); for all analyses results not shown

[e]4: 16-plex & detection by CE; selected sample set; results not shown

[f]5: Final 17-plex & detection by CE; 48 single source samples (one cDNA input) - see Supplementary Table 2; 20 human tissues (various RNA amounts) - see Suppl. Table 3; six common forensic samples (body fluids and touch DNA, two different donors each, one cDNA input) - see Supplementary Table 4; 20 blind samples including mixtures (each four replicates) - see Table 4

[g]After round 1 only one tissue candidate remained. Two amplicons for different exons were assessed to increase amplification chance

of cDNA were used as inputs for the various RT-PCRs. To analyse picogram amounts of RNAs of the FirstChoice® Human Total RNA Survey Panel, cDNAs were diluted accordingly. For the various candidate markers that were assessed by agarose gel electrophoresis RT-PCR primer concentrations were 0.2 µM. Primer concentrations of the optimized multiplex are reported in Table 1. The RT-PCRs employed amplification settings as described in [16].

RNA profiling

DNA/RNA co-isolation was performed as described by Lindenbergh et al. [16]. Tissue debris that had not fully lysed after 2 h of incubation at 56 °C was discarded using a QIA shredder column. RNA extracts were DNase-treated [16], and copy DNA (cDNA) was synthesised [16] using 10 µL RNA extract. Variable amounts (up to 5 µL) of cDNA were used as inputs for the various RT-PCRs. To analyse picogram amounts of RNAs of the FirstChoice® Human Total RNA Survey Panel, cDNAs were diluted accordingly. For the various candidate markers that were assessed by agarose gel electrophoresis RT-PCR primer concentrations were 0.2 µM. Primer concentrations of the optimized multiplex are reported in Table 1. The RT-PCRs employed amplification settings as described in [16].

When a novel candidate marker was examined, first the length of the amplified cDNA fragment was confirmed using an appropriate RNA extract. In all cases the observed fragment lengths approximated the designed sizes. Minus RT reactions were included in all analyses (*i.e.* both when assessing results on agarose gels and when performing CE analysis) and did not show signals.

When RT-PCR products were assessed by agarose gel electrophoresis, 10 µL PCR product were supplemented with 2 µL BlueJuice Loading Buffer (Life Technologies, Bleiswijk, The Netherlands) and separated on 4 % agarose gels (Multi Purpose, Roche, Almere, The Netherlands) in 1× TBE buffer (10×, Gibco, Invitrogen, Bleiswijk, The Netherlands) containing 0.5 µg/ml Ethidium Bromide (Invitrogen, Bleiswijk, The Netherlands). Fragments were separated for approximately 60 min at 80 V (PowerPac Basic™, BioRad, Ede, The Netherlands), visualised using UV-light (Chemi Genius2, Syngene, Cambridge, United Kingdom) and analysed using GeneSnap Software (Syngene, Cambridge, United Kingdom). When the RT-PCR products were analysed by CE, purified PCR products [16] were separated using POP-7 as separation matrix. In case of overloaded profiles, RT-PCR products were diluted 100- or 20-fold in water and re-injected for CE. GeneMapper ID-X version 1.1.1 (AB) was used to analyse the RNA profiles with 150 rfu as detection threshold.

RNA profile interpretation

RNA extracted from neat blood was used to show functional methodology for cDNA synthesis and PCR amplification when multiplex amplification and CE analysis is performed, as one blood marker resides in the multiplex. The 18S-rRNA marker in the multiplex has the role to indicate the presence of RNA and absence of factors inhibiting cDNA synthesis or PCR, which is especially informative when none of the other markers give a signal. Since 18S-rRNA signals can vary in strength for several reasons (*e.g.* differences in the proportion mRNA/rRNA isolated from a sample, relatively low or high sensitivity of organ-specific markers compared to the 18S-rRNA marker and stacking of 18S-rRNA signals in case of mixed samples), presence of the 18S-rRNA signal in a profile is not absolute, and thus, profiles lacking the 18S-rRNA signal can be considered for data interpretation.

DNA profiling

DNA was quantified using the highly sensitive *Alu* repeat system that provides accurate measurements down to 0.5 pg/µL, according to the protocol of Nicklas et al. [24] with minor adjustments to the primer concentrations as 200 nM *Alu* forward primer and DYZ5 reverse primer were used. DNA profiling was performed using the AmpFℓSTR® NGM™ PCR Amplification Kit (Applied Biosystems (AB), Foster City, Texas, USA). Where possible, 500 pg of genomic DNA (gDNA) was used as input. Amplification products were separated using CE on a 3130XL genetic analyser (AB) with POP-4 (AB) as separation matrix as earlier described [16]. Analysis of DNA profiles was performed with GeneMapper ID-X version 1.1.1 (AB) using 50 relative fluorescence units (rfu) as detection threshold [16].

Results and discussion

Selection of candidate markers

Candidate markers for organ typing were retrieved by a BIOGPS [21] search and included six candidates for brain, six for lung, six for liver, nine for skeletal muscle, four for heart and four for kidney (genes are depicted in Table 2, column first selection round). Additionally, two reported skin markers (LOR [16, 25] and LCE1C [13]) were included in the candidate set, resulting in a total of 37 genes to test.

Test material consisted of 36 excised autopsy tissues and 49 frozen tissue sections. These were subjected to RNA/DNA co-isolation [16]. DNA profiling learned that the quality of the autopsy samples was less than that of the frozen sections as 13 out of 36 autopsy samples resulted in partial DNA profiles while all of the 49 sections resulted in

full profiles (Table 3). The partial DNA profiles had a "skislope" appearance typical for degraded DNA and occurred mainly for lung, liver, heart and kidney samples.

For the mRNA marker candidates, tissue-orientated multiplexes were prepared containing all markers for each of the seven tissue types. The markers within each of these multiplexes had uniquely sized amplicons (between 58 to 175 nucleotides), which allowed assessment of their individual performance. Specificity of the candidate markers was determined by testing all 85 tissues with all seven tissue orientated multiplexes, which resulted in 595 amplifications. When visualising the PCR products on agarose gel, 14 markers appeared specific. These represent one to four markers for each of the organ tissues (Table 2, details in Supplementary Table 1).

To befit the standard forensic procedures, we aimed for an end-point multiplex RT-PCR assay and analysis of the fluorescently labelled products by CE. The process of developing such a multiplex included various selection rounds as summarised in Table 2. Prior to round 3, new marker candidates were retrieved from BIOGPS for the three organ types (heart, liver and kidney) for which only one marker remained after selection round 2. Finally, two apparently specific markers were obtained for each of the seven tissues (Table 2). These 14 tissue-specific markers were supplemented with one general muscle marker recognising both skeletal and heart muscle, one blood marker (HBB [16]) and one housekeeping marker (18S-rRNA [16]). This created a 17-plex that underwent optimisation for primer concentrations and marker spacing.

Table 3 Results of DNA analyses of the excised autopsy samples and frozen tissue sections.

Type of sampling	*n*	Full DNA profile	Partial DNA profile	Average DNA concentration (ng/µL)	Range of DNA concentrations (ng/µL)
Autopsy					
Frontal lobe	4	4	0	40.99	2.82 – 66.54
Occipital lobe	4	4	0	25.71	1.79 – 44.87
Cerebellum	4	3	1	38.14	2.46 – 76.50
Lung	3	0	3	2.85	1.41 – 4.03
Liver	3	0	3	0.77	0.54 – 1.03
Skeletal muscle	4	4	0	6.61	0.10 – 13.58
Heart	3	0	3	2.19	0.66 – 3.20
Kidney	3	0	3	2.08	0.39 – 5.00
Skin, hand palm	4	4	0	29.33	10.08 – 58.92
Skin, fingertip	4	4	0	33.14	27.38 – 41.76
Frozen sections					
Brain	7	7	0	15.23	5.57 – 31.42
Lung	7	7	0	67.18	6.71 – 269.09
Liver	7	7	0	3.82	2.52 – 5.15
Skeletal muscle	7	7	0	3.62	1.96 – 7.07
Heart	7	7	0	5.41	2.48 – 9.55
Kidney	7	7	0	13.77	5.20 – 30.65
Skin	7	7	0	18.47	1.81 – 49.53

Specificity and sensitivity of the organ-typing 17-plex regarding human tissues

The organ-typing 17-plex was subjected to performance testing. First, 48 frozen tissue specimens were analysed. Signals for the correct tissue-specific markers were obtained for all 48 samples (Supplementary Table 2). In some samples only one of the tissue-specific markers responded. These samples involve tissues for which generally one of the markers gives lower signals (Figure 1A). Non-specific signals of relatively low heights were sporadically observed (Supplementary Table 2). Co-expression of blood was seen for brain, liver, skeletal muscle and heart. Typical electropherograms (epgs) are shown in Figure 1A. In these epgs, peak heights vary not only for the tissue-specific markers, but also for the 18S-rRNA marker, which does not necessarily reflect a low expression of 18S-rRNA for certain tissues; some tissue-specific markers give relatively high peaks, due to which lower inputs were used for the profiles presented in Figure 1A. In those cases, the 18S-rRNA signal is detected in profiles with higher inputs. Thus, we do not regard 18S-rRNA a strict positive control required to give a signal in each analysed profile. Nevertheless, 18S-rRNA is very useful to infer whether RNA/cDNA is present, which is especially important when no other markers give peaks.

Next, RNAs included in the FirstChoice® Human Total RNA Survey Panel were analysed with the 17-plex. This panel contains 20 different human tissues including six of the tissues (brain, lung, liver, skeletal muscle, heart and kidney) targeted by the multiplex. Since these RNAs are of excellent quality and have a fixed RNA concentration, they were used to determine the sensitivity of the multiplex. For informative profiles, picogram amounts of RNA in the range of 50, 10 and 2 pg sufficed; nanogram amounts gave strong over-amplification and artefacts. The brain, liver and kidney markers required 2 pg in the multiplex PCR to present full RNA profiles, while the lung, skeletal muscle and heart markers needed 10 pg. The housekeeping marker required 50 pg RNA for most tissues (Supplementary Table 3). Blood is detected for only some of the tissues. Cross-reactions are evident for three markers: lung marker SFTPB and skeletal muscle markers TNNI2 and MYH1 all show signals for the thyroid (Supplementary Table 3). Thyroid expression of these latter two markers is reported in BIOGPS [21] albeit to lower level than in skeletal muscle.

Performance of the organ-typing 17-plex on body fluids

The performance of the organ-typing 17-plex was also assessed for cell types generally encountered in forensic context: blood, saliva, semen (from fertile and sterile donors), menstrual secretion, vaginal mucosa and skin (in the form of touched cotton patches). For each cell type, two independent samples were analysed. The organ-typing 17-plex contains one blood marker (HBB) that should respond in blood and menstrual secretion samples and two skin markers (LCE1C and LOR) expected to give signals in touch samplings. Strong signals for HBB and LCE1C were observed for the expected

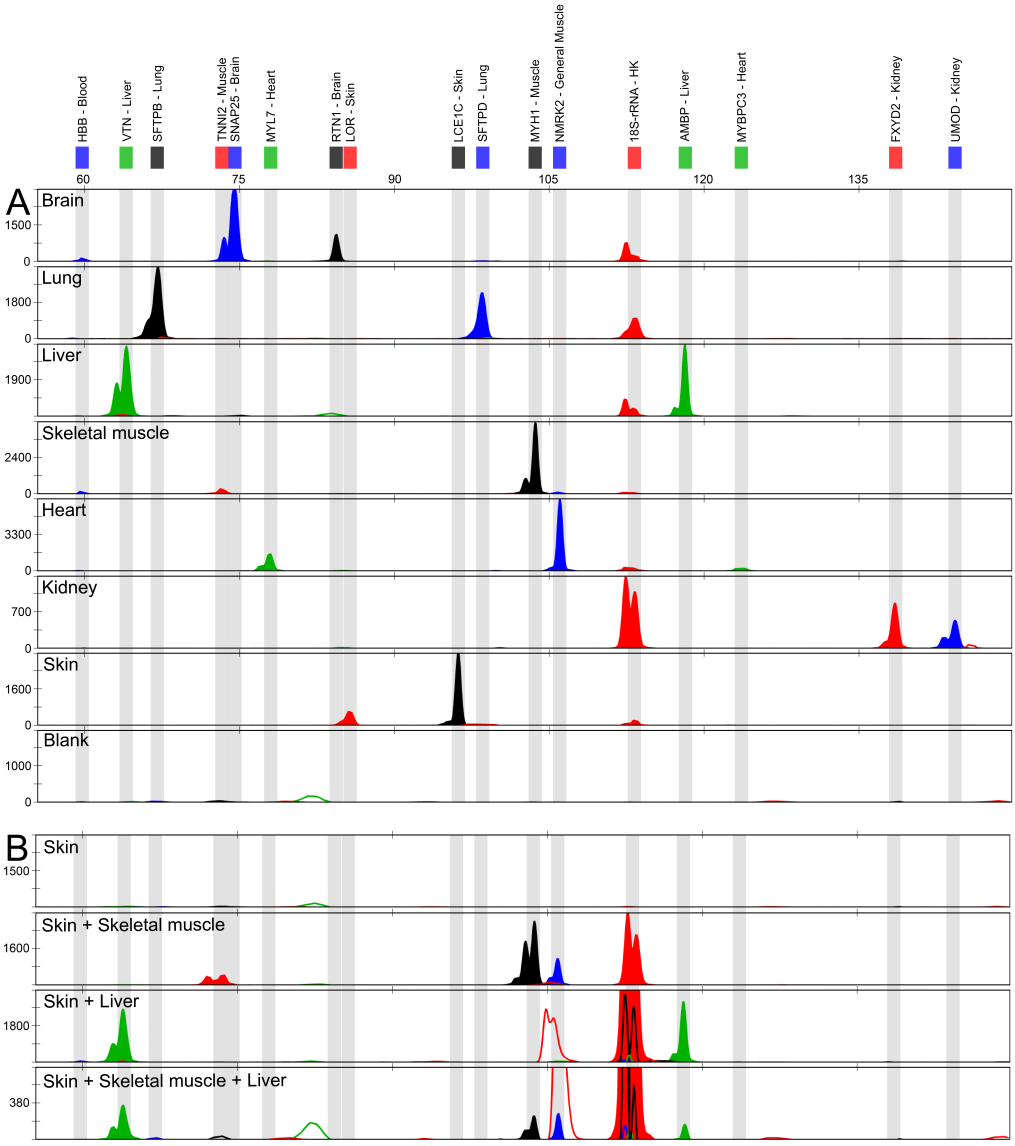

Figure 1. Typical overlay electropherograms as produced by the optimised 17-plex. A: Examples of the single source samples brain, lung, liver, skeletal muscle, heart, kidney and skin. Some tissue-specific markers generally give lower signals than their counterparts: AMBP in liver, TNNI2 in skeletal muscle, MYBPC3 in heart and LOR in skin. B: Results of mock stabbing experiments. A,B: Not filled in peaks correspond to either dye blobs which could not be removed by purification (*e.g.* at 85 nt), or artefact peaks due to over-amplification. In blanks and minus RT reactions no marker expression was observed.

sample types (Supplementary Table 4). LOR amplification failed, which may be due to the low cell content in touch specimens [16]. Additional non-specific signals (Supplementary Table 4) were sporadic and of relatively low height and we expect that these signals will not be reproduced upon replicate RNA analyses [1]. Muscle marker TNNI2 is reported to give expression in tongue [21], but apparently this does not produce TNNI2 signals in saliva.

Performance of the organ-typing 17-plex on a blind test set

A blind test set of 20 samples prepared from autopsy material was examined. Since multiple tissues may be hit upon violent actions, mixtures were included in this set. In [1] we introduced an mRNA interpretation strategy based on two components: (1) replicate RNA analyses (preferably four) and (2) interpretation rules that categorise the results as 'observed', 'sporadically observed' or 'not observed'. Scoring is based on a 'x=n/2' rule in which 'x' is the number of found and 'n' the number of theoretically possible peaks for a tissue. A tissue is scored 'observed' if $x=n/2$, 'not observed' if $x=0$ and 'sporadically observed' if $0<x<n/2$. During case interpretation, tissues scored as 'sporadically observed' are effectively regarded as 'not observed'; the separate category is used to translate all signals to the scorings. When next to other observed tissues, a score 'observed' is obtained for blood; the presence of blood is not regarded as a contribution of peripheral blood since blood may coincide in the organ tissues. This interpretation strategy was applied to the blind test set RNA data.

The scores derived from four replicate RNA profiles are presented in Table 4. In the 20 samples a total number of 49 tissues are present; 39 of these are scored 'observed', six 'sporadically observed' and four 'not observed'. For 12 samples all input tissues are considered 'observed' (Table 4). The lung, liver, heart and kidney material that was used to create this blind test set, had presented partial, degraded DNA profiles due to the condition of the cadavers from which they were excised (Table 3). Nevertheless, full RNA profiles and scorings 'observed' were obtained most times these tissues resided in the blind test set (results not shown and Table 4). Skin, heart and skeletal muscle appear most prone to marker drop out (Table 4). The skin markers are less effectively detected in the excised skin specimens than in touch specimens (Supplementary Table 4), especially when considering the DNA yields that are much higher for the excised skin specimens (Table 3) [16]. Presumably, LCE1C and LOR are predominantly expressed in upper epidermal layers that represent only a fraction of the excised skin specimens, as these comprise the epidermal, dermal and hypodermal layers plus adipose tissue. For both heart and skeletal muscle, marker drop out occurs especially for one of the two markers (MYBPC3 and TNNI2 respectively), which is in agreement with the observation that these markers required 10 pg and not 2 pg RNA to give signals in the sensitivity test (Supplementary Table 3).

Table 4 Results for the blind test set which includes mixed samples. Four RNA profiles were generated for each sample. Indicated are the number and type of tissues present in a sample ('present', marked by black cell;'-' means not present) and the scoring results using the interpretation guidelines described in [1]. The color coding is used to present the scoring results as indicated below the table.

Sample	# Tissues present[b]	Neat blood		Brain		Lung		Liver		Skeletal muscle[a]		Heart[a]		Kidney		Skin	
		present	result[c]	present	result	present	result	present	result	present	result	present	result	present	result	present	result
1	1	-		-				-		-		-		-		-	
2	1	-		-		-		-		-		-		-			
3	1	-		-		-				-		-		-		-	
4	1			-		-		-		-		-		-		-	
5	2	-		-		-				-				-		-	
6	2	-				-		-		-		-		-			
7	2	-				-		-				-		-		-	
8	2	-		-		-		-				-		-			
9	2	-		-				-				-		-		-	
10	2	-		-		-				-		-				-	
11	2	-		-				-		-		-				-	
12	2	-		-		-		-						-		-	
13	2	-		-				-		-				-		-	
14	3	-		-				-		-						-	
15	3	-		-				-				-		-			
16	3	-				-				-				-		-	
17	3	-		-						-		-				-	
18	4	-		-				-						-			
19	4	-		-						-						-	
20	7	-															

[a] General muscle marker NMRK2 not included during interpretation.

[b] Samples are ordered by number of tissues present; during analysis the samples were randomized.

[c] Blood may coincide in organ tissues; positive scores are colored green.

[d] No absent tissues were scored as observed.

	Present	Absent
Observed		none[d]
Sporadically observed		
Not observed		

Importantly, no false positive identifications, *id est* scores 'observed' for tissues not present in a sample, have occurred for this blind test set; the peaks seen for tissues not present in the samples all result in a score 'sporadically observed' which is effectively regarded 'not observed'. Blood was scored 'observed' both in the sample containing neat blood and for six samples carrying mixtures of organ tissues. The 18S-rRNA marker gave a signal in all the 80 RNA profiles. Thus, the multiplex allows assessment of mixed samples, and organ-typing results can be interpreted using the approach described in [1].

Performance of the organ-typing 17-plex on mock stabbings

To assess performance of the organ-typing multiplex in a mock violent case, an abdominal stabbing incident was mimicked. When analysing cell material present on the scalpel knife used to stab a stacking of skin, skeletal muscle and liver, signals for both liver and skeletal muscle were retrieved (Figure 1B). This means that also the lowest tissue (liver) is detected. The signal for 18S-rRNA is overloaded due to the additive effect from the multiple tissues. This hampers the use of a higher cDNA input in the 17-plex that may have facilitated improved detection of tissue-specific markers that generally give lower peaks (Figure 1A) such as TNNI2 that shows drop out for the triple layer stabbing. Skin was not detected which is probably a consequence of the vertical cut due to which hardly cells from the upper epidermal layer (where the skin markers are thought to be expressed) reside on the knife (Figure 1B). The control samples (stabbings of skin only, liver covered with skin and skeletal muscle covered with skin) neither show signals for skin, while skeletal muscle and liver were detected. This experiment shows that cells from the lower tissues remain present on the scalpel after withdrawal through the upper layers including skin.

Concluding remarks

Here, we created an end-point multiplex RT-PCR assay to infer the presence of brain, lung, liver, skeletal muscle, heart, kidney and skin. Performance tests using this organ-typing 17-plex showed high specificity and sensitivity, and the ability to identify different sources in case of mixed samples. Non-specific signals were only evident for the thyroid (from both skeletal muscle markers and one lung marker). However, not all bodily tissues were tested. Nevertheless, we do not anticipate many more non-specific signals as only very few non-tested tissues are reported in BIOGPS to give expression for the selected markers (Table 1). For degraded samples that presented partial DNA profiles, full RNA profiles were obtained which may be due to the small sizes of the amplicons. The RNA profiles generated by the 17-plex can show peak

imbalance for the different markers (Figure 1). Also, RNA profiles in general show peak height variation due to differences in gene expression for distinct donors and with changes in physiological condition. Furthermore, variation between RNA replicates occurs [26]. This variation was also observed with the organ-typing multiplex described here. Therefore, we have recommended to perform multiple RNA analyses and use interpretation guidelines as described in [1]. This strategy was successfully applied to a blind test set of 20 samples including mixtures. In conclusion, a novel tool for forensic analysis of samples from violent crimes is presented.

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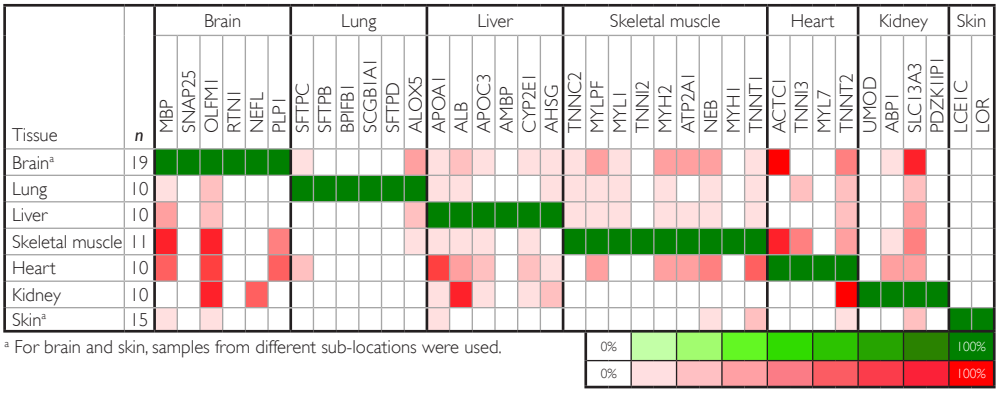
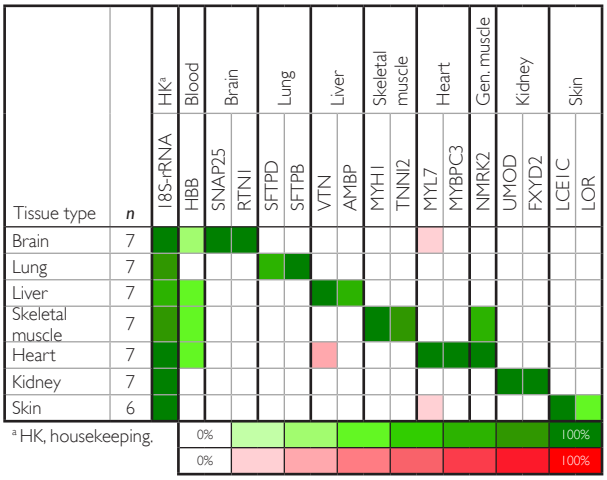
Supplementary material

Supplementary Table 1 Results of specificity tests for 37 putative organ-specific markers using 85 single sources samples. Tissue-oriented multiplexes are used and results are analysed by agarose gel electrophoresis. Darker color tones correspond to increasing percentages of positive signals. Green cells indicate signals corresponding to the target tissue, red cells signals for non-target tissues. A minimum number (*n*) of 10 donors was analysed for each tissue type.



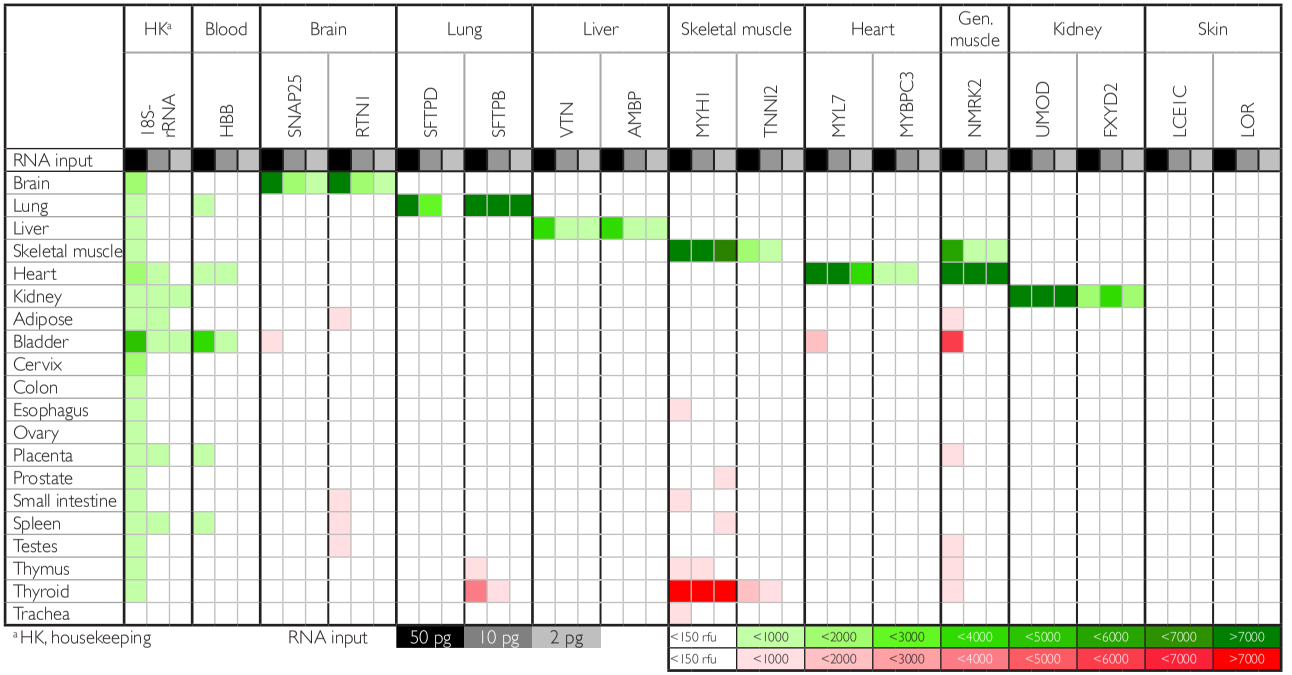
[a] For brain and skin, samples from different sub-locations were used.

Supplementary Table 2 Results of specificity tests for the markers in the organ-typing 17-plex using 48 frozen tissue sections. The number (*n*) of samples per tissue is indicated. The percentage of samples in which marker expression was observed is indicated as a color scale in which darker tones represent a higher percentage. Green cells indicate signals corresponding to target tissue; red cells signals for non-target tissues.

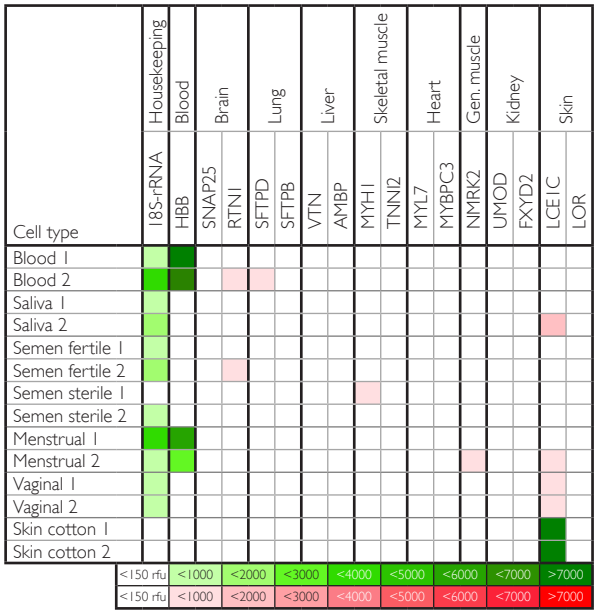


[a] HK, housekeeping.

Supplementary Table 3 Specificity and sensitivity results of the organ typing 17-plex tested on 20 different tissues of the FirstChoice® Human Total RNA Survey Panel. RNA inputs in the RT-PCR were 50, 10 or 2 pg (indicated by grey scale). Color marks indicate tissues for which signals for a marker are observed. Darker color tones indicate higher peak heights (rfu; see color scale below table). Green cells indicate signals corresponding to the target tissue, red cells signals for non-target tissues. Skin samples are not represented in the panel.



Supplementary Table 4 Results for the specificity of the markers in the organ typing 17-plex when analysing blood, saliva, semen (fertile/sterile), menstrual secretion, vaginal mucosa and touched cotton patches (two specimens of different donors each). Darker color tones indicate higher peak heights (rfu). Green cells indicate specific signals, red cells mark non-specific signals.



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