Development of an RNA-based screening assay for forensic stain identification

[1]Trisha L. Noreault-Conti, Ph.D. and [1]Eric Buel, Ph.D.
[1]Vermont Forensic Laboratory, Department of Public Safety, 103 South Main Street, Waterbury, VT 05671

Corresponding Author:

Trisha L. Noreault-Conti Ph.D.
Vermont Forensic Laboratory
103 South Main Street
Waterbury, VT 05671
TEL: 802-241-5436
FAX: 802-241-5557
E-mail: tconti@dps.state.vt.us

INTRODUCTION

In an age of countless scientific advances in molecular biology, DNA profiling has proven itself an invaluable tool in solving crimes. The potential exists, however, for the tissue origin of the suspect DNA to be called into question. For example, a semen stain containing suspect DNA can have far more serious consequences than a saliva stain. The collection of mRNAs made in any cell is unique to that cell type; thus, a differentiation could be made using mRNA as a fluid- or tissue-specific determinant. This report describes recent results using real-time PCR to identify semen and saliva. A major aim of this work is to multiplex real-time PCR assays once mRNAs are identified that clearly define specific types of stains.

Over the years, there have been countless advances made in the “back end” of biological evidence analysis. These include, but are not limited to: better DNA extraction techniques, automation using robotics, replacement of the slot blot with real-time PCR for quantitation of total (and often male) DNA, the use of a single amplification kits for the core CODIS loci, miniSTR kits for degraded DNA and expert systems for data review. Although the “front end” of sample processing has seen some changes in the formats and types of testing performed, there has not been the revolutionary change we have seen in the way the stain can be individualized with the molecular biological approaches used in DNA analysis. Major drawbacks of widely used front-end presumptive and confirmatory tests include consuming portions of the sample (in many cases which is limited), lack of confirmatory tests for saliva, limited capacity for automation and cross-reactivity with other fluids or species.

The nature of our research is to identify mRNA transcripts that will definitively identify the tissue of origin, determine if such transcripts survive the typical environmental insults that forensic samples may encounter, and to develop rapid multiplex assays to assess these molecules using small amounts of sample. One method that we are utilizing is the Plexor® System from Promega. This system allows the multiplexing of up to two RNAs in each dye channel, thus reducing the amount of sample needed and

time of analysis. Through collaboration with Promega, we set out to design a quick, one-tube seminal fluid-saliva screening assay, since analysis of these fluids is a major task for all forensic laboratories.

SEMEN AND SALIVA STAIN IDENTIFICATION

TaqMan®-based real-time PCR assays

A number of experiments have been performed to demonstrate the feasibility of stain identification using mRNA analysis. These included, but were not limited to, experiments to assess the specificity of real-time PCR assays. Using various fluid-specific genes, we have performed real-time analyses on semen and saliva using TaqMan® Gene Expression Assays from Applied Biosystems (kallikrien 3 [PSA], semenogelin 1 [SEMG1], microseminoprotein, beta- [MSMB], transglutaminase 4 [TGM4], protamine 2 [PRM2], statherin [STAT]). To test the TaqMan® sets on semen and saliva, RNA was isolated from dried blood, semen, saliva, menstrual blood, vaginal secretions, kidney, colon, adipose and skin. Control RNA from Ambion was also used for brain, heart, liver, kidney and intestine. Following reverse transcription, real-time PCR was performed with each of the TaqMan® sets. Table 1 demonstrates the specificity of the assays with reference to the tissues tested. The genes tested show varying degrees of fluid-specificity and provided a background from which further stain identification testing could be performed.

Plexor®-based real-time PCR assays

A major aim of stain identification using mRNA expression profiling is working towards multiplexing the real-time PCR assays once mRNAs are identified that clearly define specific types of stains. One methodology to achieve this goal is the Plexor® system from Promega. The number of assays performed in each reaction depends on the dye-capability of the real-time instrument that is utilized, thus reducing the amount of sample needed and time of analysis. The Plexor® qRT-PCR system takes advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis. (**http://www.promega.com/paguide/animation/selector.htm?coreName=plexor01**).

Our initial focus was to design a semen-saliva stand alone Stain ID assay, since our initial studies have identified mRNAs that are specific for these fluids. We have designed Plexor® primer sets to simultaneously detect one semen-specific (TGM4) and one saliva-specific (HTN3) mRNA in order to ascertain whether semen and/or saliva are present is a stain. Additionally, Plexor® primers sets for the housekeeper GAPDH are in the Stain ID assay. In a multiplex reaction we demonstrated that amplification of each fluid only occurred with the tissue-specific genes as expected; semen was amplified with TGM4 primers, whereas only saliva was amplified with HTN3 primers (Table 2). Additionally, artificial mixtures containing semen and saliva RNA extracts show that these primer sets are able to discriminate their target RNA in a heterogeneous sample. Experiments have shown that the Stain ID assay can identify down to 1 µl of aged seminal fluid (1 year) and saliva (3 years) – the lowest volumes tested to

date. Furthermore, there is no cross-reactivity with other commonly encountered bodily fluids such as blood, menstrual blood, vaginal secretions or urine. Therefore, at this point in time we have utilized three-dye technology to multiplex semen and saliva Plexor® primer sets into a single Stain ID assay.

DEVELOPMENTS IN NUCLEIC ACID EXTRACTION

A major obstacle which needs to be overcome in order for laboratories to invest in RNA technology is the development of a straight-forward extraction procedure. Furthermore, a crucial prerequisite to 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 method that could co-purify RNA and DNA from a single sample with a minimal number of steps would be attractive to those seeking new technologies. A number of methods describing the simultaneous isolation of DNA and RNA have been reported, however, most of these have not been optimized to deal with the reduced quality of samples encountered in forensic casework. Alternatively, the co-isolation reports using forensically relevant samples require numerous time-consuming steps that would not benefit a fast and simple stain identification assay.

A significant outcome of the Stain ID assay development was the generation of a co-isolation method for RNA and DNA extraction which produces a single extract in less than 90 minutes. A combination of the RNagents Total RNA Isolation System (Promega) with a Tris buffered phenol protocol was optimized for simultaneous extraction of the nucleic acids. The final extraction method was shown to extract sufficient quantities of quality RNA and DNA from 1 and 10 µl of semen and saliva demonstrating its utility as a dual extraction technique (Table 3). Although this method involves numerous hands-on steps, it is much faster than the TRIzol® method, and produces significantly better DNA yields. To date, it's the best co-isolation method we've tested in terms of yields and amplifiability. Importantly, the nucleic acids recovered through this extraction process are of sufficient quantity and quality for downstream analyses, such as real-time PCR and STR analysis (data not shown).

CONCLUSIONS

The biochemical approach currently used in tissue identification has undergone some changes in the past few years but essentially still relies upon the same technology where selective antibodies detect antigens to a particular source. These approaches have been simplified to save analyst time but are limited in scope; many laboratories limit tissue identifications to blood and seminal fluid only. Other tissue sources such as saliva or vaginal fluid are implied but not truly identified. We believe that the positive identification of these and other tissues can be performed in a quick and efficient manner which would allow analysts to provide a better service, more efficiently. Also with this technique, we believe tissues that are currently not routinely evaluated could be easily assessed by all laboratories so that an equality of testing could be realized across the country. Presently, some biochemical or "serological" tests are only performed in a select few laboratories. Through the development of a universal approach to tissue identification, one

could imagine a multitude of tests that could be carried out by anyone qualified to do any one of the tests. As such, a wide variety of tissues could be assessed and evaluated. As time progresses, the courts and the forensic community itself will demand tests that truly identify a tissue and allow for a better understanding of the material composing a STR pattern.

We believe that in the next few years a transition from a conventional biochemical approach to a molecular biological approach will replace routine tissue identification. Tests that are tissue specific and designed to be multiplexed could yield rapid results on minimal sample. Such testing could employ mRNA as the tissue-specific determinant. Research that moves this line of testing forward will be important to the forensic community and also to the criminal justice community in general. In conjunction with Promega, we believe we will be able to offer the first commercial RNA-based Stain ID kit for the detection of semen and saliva in forensic samples. Furthermore, the extraction procedure developed along with this assay co-isolates DNA and RNA, thus removing the requirement for separate extractions and allows DNA profiling from the exact stain in which RNA testing was performed.

TABLE 1 - Specificity of Semen, Sperm and Saliva Real-Time PCR TaqMan®-Based Assays

	SEMEN				SPERM	SALIVA	CONTROL
	PSA	SEMG1	MSMB	TGM4	PRM2	STAT	B2M
Blood	-	-	-	-	-	-	+
Semen	+	+	+	+	+	-	+
Saliva	-	-	-	-	-	+	+
Menstrual Blood	ND	-	-	-	-	ND	+
Vaginal Secretions	ND	+	-	-	-	ND	+
Kidney	-	-	-	-	-	ND	+
Colon	ND	-	-	-	-	ND	+
Adipose	ND	-	-	-	-	ND	+
Skin	ND	-	-	-	-	ND	+
Ambion Brain	ND	-	-	-	-	ND	+
Ambion Heart	ND	-	-	-	-	ND	+
Ambion Liver	ND	-	-	-	-	ND	+
Ambion Kidney	ND	-	-	-	-	ND	+
Ambion Intestine	ND	-	+	-	-	ND	+

ND = not determined

TABLE 2 - Analysis of Forensic Samples using the Plexor® Stain ID Assay

SAMPLE	SIZE	AGE (months)	HTN3 (FAM)	TGM4 (HEX)	GAPDH (ROX)
NTC	N/A	N/A	-	-	-
Positive Control	N/A	N/A	Yes	Yes	Yes
Blood	1ul	23	-	-	Yes
Blood	10ul	21	-	-	Yes
Blood/Saliva	5ul each	31	Yes	-	Yes
Blood/Semen	5ul each	28	-	Yes	Yes
Buccal (FTA)	5mm punch	27	-	-	Yes
Menstrual Blood	5x5mm cutting	27	-	-	Yes
Saliva	1ul	31	Yes	-	Yes
Saliva	10ul	29	Yes	-	Yes
Saliva/Semen	5ul each	31	Yes	Yes	Yes
Semen	1ul	7	-	Yes	Yes
Semen	10ul	7	-	Yes	Yes
Urine	1ul	31	-	-	No Call
Urine	10ul	31	-	-	Yes
Vaginal Secretions	1/4 swab	29	-	-	Yes
Kidney	10mg	17	-	-	Yes
Adipose	10mg	17	-	-	Yes
Colon	10mg	17	-	-	Yes
Skin	10mg	17	-	-	Yes
Neg Control	N/A	N/A	-	-	-

TABLE 3 - Results from Co-Extraction of DNA and RNA

SAMPLE	DNA (ng/ul)	HTN3 RNA (Ct)	TGM4 RNA (Ct)	GAPDH RNA (Ct)
10ul semen	17.3	ND	21.9	23.7
1ul semen	2.0	ND	25.0	25.0
10ul saliva	13.8	29.0	ND	29.0
1ul saliva	1.3	32.9	ND	32.4

ND = not detected