A NOVEL PAPER BASED TECHNOLOGY FOR BIOSAMPLE COLLECTION AND BIOBANKING

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Background

Biological samples, in particular whole blood have been field collected and stored in biobanks on filter paper cards for decades. Although the current filter paper technologies (Whatman FTA, 903) have been the media of choice for storing blood DNA, such dried blood spots (DBS) are resistant to full recovery and, more importantly, need to be purified before use, as the FTA chemicals inhibit protein and nucleic acid biochemistry. With DARPA support, GenTegra has developed a new class of chemically treated innovative paper, for stabilizing the DNA (GenSaver™ DNA cards), RNA (GenProtect™ RNA cards) and protein complement of whole blood. We have initiated DBS studies for whole blood and saliva to demonstrate DNA stabilization for >10yrs. In parallel, the HIV-1 and dengue virus (and host) complement of whole blood have been studied for RNA virus stabilization.

Section 1

Method for DNA stabilization in biological samples on Ahlstrom's GenSaver cards

Whole blood stabilization on GenSaver and GenSaver Color DNA cards

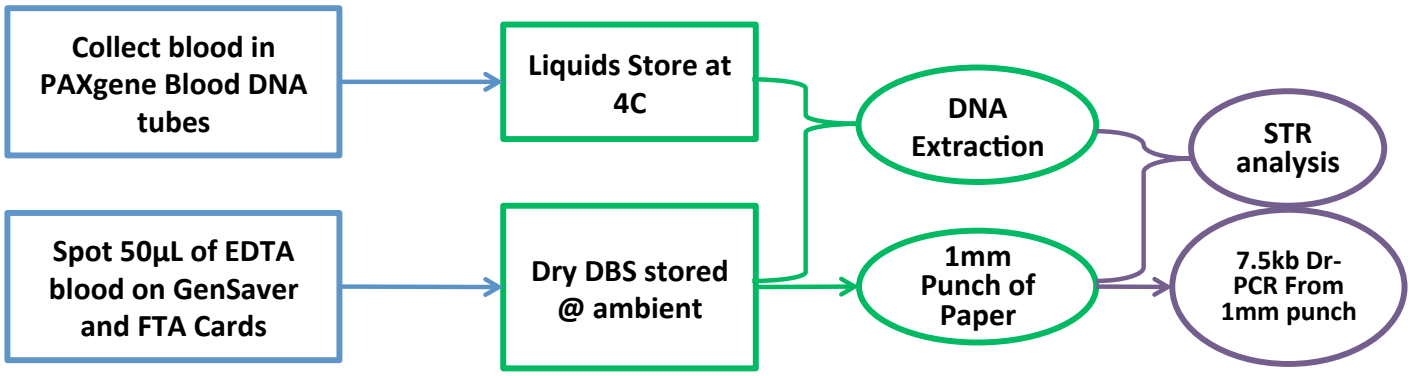
Whole blood in PAXgene® Blood DNA tubes and EDTA tubes was collected from one male and one female donor. DNA extracted from PAXgene Blood DNA tube samples using the manufacturer's suggested protocol was used as the reference sample.

50µL of Whole blood collected in EDTA tubes was spotted on the GenSaver Cards and placed at room temperature for 2 weeks.

The DBS from the GenSaver and FTA® cards were processed with the GenSolve Complete DNA kit (catalog #GSC-100). Briefly, three 6mm discs were punched out of the DBS and placed in 609µL of lysis buffer. 11µL of Proteinase K was added to the lysis solution containing the blood spots and vortexed at 56C for 1 hour. 500 µL of 100% ethanol was added to the filtrate and applied to the DNA filter column. The column was washed with 500µL each of Wash 1 and Wash 2 solutions. The DNA was eluted with 100µL of elution buffer. The amount of DNA isolated was quantified by adding 2µL of the extracted DNA to 198µL of Qubit™ HS DNA working solution and reading the fluorescence in the Qubit meter.

Ahlstrom's GenSaver™ Card

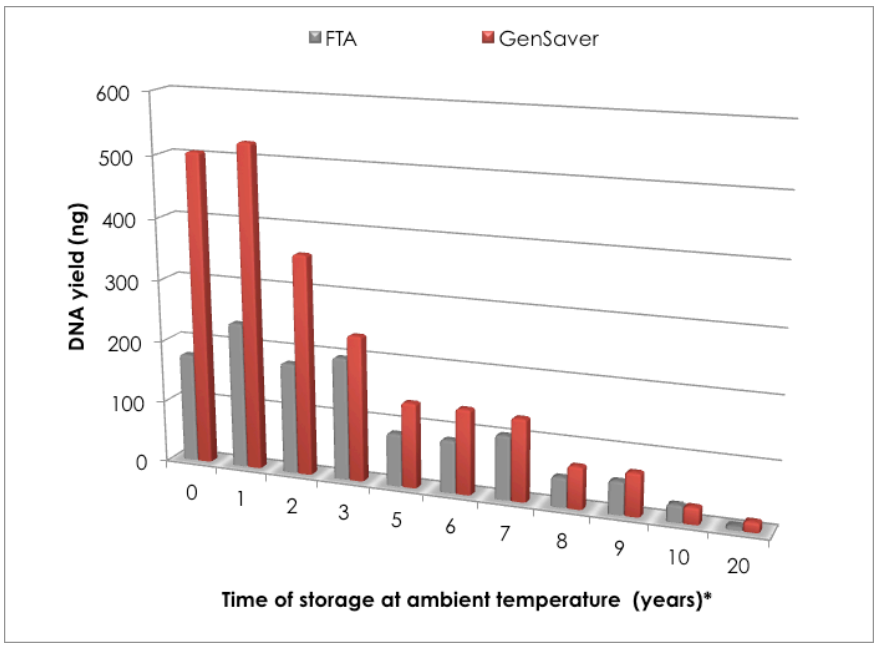
DNA Stabilization



Results for GenSaver

DNA quantity from Whole Blood DBS stored the equivalent of up to 20 years

DNA quantity was determined by excising the entire 50µL spot of DBS from GenSaver and FTA paper and the product quantified by qPCR assay.



GenSaver cards yielded good quality DNA from whole blood DBS samples even after 20 years of storage at ambient (*accelerated ageing study)

DNA yield comparison from PAXgene blood DNA to GenSaver DBS

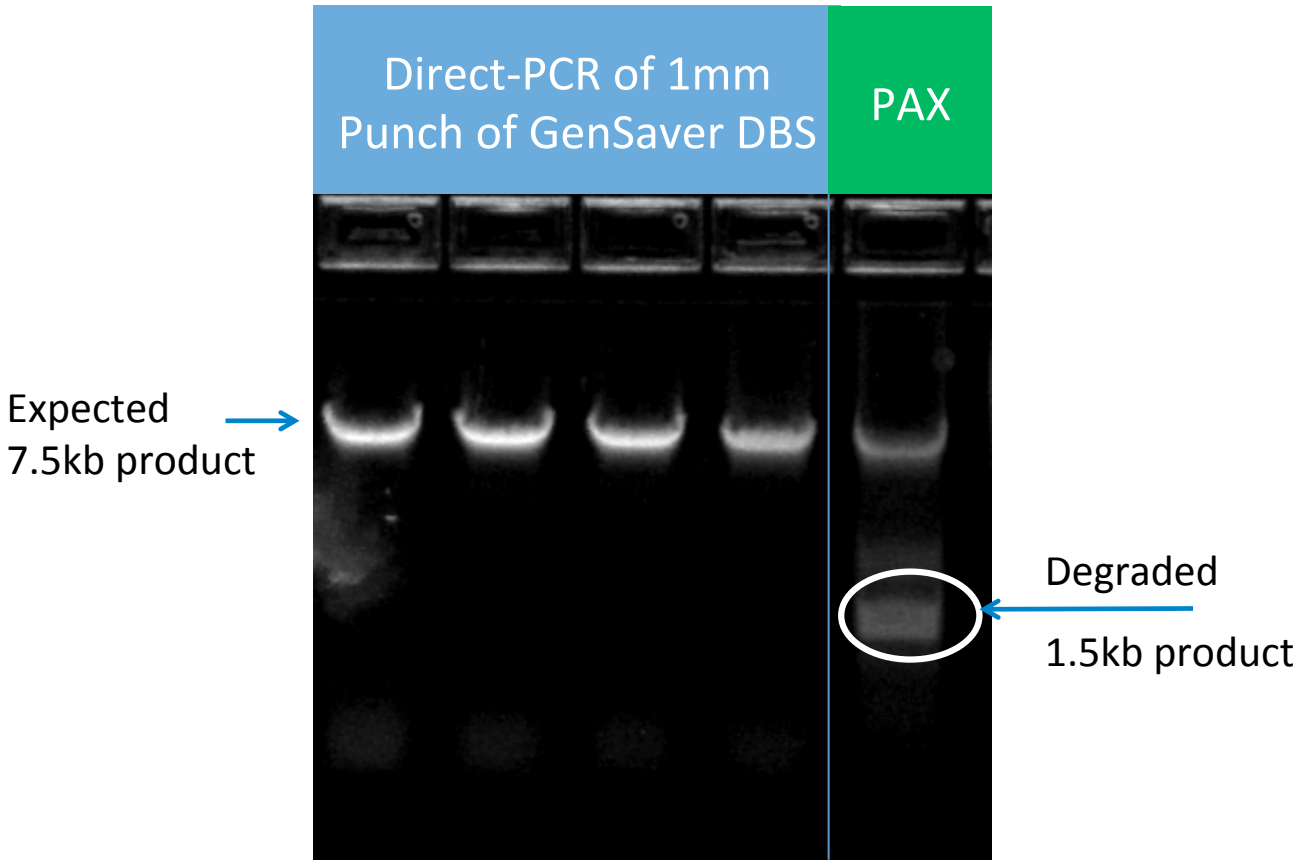
GenSaver DBS were stored at ambient and the PAXgene Blood DNA tubes were stored at 4C for 2 weeks. DNA was extracted from the DBS with GenTegra LLC's GenSolve™ Complete kit (catalog GSC-100) and eluted in 100µL of Elution buffer. DNA from whole blood stored in PAXgene Blood DNA tubes was extracted using the manufacturer's instructions. The amount of nucleic acid was quantified by Invitrogen's dsDNA Qubit quantification assay and by qPCR quantification.

Sample ID	Sample Type	ng/µL (PCR quant)	ng/µL (Qubit)	% detected by PCR
5614-P	PAXgene blood DNA whole blood	9.7	29.2	**35**
5614-G	EDTA whole blood, DBS on GenSaver	6.2	8.65	**72**
5610-P	PAXgene blood DNA whole blood	3.2	8.56	**38**
5610-G	EDTA whole blood, DBS on GenSaver	4.1	3.2	**100**

The over estimation of the quantity of nucleic acid in the PAXgene Blood DNA sample by the Qubit assay indicates significant damage of the DNA in PAXgene Blood DNA as compared to the DBS samples

7.5kb β-globin gene amplification of DNA isolated from GenSaver DBS and PAXgene Blood DNA

Direct PCR (dr-PCR) amplification from 1mm Punch of GenSaver DBS of 7.5kb fragment of single copy β-globin gene. 5ng of DNA from PAX (PAXgene Blood DNA) samples was used as the reference sample for PCR amplification of the 7.5kb fragment of β-globin gene.



Gel electrophoresis of the PCR product shows the 1.5kb degradation product present in the PAX samples indicating suboptimal stabilization of DNA in PAXgene Blood DNA chemistry

STR analysis of PAXgene Blood DNA samples and GenSaver DBS samples

STR analysis using Promega's Powerplex Fusion STR reaction mix for amplification gave 100% concordance of Direct STR (dr-STR) from 1mm Punch of GenSaver DBS vs DNA extracted from DBS and PAXgene blood DNA tube

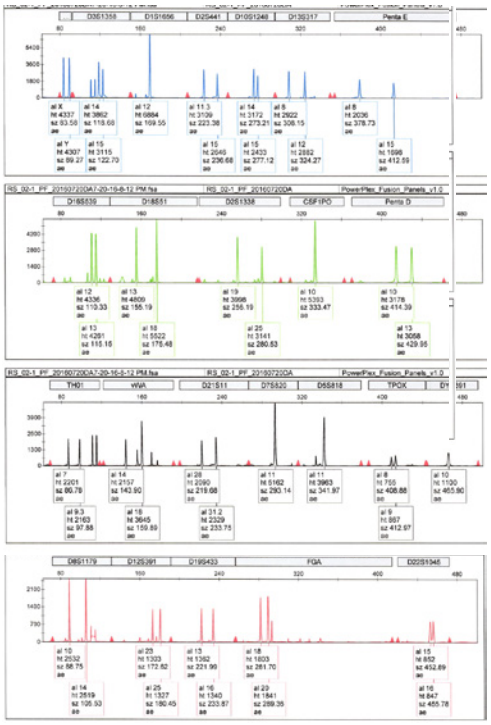
Sample	Alleles Present	Concordance	Alleles Miscalled
Isolated DNA from PAXgene Blood Donor 5610	24	100%	0
Isolated DNA from GenSaver Donor 5610	24	100%	0
Direct-STR from GenSaver Donor 5610	24	100%	0
Isolated DNA from PAXgene Blood Donor 5614	23	100%	0
Isolated DNA from GenSaver Donor 5614	23	100%	0
Direct-STR from GenSaver Donor 5614	23	100%	0

STR profile comparison of DNA extracted from PAXgene Blood DNA tube whole blood samples and GenSaver DBS to direct STR (dr-STR) amplification from 1mm punch of GenSaver DBS

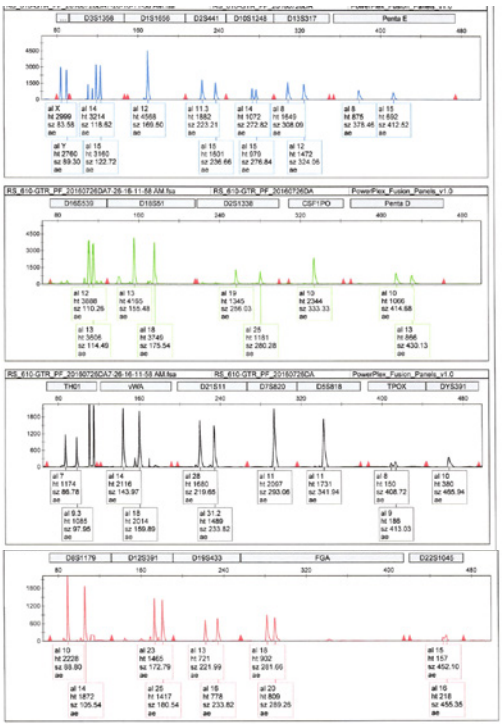
		Isolated DNA				Direct Amplification	
		5614-P	5614-G DBS	5610-P	5610-G DBS	**5614-dr STR**	**5610-dr STR**
1	Amelogenin	X	X	X, Y	X, Y	X	XY
2	D3S1358	14, 17	14, 17	14, 15	14, 15	14, 17	14, 15
3	D1S1656	13	13	12	12	13	12
4	D2S441	10, 11	10, 11	11.3, 15	11.3, 15	10, 11	11.3, 15
5	D10S1248	12, 16	12, 16	14, 15	14, 15	12, 16	14, 15
6	D13S317	11, 12	11, 12	8, 12	8, 12	11, 12	8, 12
7	Penta E	13, 18	13, 18	8, 15	8, 15	13, 18	8, 15
8	D16S539	11, 12	11, 12	12, 13	12, 13	11, 12	12, 13
9	D18S51	12, 17	12, 17	13, 18	13, 18	12, 17	13, 18
10	D2S1338	16, 20	16, 20	19, 25	19, 25	16, 20	19, 25
11	CSF1PO	11, 12	11, 12	10	10	11, 12	10
12	Penta D	11, 12	11, 12	10, 13	10, 13	11, 12	10, 13
13	TH01	8	8	7, 9.3	7, 9.3	8	7, 9.3
14	vWA	14, 16	14, 16	14, 18	14, 18	14, 16	14, 18
15	D21S11	29, 30	29, 30	28, 31.2	28, 31.2	29, 30	28, 31.2
16	D7S820	10, 11	10, 11	11	11	10, 11	11
17	D5S818	11, 14	11, 14	11	11	11, 14	11
18	TPOX	8, 9	8, 9	8, 9	8, 9	8, 9	8, 9
19	DYS391*	-	-	10	10	-	10
20	D8S1179	13	13	10, 14	10, 14	13	10, 14
21	D12S391	18, 22	18, 22	23, 25	23, 25	18, 22	23, 25
22	D19S433	12, 13	12, 13	13, 16	13, 16	12, 13	13, 16
23	FGA	21, 23	21, 23	18, 20	18, 20	21, 23	18, 20
24	D22S1045	15, 17	15, 17	15, 16	15, 16	15, 17	15, 16

STR profiles are in 100% concordance between the isolated DNA samples and the dr-STR samples. The fluorescence units of the electropherogram for the dr-STR samples rivals that of the isolated DNA samples. The advantage of dr-STR is that the "Chain of custody" is maintained since there are very few hands-on steps unlike that needed for DNA isolation from DBS

STR of donor 5610-P

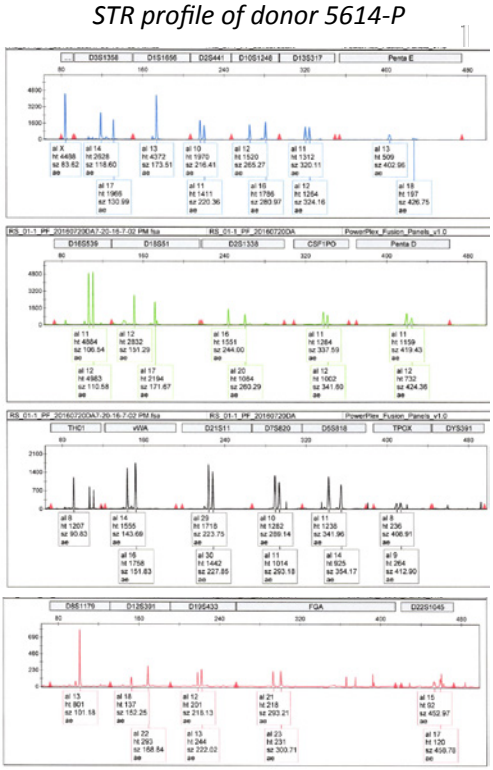


***Direct-STR** of donor 5610-G DBS*

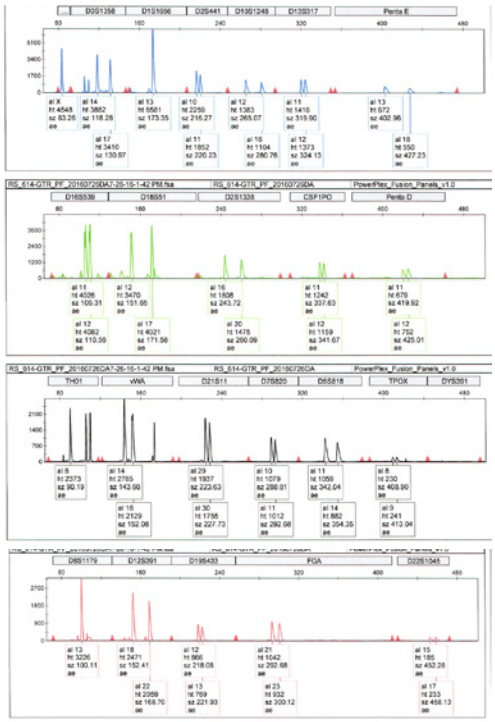


STR Profile donor 5610 (Male): dr-STR of 1mm punch of GenSaver DBS (5610-G) compares in signal intensity and profile to STR profile from PAXgene Blood DNA (5610-P)

STR profile of donor 5614-P



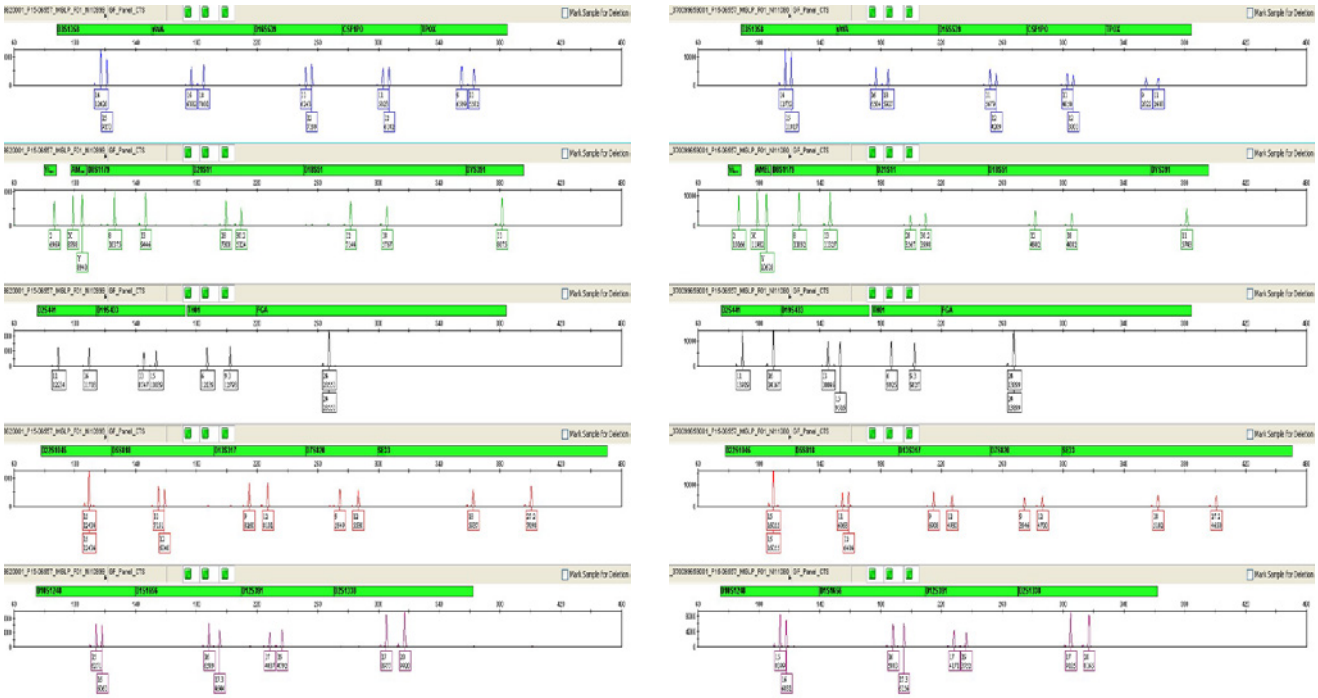
***Direct-STR** profile of donor 5614-G DBS*



STR Profile donor 5614 (Female): dr-STR of 1mm punch of GenSaver DBS (5614-G) compares in signal intensity and profile to STR profile from PAXgene Blood DNA (5614-P)

GenSaver DBS at time Zero

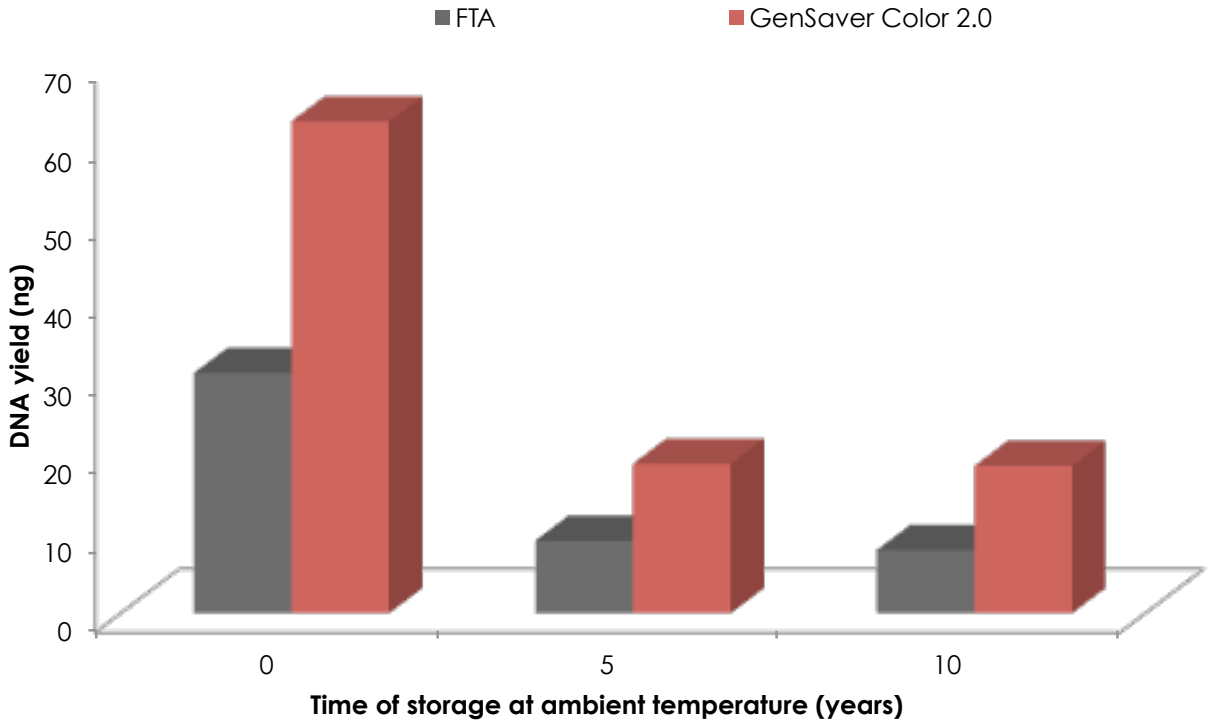
GenSaver DBS at time = 2.5 years



STR analysis of DBS stored at ambient temperature for 2.5 years (accelerated study) show 100% concordance with STR profile of fresh DBS samples

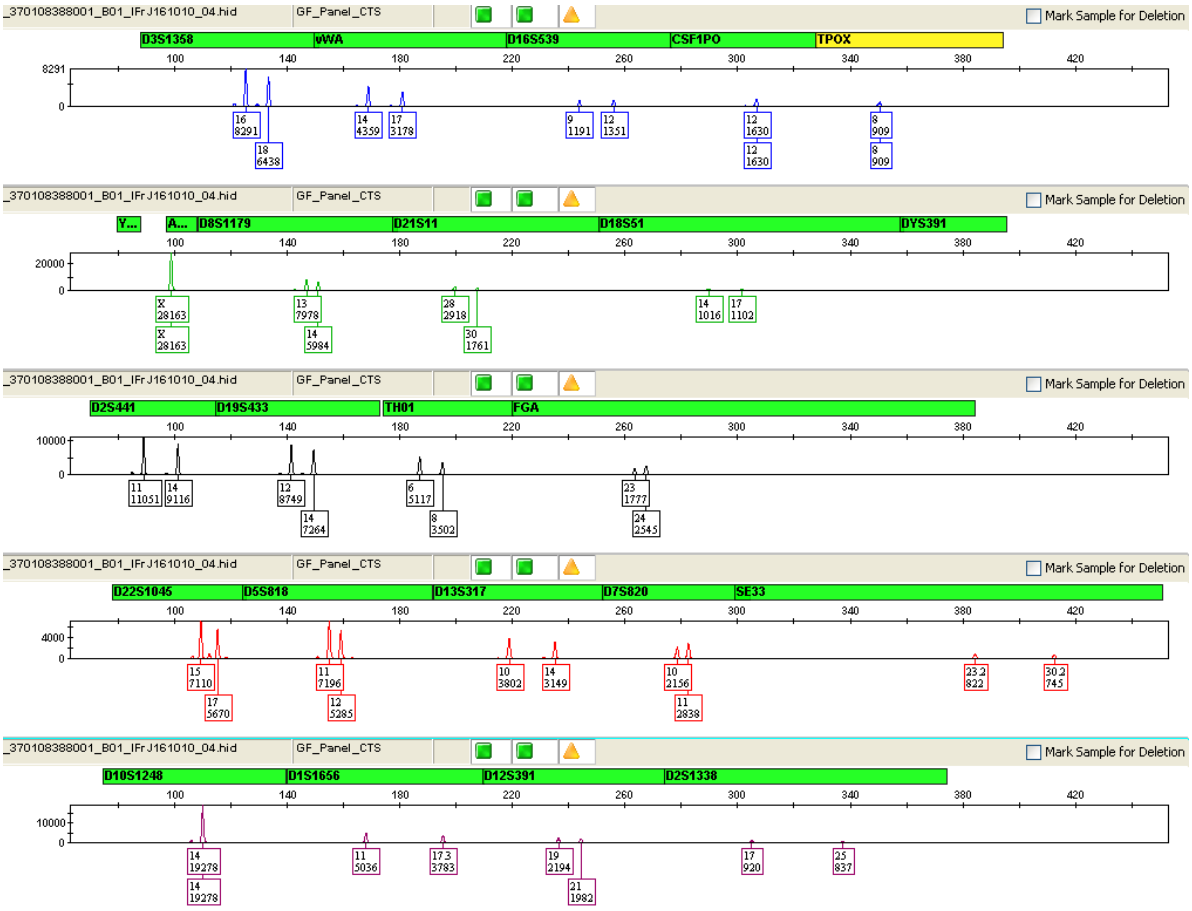
10-year Ambient storage of Saliva on FTA and GenSaver Color 2.0 cards

qPCR quantification of DNA yielded from saliva samples spotted GenSaver color 2.0 cards and subject to an accelerated time study yielded consistently 2x more than FTA stored under same conditions.



The higher yield for GenSaver color is not sample specific as blood samples gave the same outcome, we routinely get yields of 100% more DNA with DBS from GenSaver compared to FTA paper. Both the isolated DNA's gave good quality STR profiles

STR profile of DNA from 10-year saliva samples isolated from GenSaver color 2.0 DBS

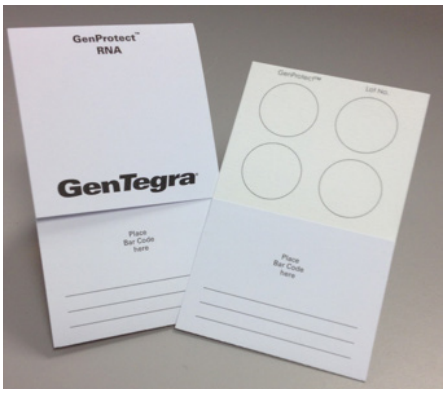


GenSaver Color 2.0 cards can be used for storing saliva at ambient for >10 years (20 year accelerated aging ongoing). DNA isolated from dried Saliva samples stored on GenSaver cards at ambient for equivalent of up to 10 years (accelerated aging) gave good quality STR profile

Section 2

Method for viral RNA stabilization on GenProtect™ RNA cards

GenProtec™ RNA cards are produced with GenTegra's proprietary RNA stabilization chemistry and are presently in beta testing. If interested in being part of the beta test group, please contact GenTegra at sales@gentegra.com or jamesn@gentegra.com

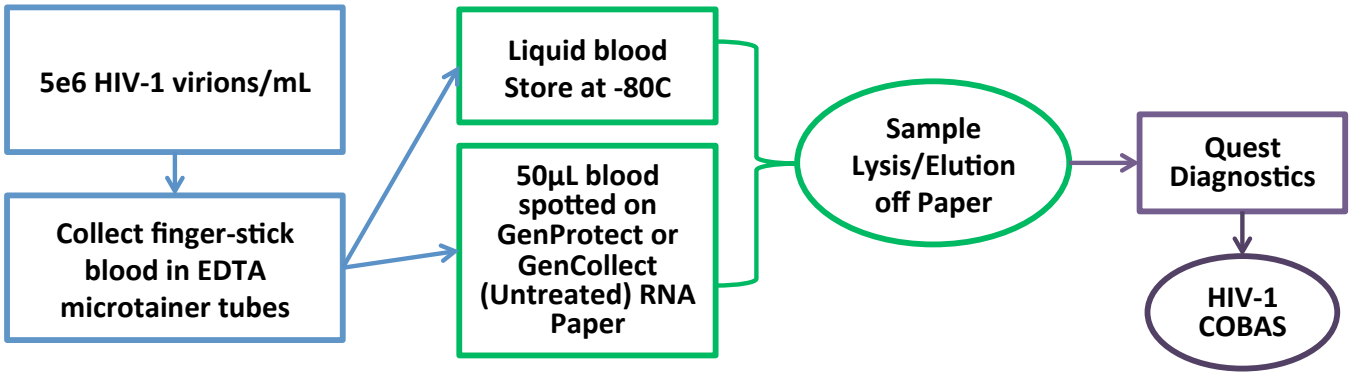


Stabilization of HIV in whole blood on GenProtect RNA card

5e6 HIV-1 virions/mL was added to 50µL of finger prick blood collected in microtainer tubes and spotted on GenProtect RNA paper or GenCollect (untreated) paper and dried at ambient. 50µL of matched whole blood control sample was placed at -80C.

The eluted DBS samples and the control liquid blood samples were sent to Quest Diagnostics for analysis of the HIV viral load on cobas®. Briefly, the entire 50µL DBS was cut and eluted with Lysis buffer from Qiagen DSP Viral RNA mini kit. The DBS lysate and the whole blood sample were shipped to Quest on dry ice for quantitative reverse transcription HIV viral load assay.

GenProtect™ RNA Stabilization

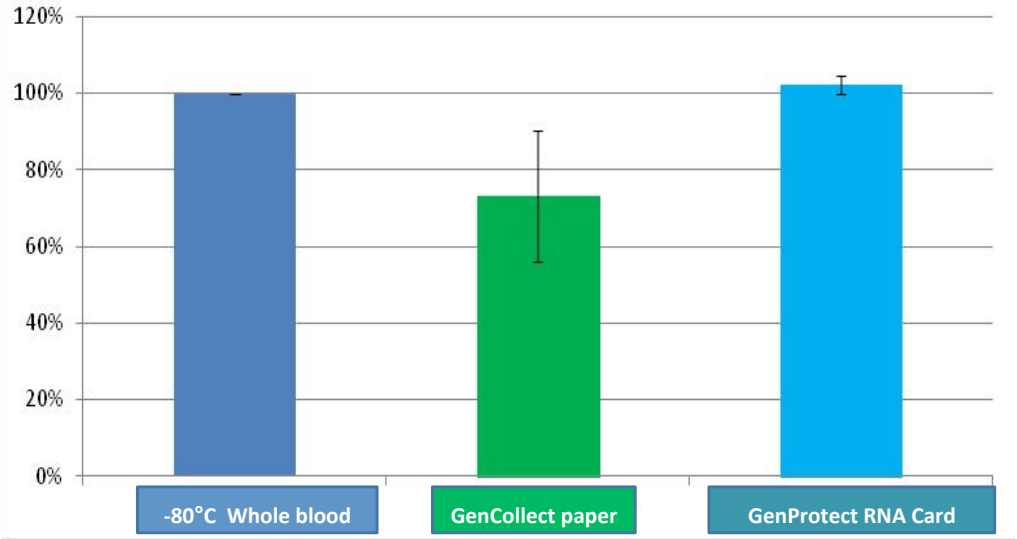


For direct quantitative Reverse Transcription –Polymerase Chain Reaction (dr-RT PCR): One 1mm punch from the DBS was added directly to the RT-PCR amplification master mix. 0.57µL from the corresponding matched whole blood sample (-80C) was used directly in qRT-PCR. 10µL of the amplified product was used in the down-stream qPCR assays.

Results for GenProtect RNA cards

HIV-1 Stability on GenProtect RNA cards

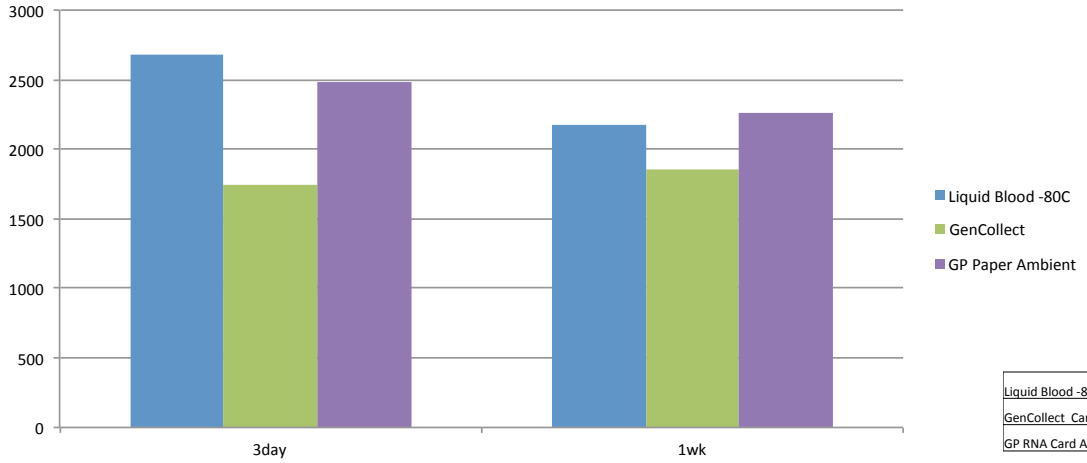
100µL of whole blood spiked with HIV viral particles was spotted on either GenProtect RNA cards or GenCollect cards. The control sample was spiked whole blood stored at -80C. The paper samples were stored at ambient for 24 hours before testing by a one step dr-RT PCR HIV assay for the pol gene.



Direct reverse transcription PCR (dr-RT PCR) performed in triplicates on a 1.2mm Punch each of DBS from GenProtect RNA card and GenCollect card and 0.57µL (equivalent volume to 1.2mmx0.125mm DBS punch) of whole blood show that GenProtect RNA card chemistry protects the viral particles as well as the frozen -80C sample

Stability of HIV viral particles at ambient in Liquid Blood on untreated GenCollect card and GenProtect (GP) RNA card

100µL whole blood spiked with HIV viral particles was spotted on GenProtect RNA card and GenCollect card. The reference sample was whole blood stored at -80C. The cards were stored at ambient for up to 1 week before isolating the viral RNA using the Qiagen DSP Viral RNA mini kit from the whole blood and DBS samples.



	3day	1wk
	HIV-1/mL	HIV-1/mL
Liquid Blood -80°C	2680	2173
GenCollect Card Ambient	1743	1850
GP RNA Card Ambient	2479	2261

The cobas HIV I assay analysis (Quest Diagnostics) show that the samples stabilized on the GenProtect RNA card have equivalent stability to whole blood samples stored at -80C further validating GenProtect RNA card as a convenient alternative for collecting samples to determine viral load

Dengue Virus 2 amplification (CDC assay) of DBS on GenProtect Cards

Stabilizing effect of GenProtect RNA card on Dengue virus was tested by ATCC. Briefly, 50 µL of Dengue virus 2 (titer 10^-6) was applied to GenProtect RNA card. The cards were air dried at room temperature in a Bio-Safety Cabinet overnight for ~18 hours. The RNA from the dr-RT PCR HIV assay for the pol gene. cards was extracted with Qiagen DSP Viral RNA mini kit. RNA was eluted in 60µL of TE buffer pH 7.0, aliquoted and stored at -80C until molecular testing. An aliquot of the Dengue Virus 2 test sample stored at -80C was used as reference. qRT-PCR was performed on the samples in triplicates using the **CDC approved Dengue Virus assay.**

Sample description.	Average Ct	ΔCt
Negative control (RT-PCR grade water)	38.33	
Dengue Virus 2 (Reference test sample neat)	18.06	0.00
Dengue Virus 2 applied to GenProtect RNA card	15.68	-2.38

Initial testing of Dengue Virus 2 on GenProtect RNA cards imply that the viral RNA is stabilized on the GenProtect RNA card and readily available for extraction compared to the reference test samples

Conclusion

We conclude from our study that Ahlstrom's GenSaver card and GenTegra's GenProtect RNA card have met our goals for a product that is simple to use and suitable for high volume economical collection of biological samples capable of:

- **Stabilizing RNA and DNA in whole blood and saliva suitable for PCR, STR, viral RNA quantification, NGS**
- **Direct qPCR and Direct qRT-PCR from a 1mm punch of DBS (bypass sample preparation step)**
- **Convenience of collecting biological samples at ambient**
- **Convenience of storage for up to 20 years at ambient**
- **Convenience of shipping at ambient**

The carbon foot print for long term storage of biological samples on paper cards is essentially zero when compared to -80C which is 11,670 Lbs. of CO2/year; making storage on cards an economical and ecofriendly option for most biological labs.

GenTegra LLC partnered with Ahlstrom in the commercial development of these new products. The results of this two-year partnership are the Ahlstrom GenSaver™ card and the GenTegra GenProtect RNA card. These are high quality Ahlstrom paper impregnated with GenTegra's Active Chemical Protection™ formulations.

GenTegra®

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