Evaluation of a New Commercial Kit for Detection of Human Herpesvirus 8 (HHV-8) in Saliva.

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Introduction: Salivary Detection of HHV-8

Saliva is a window into the body's health, and combined with PCR is a powerful tool for the detection of many viruses. With the reservoir of HHV-8 being the oropharynx viral transmission is via saliva making detection, and treatment monitoring, ideal in whole mouth fluid (WMF). Traditionally, salivary detection of HHV-8 has involved the collection of WMF. Samples are frozen without additives or in Qiagen's RNAprotect solution. Throat epithelial cells can be collected with throat gargles with 10 mL of PBS, which is then preserved by freezing. All of these sample types are then extracted via spin columns before examination by PCR. While these methods work well in developed nations that have sufficient resources they are not useful in field studies in developing nations or resource poor settings. DNA·Genotek offers a commercial salivary collection kit useful for examining human genomic DNA, but are expanding into microbial diagnostics. Our study evaluated the DNA·Genotek prototype kit (P-021) designed to stabilise microbial DNA, in both *in vitro* spiking studies and a Kenyan field trial to compare the DNA·Genotek kit against traditional methods to quantitate HHV-8 oral shedding in HIV-positive patients in Kenya and determine HHV-8 subtypes.

Methods:

Effect of Storage Duration on WMF using the DNA·Genotek Kit

- WMF was spiked with HHV-8 produced from BCBL-1 cell lines that are HHV-8 positive/EBV negative
  - Free HHV-8 virion ($4.38x10^8$ copies/mL) was purified from the supernatant while cell-associated HHV-8 ($1.33x10^{11}$ copies/mL) was obtained from the cell pellets
- Spiked WMF from 3 subjects were stored in the DNA·Genotek P-021 up to 9 months and examined by qPCR for HHV-8 ORF73 & ORF26
  - Samples were extracted via the DNA·Genotek method, which involves ethanol precipitation and purification of DNA
  - All HHV-8 concentrations were normalised to copes HHV-8/mL WMF

Kenyan Field Trial

- WMF and Phosphate Buffered Saline Throat Gargles (PBS-TG) were collected from 10 Kenyan HIV-positive patients on HAART (males: females, 2:8) presenting at the School of Dental Science, University of Nairobi in Nairobi, Kenya
  - Samples were stored frozen and in DNA·Genotek P-021 kits at room temperature in Kenya
  - Specimens were processed 14 months later in Australia.
- Frozen samples were extracted via Qiagen DNeasy Blood and Tissue Kit while DNA·Genotek P-021 kits were ethanol precipitated.
- DNA concentration and purity was determined via NanoDrop, while viral loads were determined by qPCR for HHV-8 ORF73 & ORF26

Sample Processing using the DNA·Genotek collection Kit

- 10-fold serial dilutions of pGEM-T/ORF73 & pGEM-T/ORF26 performed in TE Buffer and WMF (stored frozen & in DNA·Genotek P-021 kits)
  - qPCR for HHV-8 ORF73 & ORF26 was performed directly on spiked TE Buffer
  - Spiked WMF stored frozen was extracted via Qiagen DNeasy Blood and Tissue Kit
  - Spiked WMF stored in DNA·Genotek P-021 kits was extracted via ethanol precipitation and Qiagen DNeasy Blood and Tissue Kit
- qPCR for HHV-8 ORF73 & ORF26 was performed on all samples in triplicate to determine efficiency, linearity and dynamic range

Effect of Storage Duration on WMF using the DNA·Genotek Kit:

- Viral loads were stable in the DNA·Genotek P-021 kits for 6 months for cell-associated HHV-8 but 9 months for free HHV-8 virion
- 31% decrease in cell-associated HHV-8, but only 9.9% decrease in free HHV-8 virion from 6 months to 9 months
- % loss of cell-associated HHV-8 (max loss: 48.27%) is much higher than free HHV-8 virion (max loss: 26.27%)
  - Difference possibly due to the ethanol precipitation method discarding cell-associated virus with the cell pellet

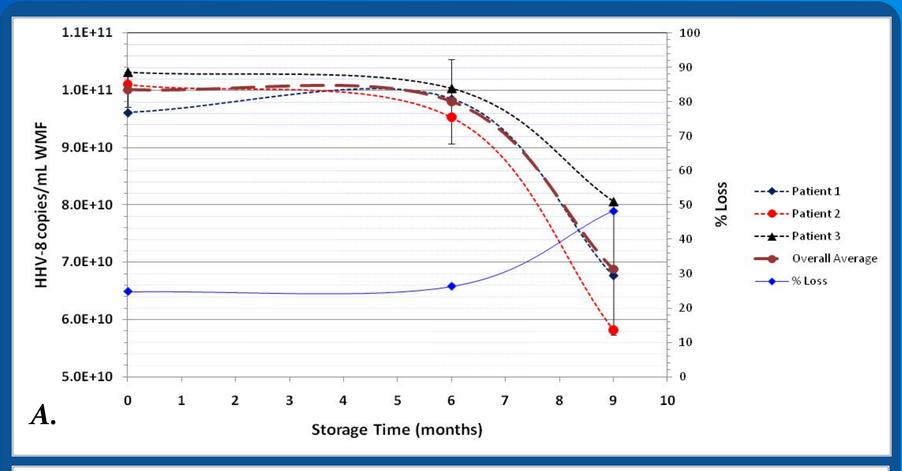

Figure 1: *HHV-8 concentration in DNA·Genotek kits spiked 1:10 with (A)* $1.33x10^{11}$ copies/mL *cell-associated HHV-8 or (B)* $4.38x10^8$ copies/mL of free HHV-8 virion *over 9 months.*

DNA·Genotek Kit Processing Methods:

- Standard curves produced in spiked WMF stored in the DNA·Genotek kits and purified via ethanol precipitation had poor efficiency and linearity
- Extraction of the DNA·Genotek kits with Qiagen spin columns displayed excellent efficiency and linearity
  - Efficiency and linearity (R^2) is as good as plasmid diluted in TE Buffer and WMF
  - Linear range and lower limit of detection is slightly better than TE Buffer and WMF for both assays.

Kenyan Field Study:

- All 10 Kenyan HIV-positive patients had good oral health with no evidence of tumours or other soft tissue lesions
- 30% had hyposalivation (average: 0.227 mL/min; range: 0.199-0.286 mL/min)
- Total DNA concentration: DNA·Genotek Kits > WMF > PBS-TG (Table 1)
  - When extraction volumes were normalized to 100 µL WMF the total DNA concentration were comparable frozen WMF & DNA·Genotek kits (Confirmed by GAPDH qPCR)
- DNA purity was excellent in all samples no matter how they were stored or extracted
- HHV-8 was detected in all samples , but only in low concentrations (Table 2)
  - WMF had a higher positive detection rate due to the slightly higher viral loads as a result of the DNA·Genotek kits being extracted via ethanol precipitation of the supernatant with the pellets being discarded
  - PBS-TG had the lowest positive detection rated possibly due to the huge dilutions factor when using 10 mL PBS

Table 1: Total DNA concentration (ng/µL) and purity of DNA extracted from the Kenyan oral samples frozen and in the DNA·Genotek P-021 kits for 14 months.

	Frozen Samples				DNA·Genotek Kit					
	WMF		PBS-TG		WMF			PBS-TG		
	[DNA]/100 µL (ng/µL)	Purity	[DNA]/100 µL (ng/µL)	Purity	[DNA]/Total (ng/µL)	[DNA]/100 µL (ng/µL)	Purity	[DNA]/Total (ng/µL)	[DNA]/100 µL (ng/µL)	Purity
Griff 5	25.40	1.90	5.66	1.72	120.82	48.33	1.99	7.29	2.92	1.90
Griff 6	18.82	1.67	3.85	2.93	33.42	13.37	1.81	4.50	1.80	1.77
Griff 7	13.31	1.85	4.74	2.72	50.94	20.38	2.11	3.17	1.27	1.06
Griff 8	27.20	1.70	4.09	1.98	50.64	20.26	1.93	2.63	1.05	1.79
Griff 9	30.44	1.74	4.78	2.45	99.72	39.89	1.95	2.90	1.16	2.63
Griff 10	68.55	1.90	6.08	2.15	248.80	99.52	1.94	6.39	2.56	1.29
Griff 11	20.08	1.89	6.73	2.03	120.73	48.29	1.99	7.11	2.84	1.51
Griff 12	30.13	2.06	6.14	1.66	130.27	52.11	2.05	17.05	6.82	1.92
Griff 13	19.22	1.95	4.28	1.59	58.15	23.26	1.93	3.56	1.42	2.86
Griff 14	13.51	1.69	3.86	1.74	55.99	22.40	1.99	10.90	4.36	2.19
Average	26.67	1.84	5.02	2.10	96.95	38.78	1.97	6.55	2.62	1.89
St. Dev	15.97	0.13	1.05	0.46	63.94	25.58	0.08	4.51	1.81	0.56

Table 2: Normalized HHV-8 viral loads in Kenyan oral samples that were frozen and stored in the DNA·Genotek kit presented as HHV-8 copies/mL WMF. Values of "ND" indicate that HHV-8 was "not detected" in these samples.

Frozen Samples								
	WMF				PBS Throat Gargle			
Serial #	HHV-8 ORF73	HHV-8 ORF26	Average Viral Load	GAPDH	HHV-8 ORF73	HHV-8 ORF26	Average Viral Load	GAPDH
Griff 5	4.92E+04	3.90E+05	2.19E+05	4.48E+08	ND	ND	ND	1.16E+08
Griff 6	3.39E+06	2.97E+05	1.84E+06	1.02E+09	1.61E+06	4.38E+05	1.02E+06	6.05E+07
Griff 7	ND	ND	ND	4.16E+08	2.13E+06	2.39E+05	1.18E+06	1.49E+07
Griff 8	3.61E+06	7.60E+05	2.18E+06	9.95E+08	ND	ND	ND	5.65E+07
Griff 9	2.14E+06	ND	2.14E+06	1.41E+09	ND	ND	ND	9.00E+07
Griff 10	6.00E+06	1.51E+06	3.75E+06	3.17E+09	ND	ND	ND	1.32E+08
Griff 11	2.70E+06	ND	2.70E+06	3.49E+08	ND	ND	ND	7.70E+07
Griff 12	1.16E+06	2.02E+05	6.79E+05	1.03E+09	ND	ND	ND	5.35E+07
Griff 13	3.95E+05	3.39E+05	3.67E+05	4.61E+08	1.18E+06	3.51E+05	7.66E+05	1.26E+08
Griff 14	2.80E+06	1.53E+05	1.47E+06	4.70E+08	3.67E+06	ND	3.67E+06	1.54E+07
	Average GAPDH (Total)			9.59E+08	Average GAPDH (Total)			7.41E+07
DNA Genotek Kit								
Serial #	WMF				PBS Throat Gargle			
	HHV-8 ORF73	HHV-8 ORF26	Average Viral Load	GAPDH	HHV-8 ORF73	HHV-8 ORF26	Average Viral Load	GAPDH
Griff 5	ND	ND	ND	9.78E+08	ND	ND	ND	1.13E+08
Griff 6	ND	ND	ND	5.54E+08	ND	ND	ND	4.18E+07
Griff 7	1.67E+06	6.64E+05	1.17E+06	5.64E+08	ND	ND	ND	1.09E+07
Griff 8	ND	4.26E+05	4.26E+05	1.06E+09	ND	ND	ND	2.94E+07
Griff 9	ND	ND	ND	8.46E+08	ND	2.96E+05	2.96E+05	5.72E+07
Griff 10	ND	ND	ND	3.52E+09	2.18E+05	ND	ND	1.06E+08
Griff 11	ND	ND	ND	6.22E+08	ND	1.89E+05	1.89E+05	1.68E+08
Griff 12	ND	3.10E+05	3.10E+05	1.67E+09	2.34E+06	2.28E+06	2.31E+06	1.48E+07
Griff 13	ND	7.56E+04	7.56E+04	8.60E+08	ND	1.18E+05	1.18E+05	1.44E+08
Griff 14	ND	3.80E+05	3.80E+05	6.00E+08	ND	ND	ND	2.56E+07
	Average GAPDH (Total)			1.13E+09	Average GAPDH (Total)			7.10E+07

Table 3: qPCR dynamics including efficiency, R^2, and linear rang e for the HHV-8 ORF73 & ORF26 qPCR assays on pGEM-T/ORF73 & pGEM-T/ORF26 plasmids diluted in TE Buffer and WMF stored and extracted with 2 different methods.

Sample Medium and Processing Methods	Efficiency (%)	R^2 Value	Linearity Range
HHV-8 ORF73 qPCR assay			
TE Buffer	97.544	0.99664	6.34E+03 to 6.34E+10
WMF (23/05/2011)	95.95	0.99887	2.66E+02 to 1.45E+09
DNA·Genotek (EtOH precipitation)	164.22	0.89071	Non-linear
DNA·Genotek (Qiagen Spin Columns)	104.52	0.99542	2.44E+01 to 1.12E+09
HHV-8 ORF26 qPCR assay			
TE Buffer	100.4	0.99407	1.95E+02 to 1.95E+10
WMF (23/05/2011)	99.13	0.98179	3.39E+01 to 1.12E+09
DNA·Genotek (EtOH precipitation)	167.31	0.84137	Non-linear
DNA·Genotek (Qiagen Spin Columns)	97.46	0.99625	5.89E+01 to 1.56E+09

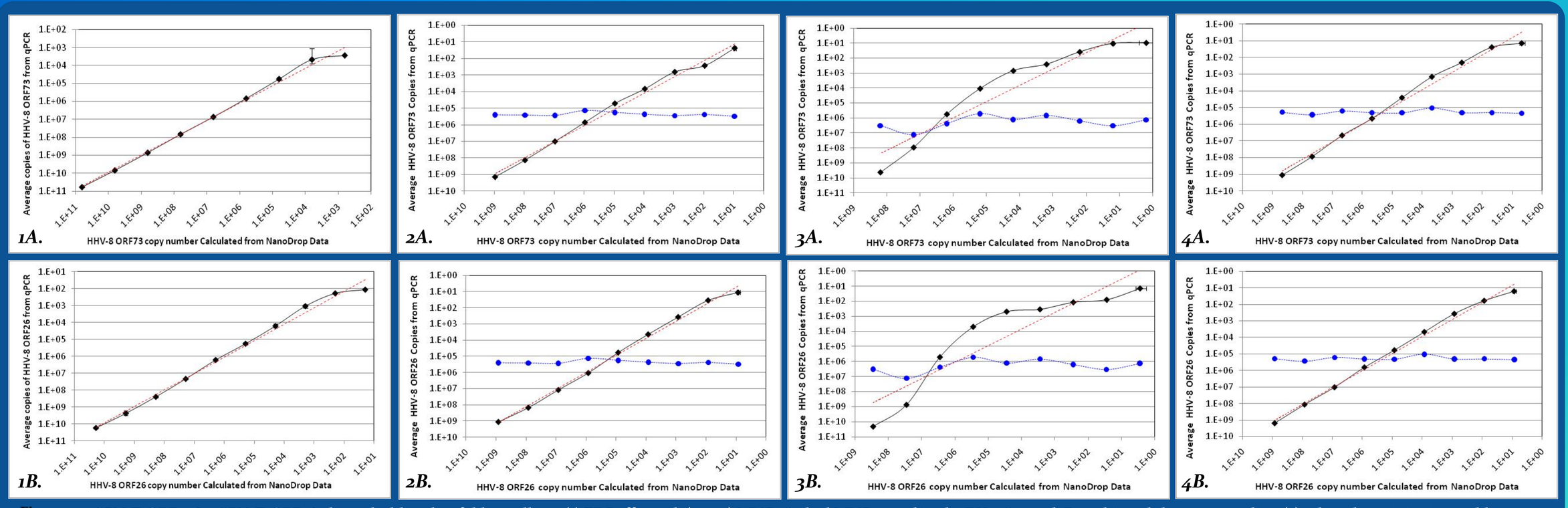

Figure 2: pGEM-T/ORF73 & pGEM-T/ORF26 plasmids diluted 10-fold serially in (1) TE Buffer and (2, 3, 4) WMF. Spiked WMF stored in the DNA·Genotek P-021 kit and then extracted via (3) ethanol precipitation yields a non-linear standard curve for both for (A) HHV-8 ORF73 and (B) HHV-8 ORF26 qPCR assays. When the same samples stored in the DNA·Genotek P-021 kit are extracted via (4) via Qiagen spin columns the linear range and sensitivity is as good or better than plasmids diluted in (1) TE Buffer or (2) WMF and extracted immediately with Qiagen spin columns. GAPDH qPCR (blue line) show that cellular DNA is similar in all samples proving that the differences in HHV-8 copy number is due to the serial dilutions and not a difference in amount of sample extracted.

Conclusions:

1. DNA·Genotek kits are excellent for preserving oral specimens collected in the field for up to 14 months.
2. DNA·Genotek kits produce high quality DNA with yields as high or higher than frozen WMF.
3. Standard curves produced from spiked WMF stored in the DNA·Genotek kits and extracted with the Qiagen DNeasy Blood and Tissue Kit are as good or better than plasmids diluted in TE Buffer or WMF extracted with Qiagen spin columns.
4. 24.4 copies HHV-8/µL can be detected when the DNA·Genotek kits are extracted with spin columns.

The DNA·Genotek system has been a great asset in the detection of HHV-8 in our field studies in Kenya where room temperature can exceed 30°C. When extracted with spin columns serial dilutions show near perfect linearity down to 24.4 copies of HHV-8/µL.

References:

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