STAIN IDENTIFICATION

The Use of Real-Time PCR for Forensic Stain Identification

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INTRODUCTION

With innovative molecular biological techniques becoming the norm in the forensic laboratory, it is plausible that traditional serological testing methods used to identify stains will be replaced with molecular biological techniques. Traditional serological approaches for stain identification often involve a presumptive color test, followed by a confirmatory test that typically uses a specific antibody that complexes with a known protein, such as hemoglobin for human blood and P30 or prostatic antigen for seminal fluid. While these testing methods have improved in simplicity and selectivity over the years, several problems still exist, such as the possibility of cross-reactivity with other species and the lack of specificity for particular fluids.

New tests that are fluid-specific and can be multiplexed would yield rapid results with a minimal amount of sample. Such testing could employ mRNA as the specific determinant. While the DNA of all tissues from an individual is essentially identical, the mRNA spectrum made by the different cells in each tissue is very diverse. Each tissue or cell type makes a unique constellation of mRNAs, some specific for only that tissue or cell type. Some body fluids, such as blood, contain cells as part of their function, while other fluids, such as urine, contain cells that have been shed from their tissue of origin. Therefore, analysis of the "RNA profile" in a sample may uniquely identify the fluid of origin.

The nature of this research is to identify mRNA transcripts that will distinguish the fluid of origin, determine if such transcripts survive the typical environmental insults that forensic samples may encounter, and develop rapid multiplex assays to assess these molecules using small amounts of sample.

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BLOOD AND SEMEN STAIN IDENTIFICATION USING TAQMAN®-BASED REAL-TIME PCR ASSAYS

We performed a number of experiments to demonstrate the feasibility of stain identification using mRNA analysis. These included experiments to assess the specificity and sensitivity of real-time PCR assays, as well as the stability of mRNA over time. Using various blood- and seminal fluid-specific gene targets, we performed real-time analyses on blood and semen using TaqMan® gene expression assays from Applied Biosystems (CD3 antigen, gamma polypeptide [CD3G], hemoglobin beta [HBB], beta spectrin [SPTB], protamine 2 [PRM2], kallikrien 3 [PSA] and semenogelin 1 [SEMG1]).

Initially we tested the TaqMan® assay specificities following isolation of RNA from 10µl of dried blood, semen or saliva. Following reverse transcription, we performed real-time PCR with each of the TaqMan® sets. Table 1 demonstrates the specificity of the assays with the three fluids tested. Three blood-specific assays (CD3G, HBB, SPTB) yielded amplified products only from blood, the three seminal fluid-specific assays (PRM2, PSA, SEMG1) yielded amplified products only from semen, and no detectable products were obtained from saliva using the blood or seminal fluid assays.

Next, we determined assay sensitivities using a range of fluid volumes spotted and dried on cloth. Table 2 shows the dynamic range of the blood and seminal fluid

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assays. Blood stains as small as 0.0001µl were amplified with the HBB assay (data not shown), and amplification from 0.1µl of semen spotted onto cloth occurred with the SEMG1 and PRM2 assays.

Lastly, and most importantly, we performed experiments to demonstrate the stability of mRNA over time. When molecular biologists began isolating mRNA for experiments, it was thought that mRNA was ephemeral and samples needed to be processed rapidly. However, due to new techniques and the recent increase in knowledge, mRNA has been shown to be relatively stable. We amplified mRNA isolated from dried blood spots stored at room temperature for various times (1µl for 3–510 days and 10µl for 3–864 days) using the HBB, CD3G and SPTB assays. The older samples showed only a minor decrease in amplification (Table 3). We also analyzed mRNA isolated from dried semen stored at room temperature for various times (1µl for 16–807 days and 20µl for 16–791 days). These experiments showed that the assays tested (PRM2, PSA and SEMG1) produced amplifiable mRNA from stains stored over a period of 26 months (Table 3).

Table 1. Specificities of Blood, Semen and Saliva Real-Time PCR, TaqMan®-Based Assays.

Stain	Assay	C_t Value
10µl blood	CD3G	29.04
10µl semen	CD3G	ND
10µl saliva	CD3G	ND
10µl blood	HBB	17.27
10µl semen	HBB	ND
10µl saliva	HBB	ND
10µl blood	SPTB	31.50
10µl semen	SPTB	ND
10µl saliva	SPTB	ND
10µl blood	PRM2	ND
10µl semen	PRM2	27.54
10µl saliva	PRM2	ND
10µl blood	PSA	ND
10µ semen	PSA	37.98
10µl saliva	PSA	ND
10µl blood	SEMG1	ND
10µl semen	SEMG1	33.34
10µl saliva	SEMG1	ND

ND = Not detected

These experiments indicate that biological stains can be identified by determining which mRNAs they contain. The genes tested thus far show initial fluid specificity. mRNAs extracted from dried stains ranging from 3 days to 3 years of age have been successfully amplified, demonstrating the stability of mRNA over time.

BLOOD AND SEMEN STAIN IDENTIFICATION USING PLEXOR™-BASED REAL-TIME PCR ASSAYS

Once mRNAs that clearly define specific types of stains are determined, a major goal of stain identification using mRNA profiling is multiplexing real-time PCR assays. One methodology to achieve this goal is the Plexor™ qRT-PCR System[a–c] from Promega. Depending on the dye capability of the real-time instrument used, this system allows multiplexing of up to six mRNAs in one assay, thus reducing the amount of sample needed and time required for analysis. The Plexor™ qRT-PCR System takes advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis (A Plexor™ System animation is available at: www.promega.com/paguide/animation/selector.htm?coreName=plexor01).

Our focus was to design a blood-semen standalone assay, since our studies identified mRNAs that are specific for these fluids. We designed Plexor™ primers to simultaneously detect HBB, PSA and PRM2 to identify whether blood or semen is present in a stain. In a multiplex reaction we demonstrated that amplification of fluid-specific genes only occurred with the expected fluid; blood was amplified with HBB primers, whereas only semen was amplified with PSA and PRM2 primers (Table 4). A mixture of blood and semen RNA extracts showed that these primer sets were able to discriminate their target RNA in a heterogeneous sample. We believe the C_t values in the no-template control and no-RT reactions represent nonspecific amplification due to primer interactions or minor amplification from DNA contamination. However, the major difference between the negative controls and the samples (>6 C_t) shows the promise of the assay to clearly define stain types in a multiplex.

Table 2. Sensitivities of Blood and Semen Real-Time PCR, TaqMan®-Based Assays.

Stain	Assay	C_t Value
0.1µl blood	CD3G	ND
1µl blood	CD3G	33.41
5µl blood	CD3G	29.63
10µl blood	CD3G	28.69
20µl blood	CD3G	28.62
0.1µl blood	HBB	24.31
1µl blood	HBB	21.6
5µl blood	HBB	19.08
10µl blood	HBB	16.67
20µl blood	HBB	15.84
0.1µl blood	SPTB	ND
1µl blood	SPTB	35.00
5µl blood	SPTB	34.35
10µl blood	SPTB	31.56
20µl blood	SPTB	31.24
0.1µl semen	PRM2	33.12
1µl semen	PRM2	29.42
5µl semen	PRM2	29.09
10µl semen	PRM2	27.68
20µl semen	PRM2	27.83
0.1µl semen	PSA	ND
1µl semen	PSA	32.85
5µl semen	PSA	35.53
10µl semen	PSA	37.68
20µl semen	PSA	39.15
0.1µl semen	SEMG1	34.31
1µl semen	SEMG1	32.97
5µl semen	SEMG1	35.76
10µl semen	SEMG1	34.07
20µl semen	SEMG1	32.43

ND = Not detected

The success of the blood-semen assay using the Plexor™ platform led to experiments to develop a quick, one-tube seminal fluid assay because seminal fluid analysis is a major task for all forensic laboratories. For semen, we are interested in genes specific for sperm and prostatic components. We

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Table 3. Aged Blood and Semen in Real-Time PCR, TaqMan®-Based Assays.

Stain	Age (days)	CD3G C_t Value	HBB C_t Value	SPTB C_t Value
1µl blood	3	31.97	19.94	–
1µl blood	23	33.40	19.99	36.19
1µl blood	461	–	23.30	–
1µl blood	496	35.04	23.52	–
1µl blood	510	33.90	23.15	36.34
10µl blood	3	29.34	17.68	32.01
10µl blood	420	31.65	19.94	33.69
10µl blood	490	31.53	20.27	34.07
10µl blood	855	32.73	20.15	34.51
10µl blood	864	32.12	20.51	33.21

Stain	Age (days)	PRM2 C_t Value	PSA C_t Value	SEMG1 C_t Value
1µl semen	16	34.61	36.78	34.60
1µl semen	461	30.35	33.51	34.03
1µl semen	507	27.41	34.58	33.48
1µl semen	699	30.95	35.46	34.59
1µl semen	807	36.90	36.13	38.69
20µl semen	16	27.92	ND	33.61
20µl semen	507	20.10	28.04	27.53
20µl semen	607	22.19	26.00	27.50
20µl semen	777	21.34	30.89	30.49
20µl semen	791	22.94	31.38	31.56

ND = Not detected

Table 4. Blood and Semen Detected by Real-Time PCR, Plexor™-Based Multiplex Assay.

Sample	HBB (FAM)	PSA (HEX)	PRM2 (ROX)
NTC	ND	ND	31.4
Blood (no RT)	ND	ND	30.2
Semen (no RT)	30.3	32.0	32.0
1:1 mixture (No RT)	31.5	ND	33.2
Blood	**18.5**	ND	30.6
Semen	29.7	**25.3**	**21.4**
1:1 mixture	**20.3**	**26.1**	**21.5**

ND = Not detected
NTC = No-template control
RT = Reverse transcription

have performed preliminary work using the Plexor™ System with our current real-time instrumentation and believe we will be able to offer a real-time PCR seminal fluid assay to the forensic community. An assay that determines whether the stain 1) is seminal fluid and 2) contains sperm could alleviate the need to perform extensive microscopy to identify sperm.

THE FUTURE OF RNA IN STAIN IDENTIFICATION

As demand for sample analysis increases, forensic laboratories continue to balance manpower and cost issues with the value of analysis when evaluating new techniques. Laboratories investing in RNA technology will require a straightforward extraction procedure. Furthermore, a crucial prerequisite for these analyses is the development of a DNA/RNA co-extraction method to minimize sample requirements and eliminate the need for two separate extractions. A number of methods for simultaneous isolation of DNA and RNA have been reported; however, most of these are not optimal for dealing with the reduced quality of samples encountered in forensic casework. Alternatively, co-isolation reports using forensically relevant samples require numerous time-consuming steps. A preferred co-extraction method would provide a high yield and amplification efficiency, be simple and efficient, and involve nonhazardous reagents.

The purpose of RNA-based stain identification research is to develop a simple extraction and analysis method that allows quick, unequivocal identification of biological stains. With these proposed assays, a single stain will result in amplification of only the mRNA corresponding to its fluid type; a mixture, however, should amplify the representative mRNAs for the various fluids. The assays will function more qualitatively than quantitatively, so although they may indicate which fluids are present, they may not give the exact ratio of each fluid present. It is of greater concern to know what kind of mixture exists than to know the exact amount of each fluid present. Furthermore, a major advantage of these assays is that a single test will be used to classify the sample as blood, semen, vaginal secretions, etc., drastically reducing the number of identification analyses performed prior to DNA profiling (if required).

CONCLUSIONS

The implementation of DNA analysis in the forensic laboratory has been a tremendous benefit to the criminal justice community. However, as technologies progress, there are more cases and more items per case requiring DNA analysis, and unfortunately, the manpower and resources have not kept up with demand. Research into stain identification using real-time PCR seeks to find faster and more efficient methods to work DNA cases. The goal is to develop methods that broaden real-time PCR applications and investigate new technologies that could radically change the analysis of biological stains. The biochemical approaches currently used in fluid identification are limited in scope and often imply, but not truly identify, the fluid source. The evaluation of mRNA through real-time PCR is a technique that can offer a level of confidence and expand our knowledge of the materials we routinely examine. This research into new technologies will demonstrate the power of multiplexing for forensic analysis.