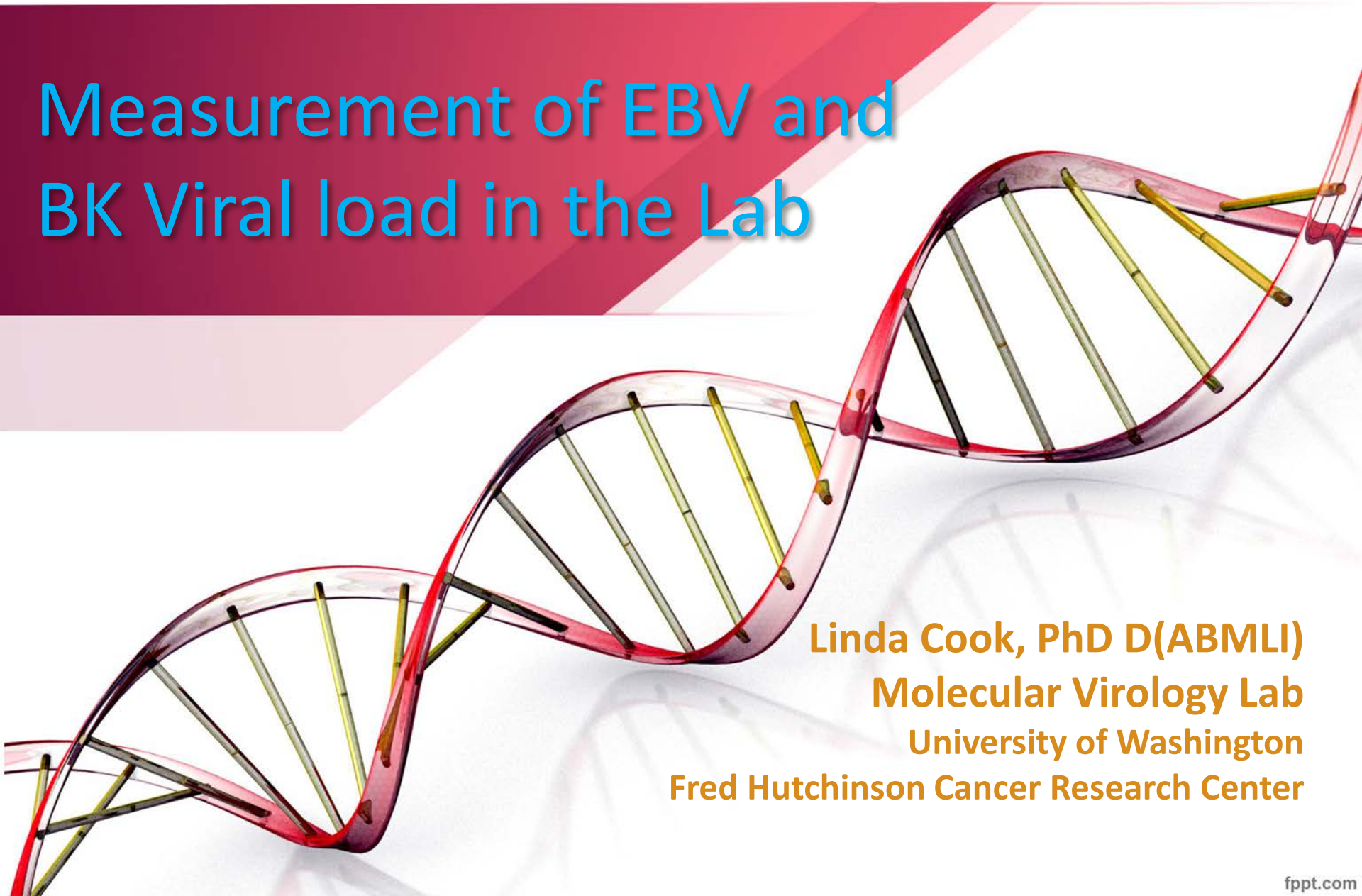
Measurement of EBV and BK Viral load in the Lab



Linda Cook, PhD D(ABMLI)
Molecular Virology Lab
University of Washington
Fred Hutchinson Cancer Research Center

EBV Associated Diseases and Malignancies

- Primary Infection (asymptomatic)
- Acute Infectious Mononucleosis
- Neurologic syndromes
- Hematologic abnormalities
- Lymphoproliferative Disorders
  - Hemophagocytic Lymphohistiocytosis, Lymphomatoid Granulomatosis, Chronic active EBV (CAEBV), XLP Syndrome, PTDL

- Burkitt's Lymphoma
- HIV associated lymphoma
- Leiomyoma and Leiomyosarcoma
- Hodgkin's disease
- Nasopharyngeal Carcinoma
- T-cell lymphoma

EBV Infections

- Primary Infection –
  - 25-50% of peripheral blood memory cells become latently infected

- Chronic Infections
  - 1 infected B cell in 10^5 to 10^6 cells
  - 1-20 episomes per cell
  - Transcriptionally quiescent

Standardization Issues?

1) Sample Type
2) PCR design issues
3) Other Standardization Issues

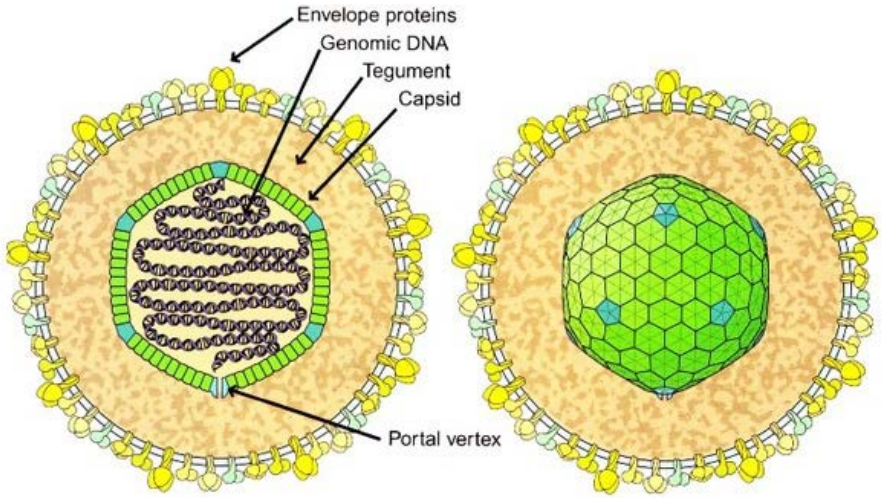
SAMPLE SELECTION

PATIENT SAMPLE?

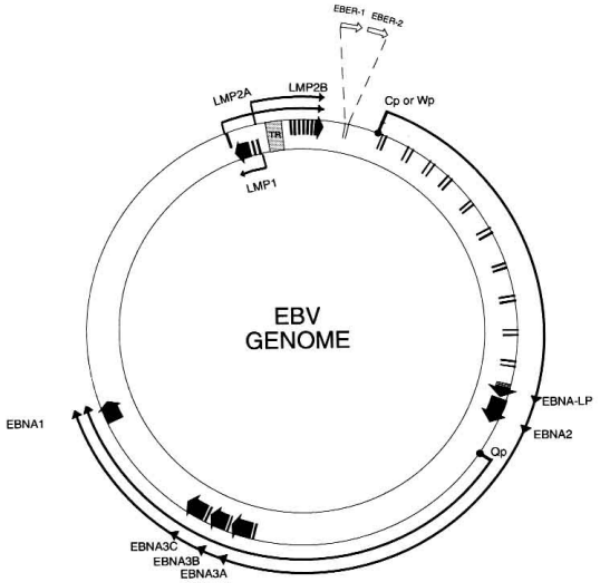
STANDARD MATERIAL?

Measuring EBV

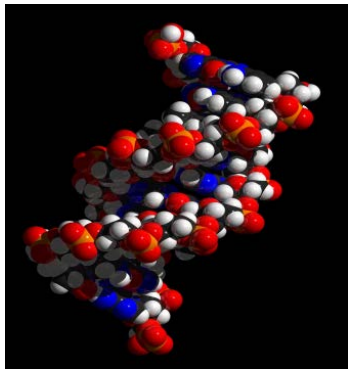
Intact Virions



172,000 bp, about 85 genes

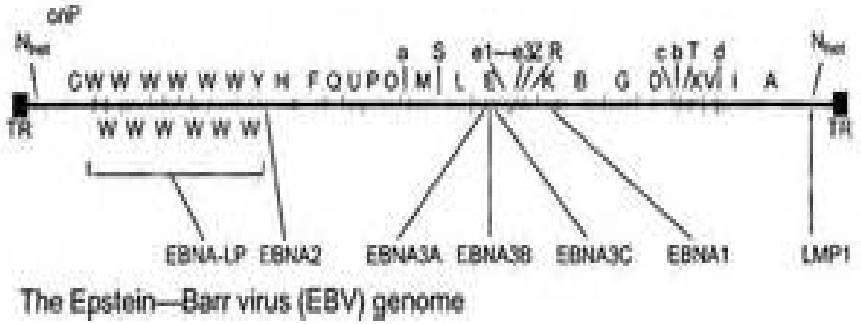


Episomes – cellular
(multiple copies)



DNA Fragments

Whole Genome - linear

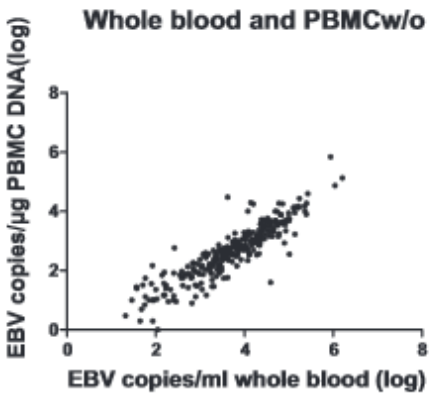


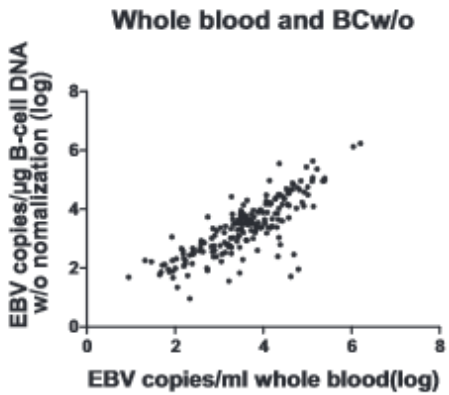
The Epstein—Barr virus (EBV) genome

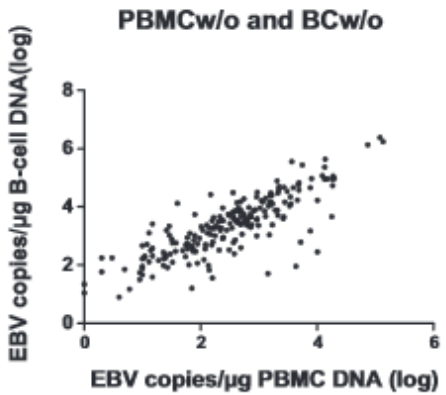
Sample Types?

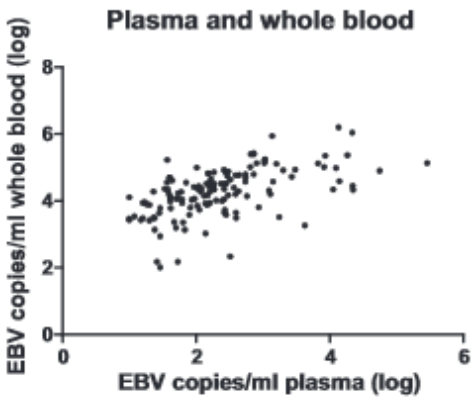
- Whole Blood
- Plasma
- White blood cells
- Lymphocytes
- CSF
- Tissues and lymph node biopsies
- Nasopharyngeal swabs, other swabs
- Saliva
- Trans-oral brush biopsies

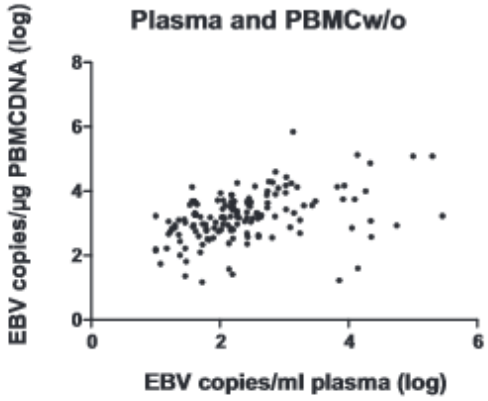
Sample Type Differences

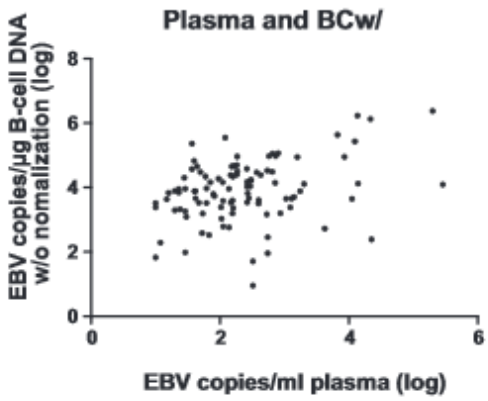












Ruf S, et al J Clin Virolo 53:186, 2012

Kimura, Rev Med Virol 2008:18, 305

Table 3. Optimal specimens for measuring viral load in each Epstein–Barr virus (EBV)-associated disease

Disease	Infected cells	Infection pattern	Specimens for measuring viral load		
			Plasma or serum	Mononuclear cells	Whole blood
Infectious mononucleosis	Plasma cells B cells	Lytic infection Latency III	Desirable	Not recommended	Not recommended
Post-transplant lymphoproliferative disorder	B cells	Latency III	Controversial	Desirable	Preferable
Hodgkin's lymphoma	Hodgkin cells (B cell origin)	Latency II	Desirable	Not recommended	ND
Chronic active EBV infection	T or NK cells	Latency II	Useful for prognosis	Desirable for diagnosis	ND
Nasopharyngeal carcinoma	Squamous cells	Latency II (Lytic infection)	Desirable	Not recommended	ND

NK, natural killer; ND, no or little data available.

Kanakry, et al 2016

2,146 patient studied, 535 EBV patients with at least 1 positive result; Compared plasma to PBMC sample types.

Of 105 with active EBV+ disease, plasma was positive 99% of the time, PBMC only 54%.

Key Points

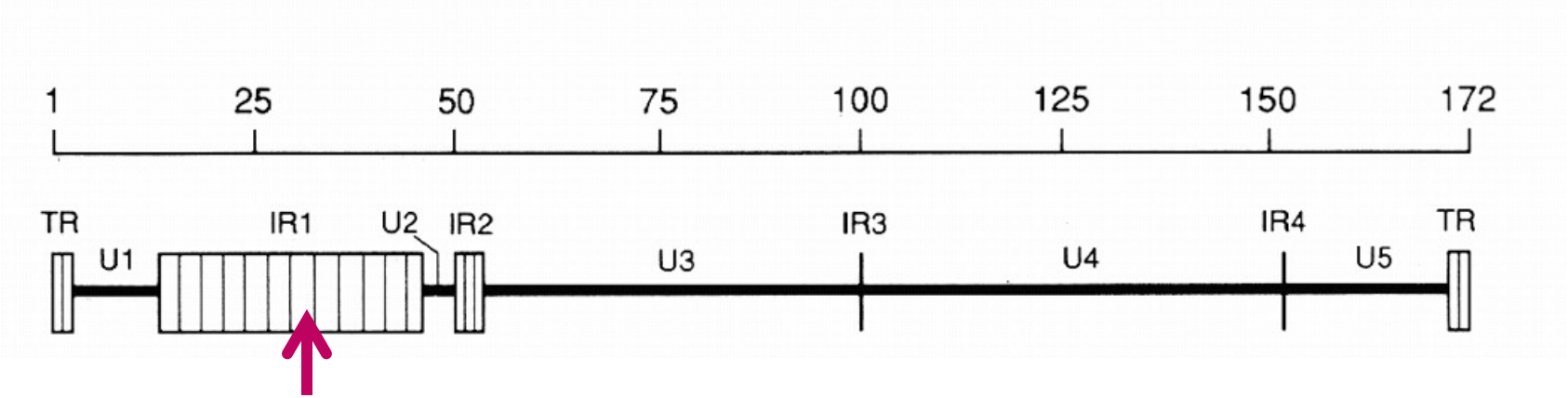
- Cell-free (plasma) EBV DNA performs better than cellular EBV DNA as a marker of a broad range of EBV^+ diseases.
- Within a largely immunocompromised and hospitalized cohort, detection of EBV DNA in plasma is uncommon in the absence of EBV^+ disease.

PCR Assay Design

No large primer set comparison studies have been published

Genome Location of Amplicon

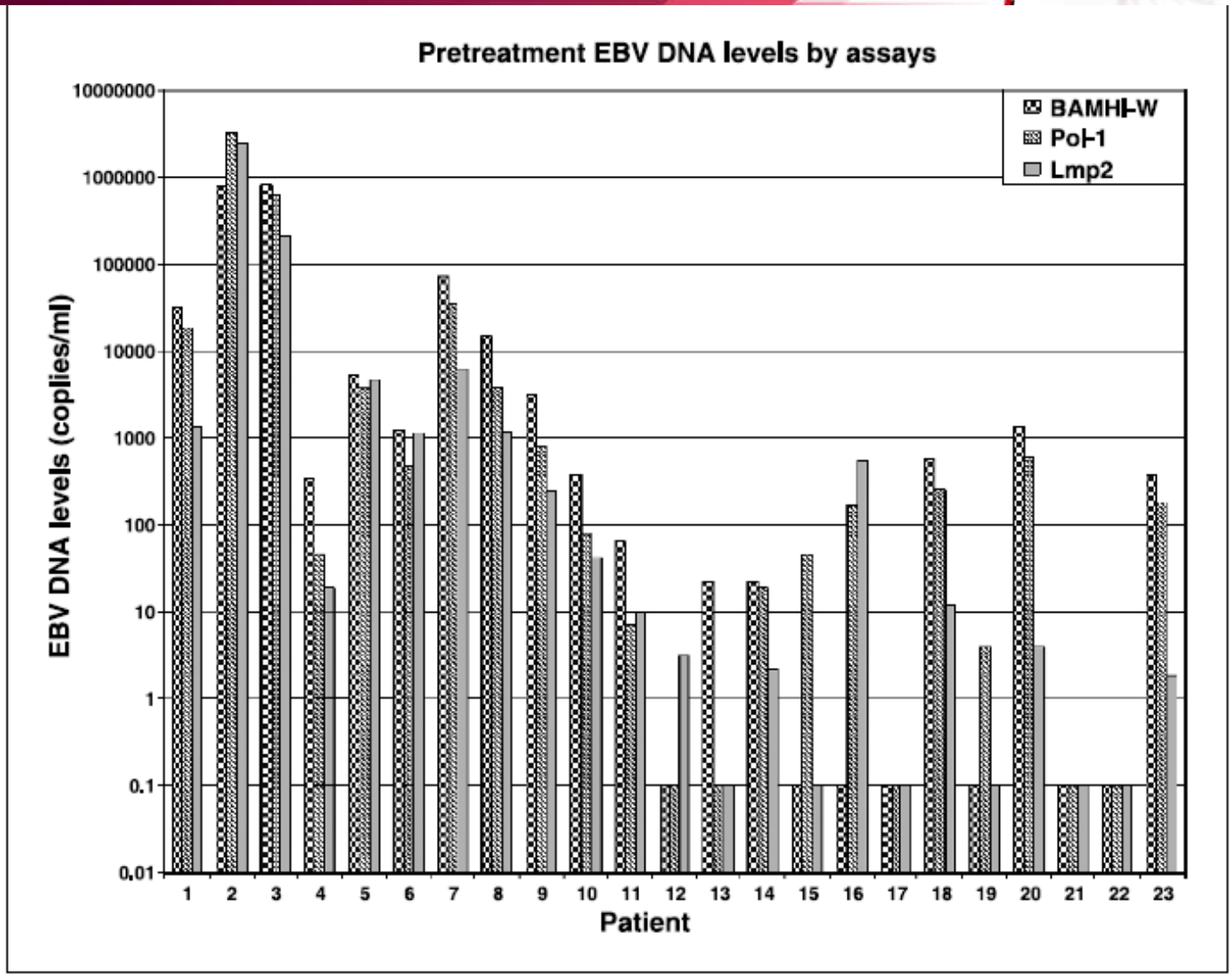
Sensitivity vs Accurate Quantity?



PCR in the BamHI-W region in the EBNA encoding region of the genome.
NGS studies = clinical isolates average 7, more consistent than culture strains.

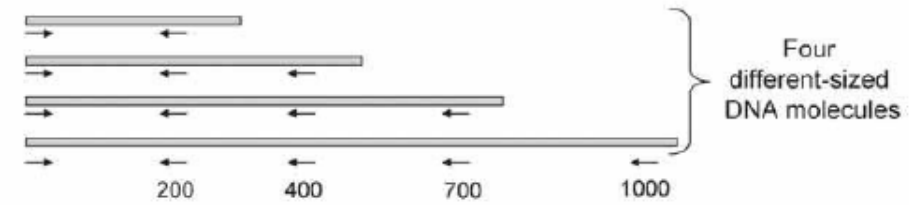
Many other regions of this large genome have been targets of PCR assays. These give a better 1:1 relationship of virus quant to PCR signal but have lower sensitivity for detection of EBV..

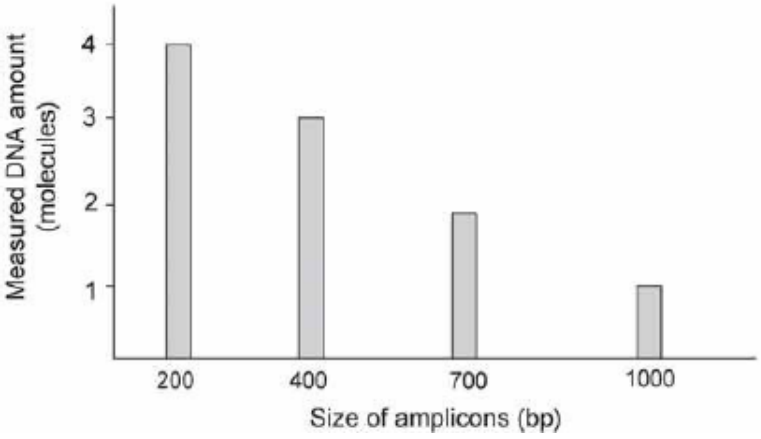
3 Assay Comparison

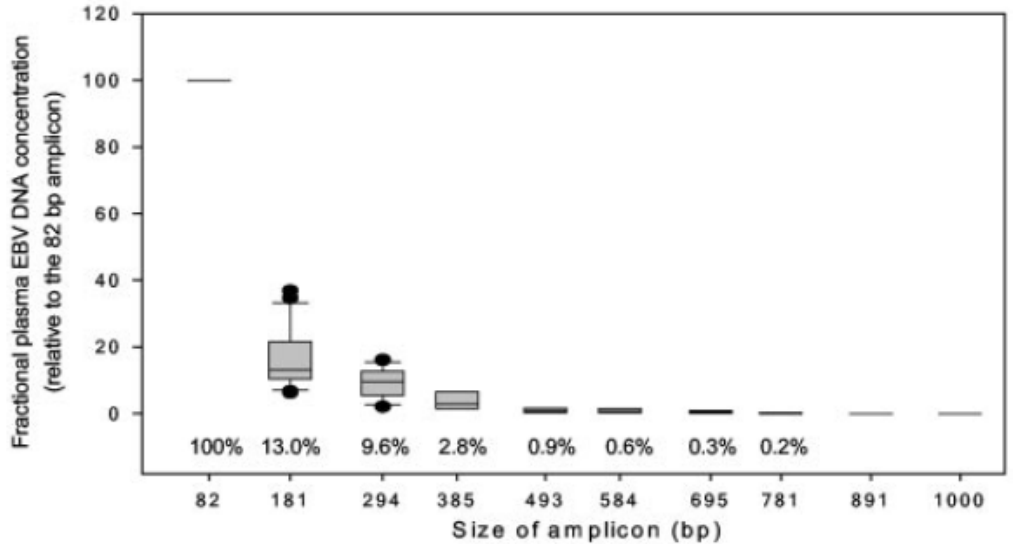


Le Q-T
Clin Can
Res 2005:
11:5700

PCR Detection of fragmented DNA







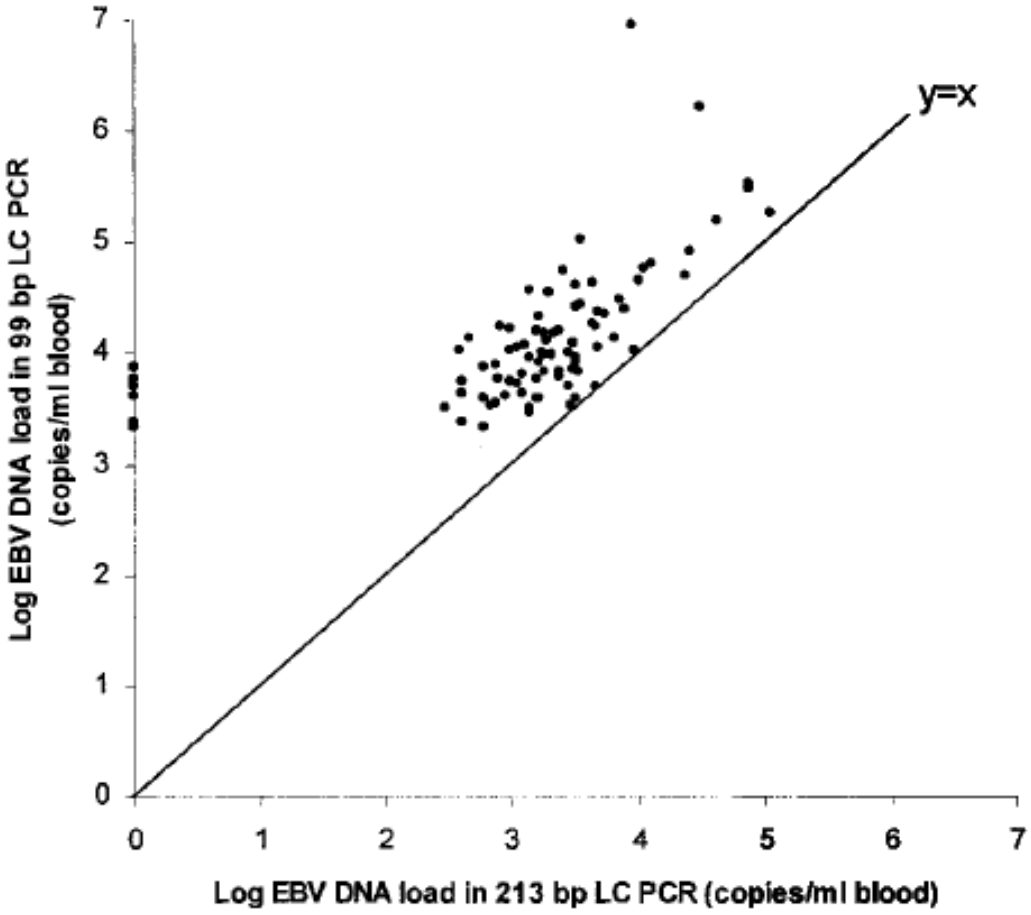
Chan KCA and Lo YMD, Methods in Mol Biol 336:111, 2006; Cancer Res 63:2028, 2003

PCR Amplicon Size

Whole blood samples – Authors reasoned that cell lysis by Apoptosis yields DNA fragments approximately 150 bp in size, so a smaller amplicon may give higher levels of detection.

Compared 99bp amplicon to 213 bp amplicon (same genome region).

Smaller amplicon picked up 20 (13%) more positive patients.



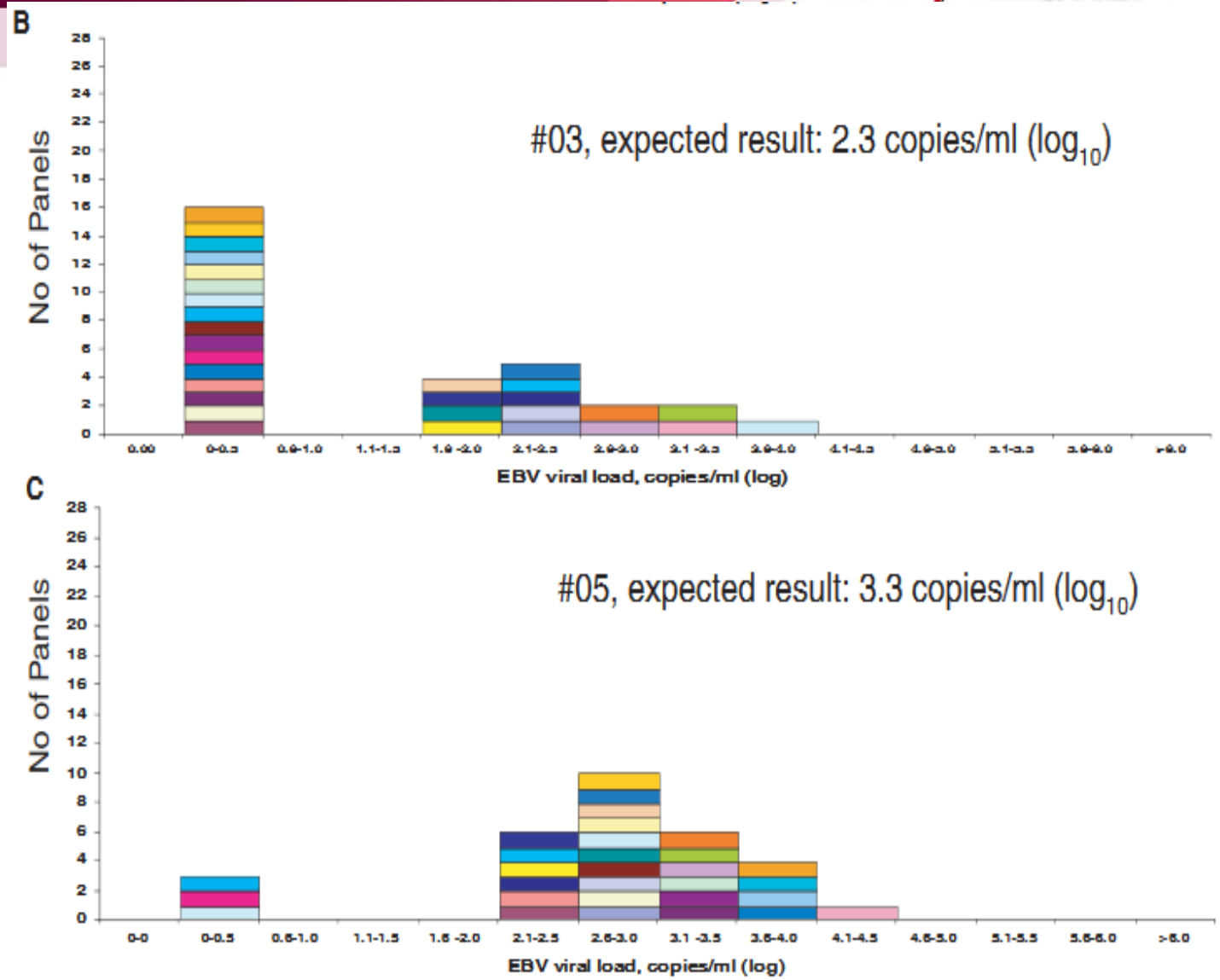
Stevens, SJ et al J Clin Micro 43:3066, 2005

Comparison Studies

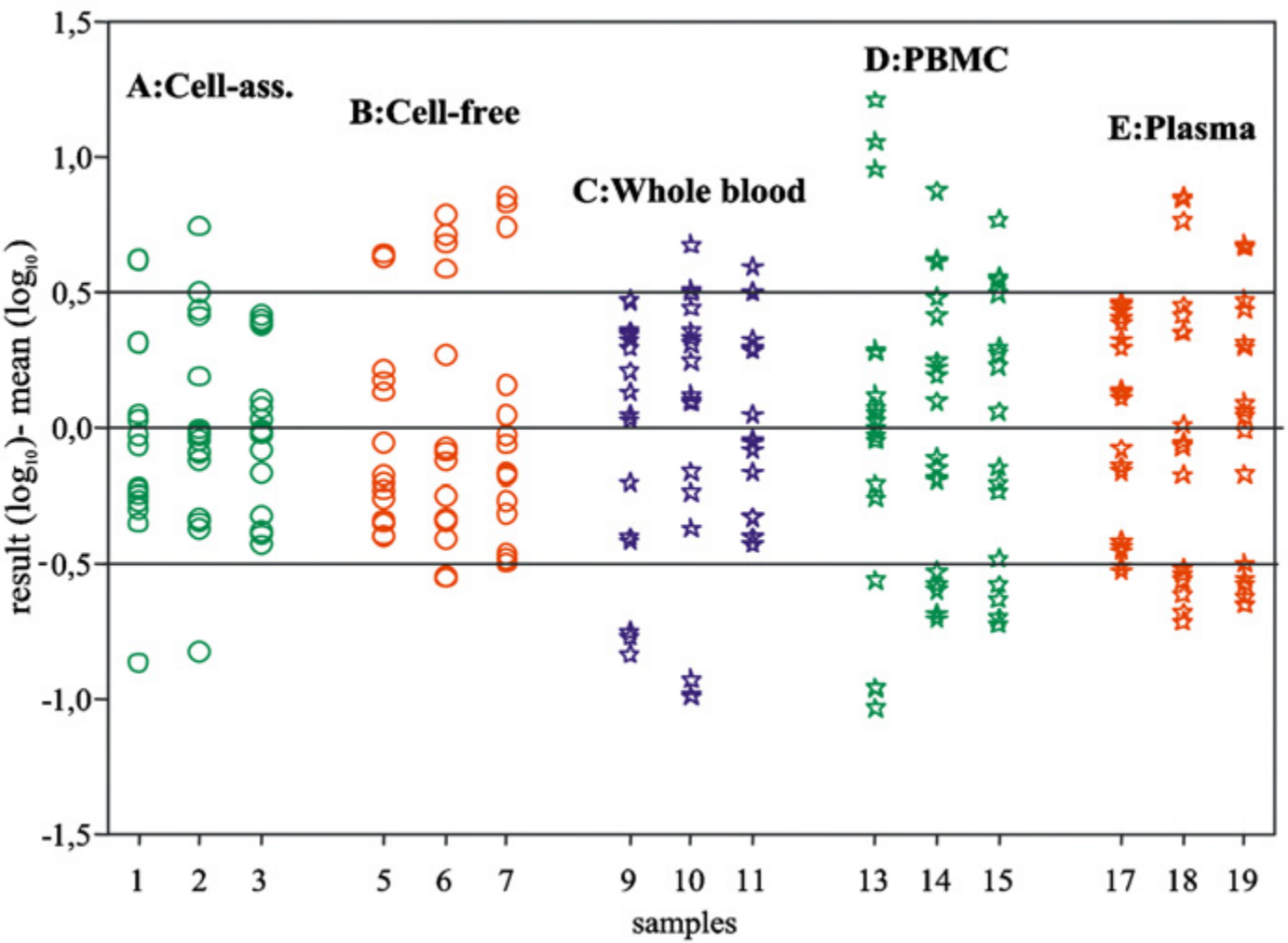
Reference Materials

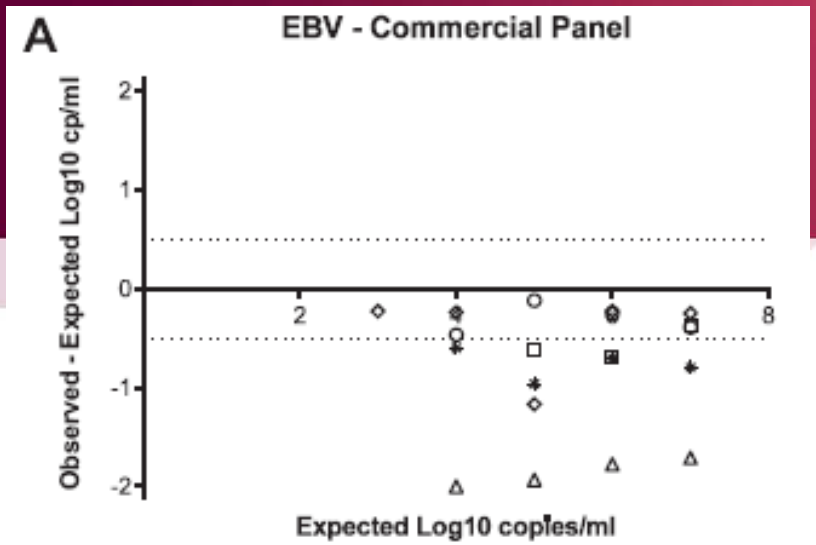
- 1) WHO International Standard NIBSC 09/260
  - EBV95-8 strain
- 2) EBV Plasma Panels
    - Virus particles, 5 members ranging from 10e2 – 10e6 IU/mL
- 3) Quantitated Viral DNA
    - EBV B95-8 Strain, 1 vial of quantified DNA

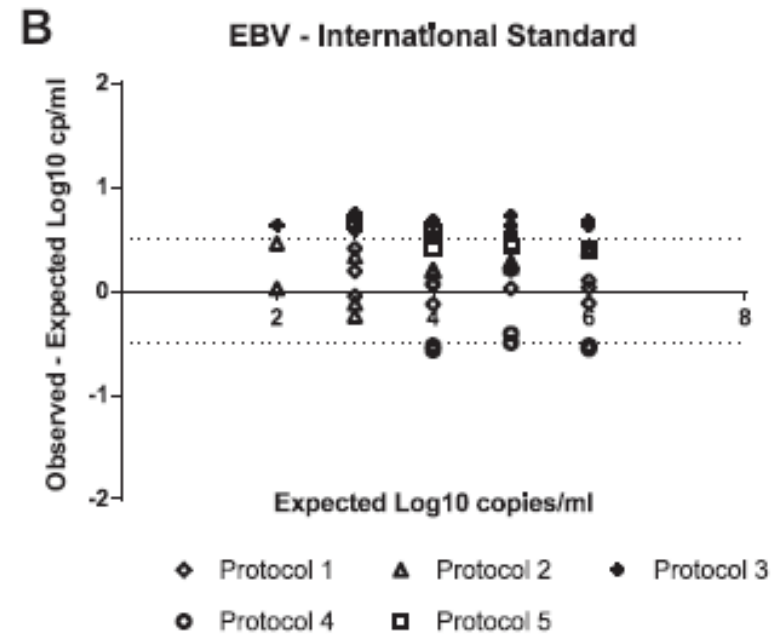
Preiksaitis 2009 Study



Abbate 2011 Study



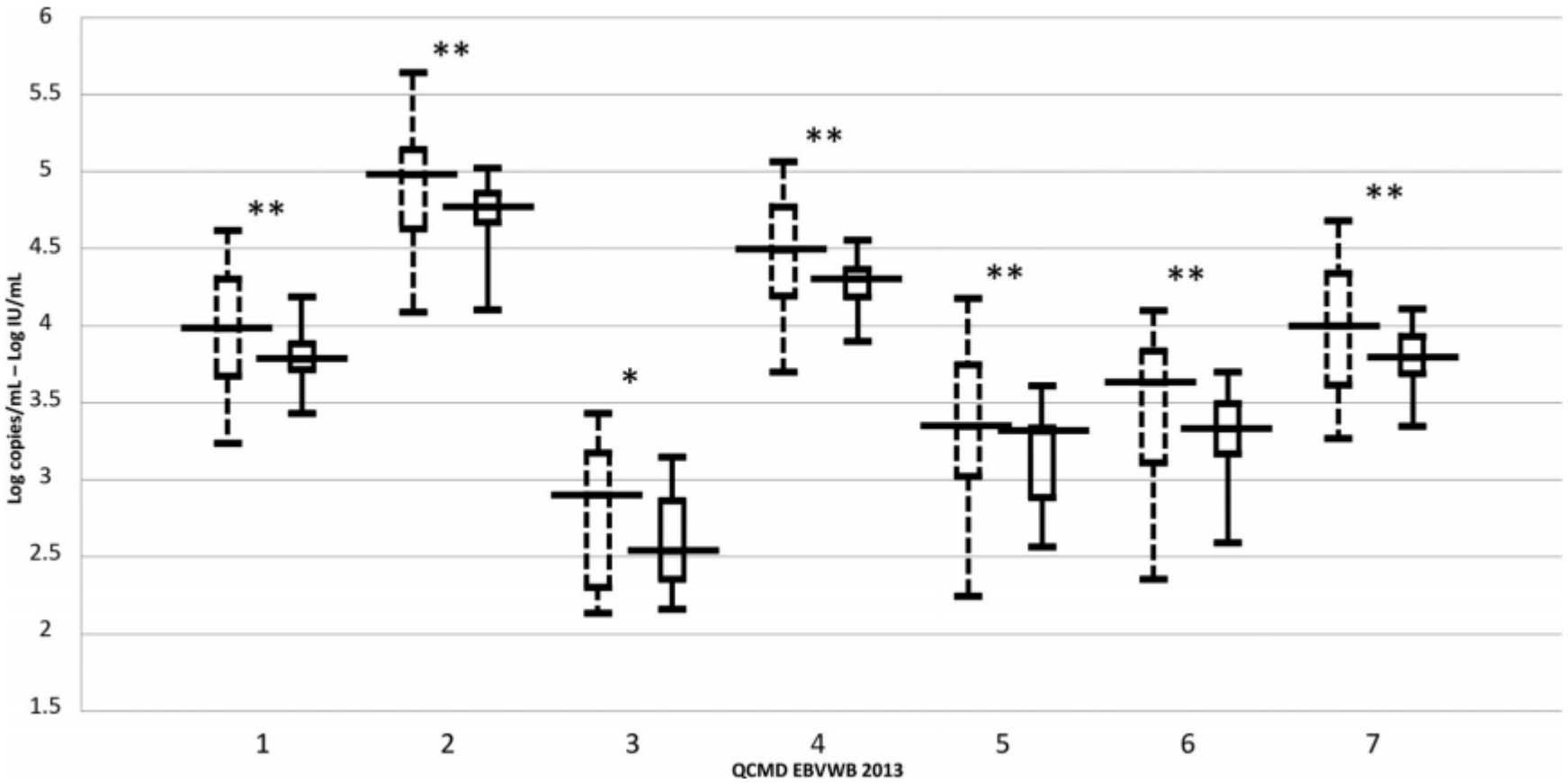




Results had broad spreads across the different extraction/PCR instruments, were improved somewhat by utilizing the International Standard material.

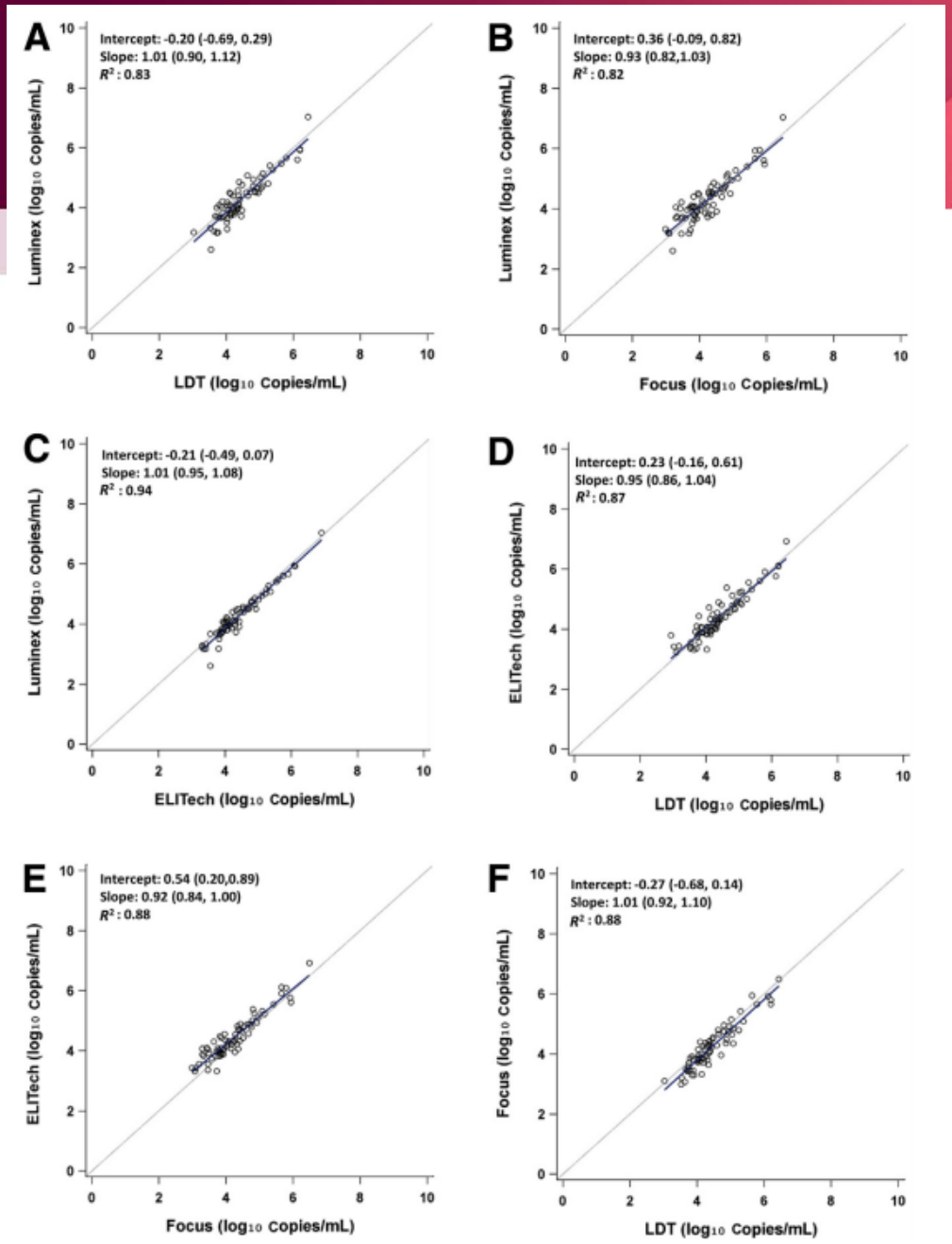
Danziger-Isakov RJ Clin Transplant 2014:28:1416

Cross-Lab Comparison –
12 French Labs – Semenova 2016



Whole Blood Samples

Dotted Boxes – Copies/ mL, Solid Boxes – results after IU/mL standardization



Buelow, et al 2016

Comparison of 4 commercially available reagents (3 ASRs, 1 LDT) – detecting EBV DNA spiked into whole blood samples

“Harmonization” Efforts – Le et al 2013

- Support for Radiation Therapy Oncology Group studies
- 4 labs – international sites
  - Stanford, Chinese University of Hong Kong
  - National Taiwan University Hospital
  - Chang-Gung Memorial Hospital
- 40 patient samples analyzed 2 times at each site
- Variables identified
  - Calibrators
  - Master Mix
  - DNA Extraction method

Proficiency Materials

- College of American Pathologists

  VLS Survey – 2015 survey 1st to give quantitative results

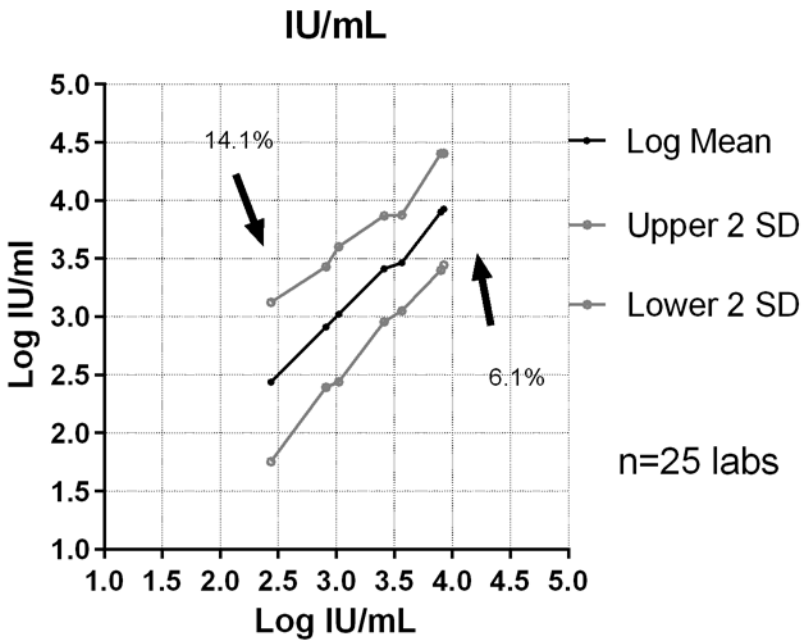
- QCMD
  - European/British company makes a wide variety of proficiency testing materials including EBV
    - Annual, 10 samples (1 negative) range of quantities
    - 2015 – split samples into 2 shipments

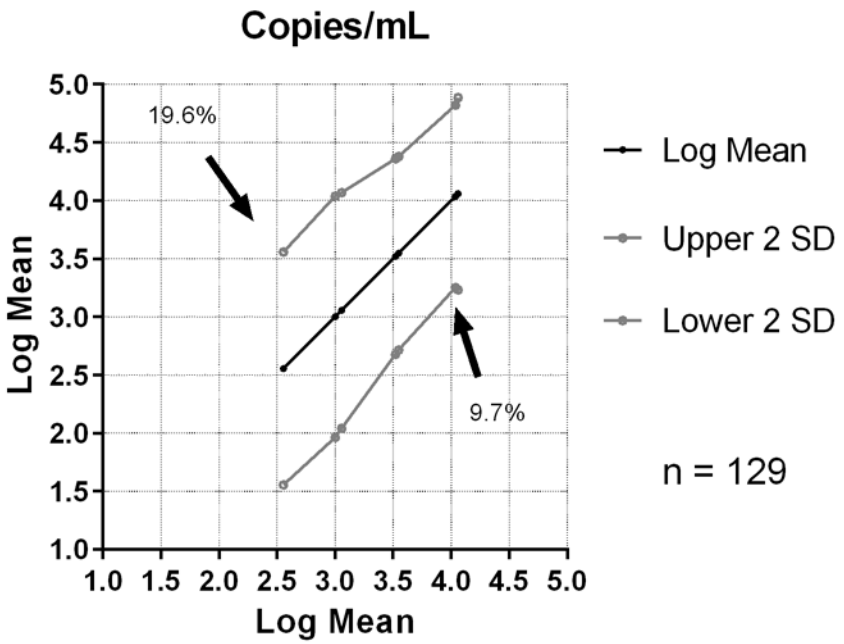
CAP Results –

1st 2 Survey Sets 2015

Sample	Units	Log Mean	S.D.	2 S.D Range	# Negative
#2-05	IU/mL (n=18)	1.88	0.965	0.05 - 3.81	39%
	c/ml (n=60)	2.04	1.24	0.00 – 4.52	
#2-06	IU/mL (n=30)	3.41	0.33	2.75 - 4.07	
	c/ml (n=122)	3.60	0.452	2.72 - 4.52	
#2-15	IU/mL (n=22)	2.62	0.32	1.98 – 3.26	20.5%
	c/ml (n= 82)	2.74	0.78	1.18 – 4.30	
#2-16	IU/mL (n=33)	3.55	0.40	2.75 - 4.35	
	c/ml (n=137)	3.68	0.52	2.64 – 4.72	

QCMD 2014 Data





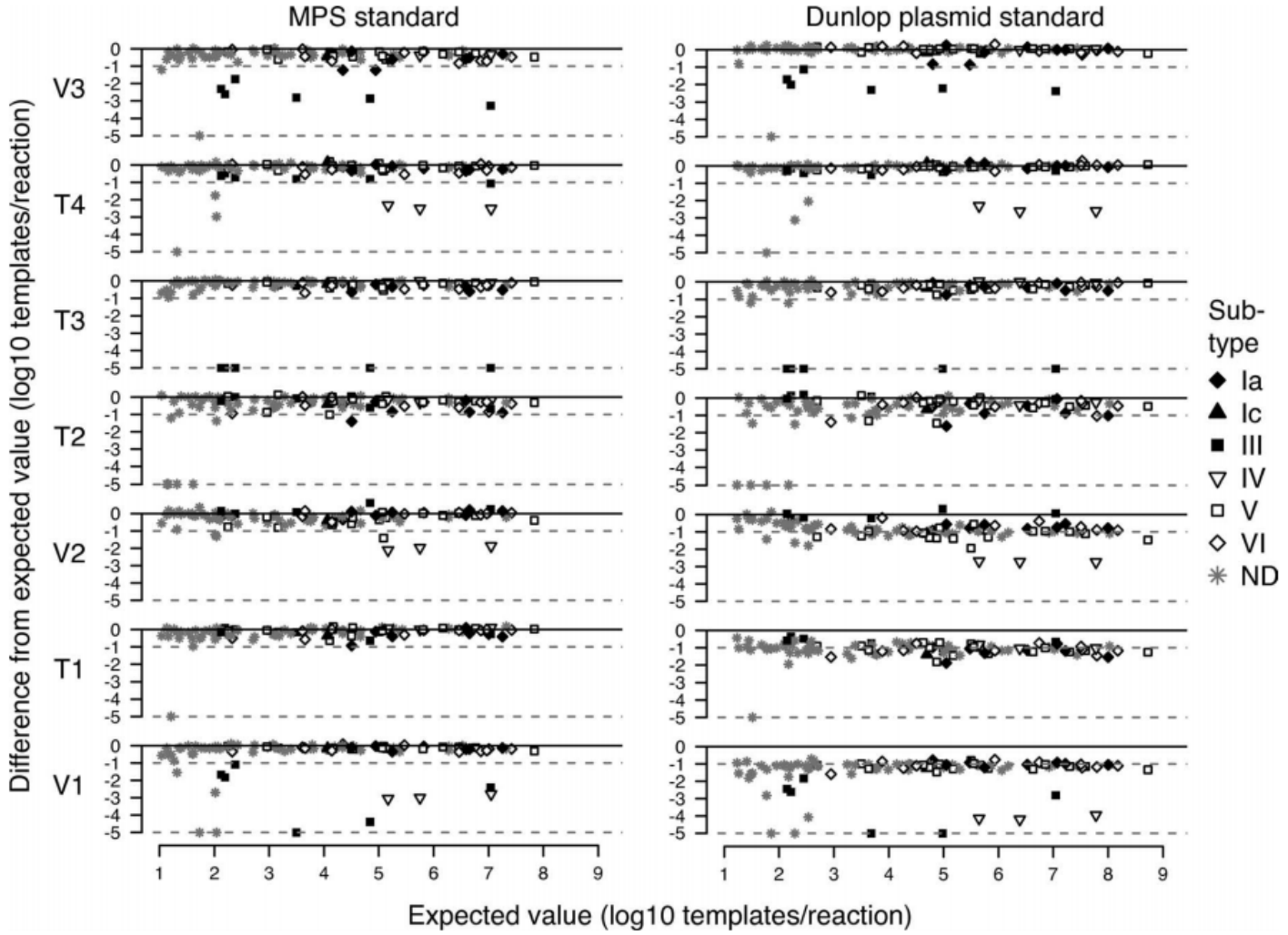
BK Virus

Samples
Primer / Probe Issues
Comparison Studies
Standard Issues

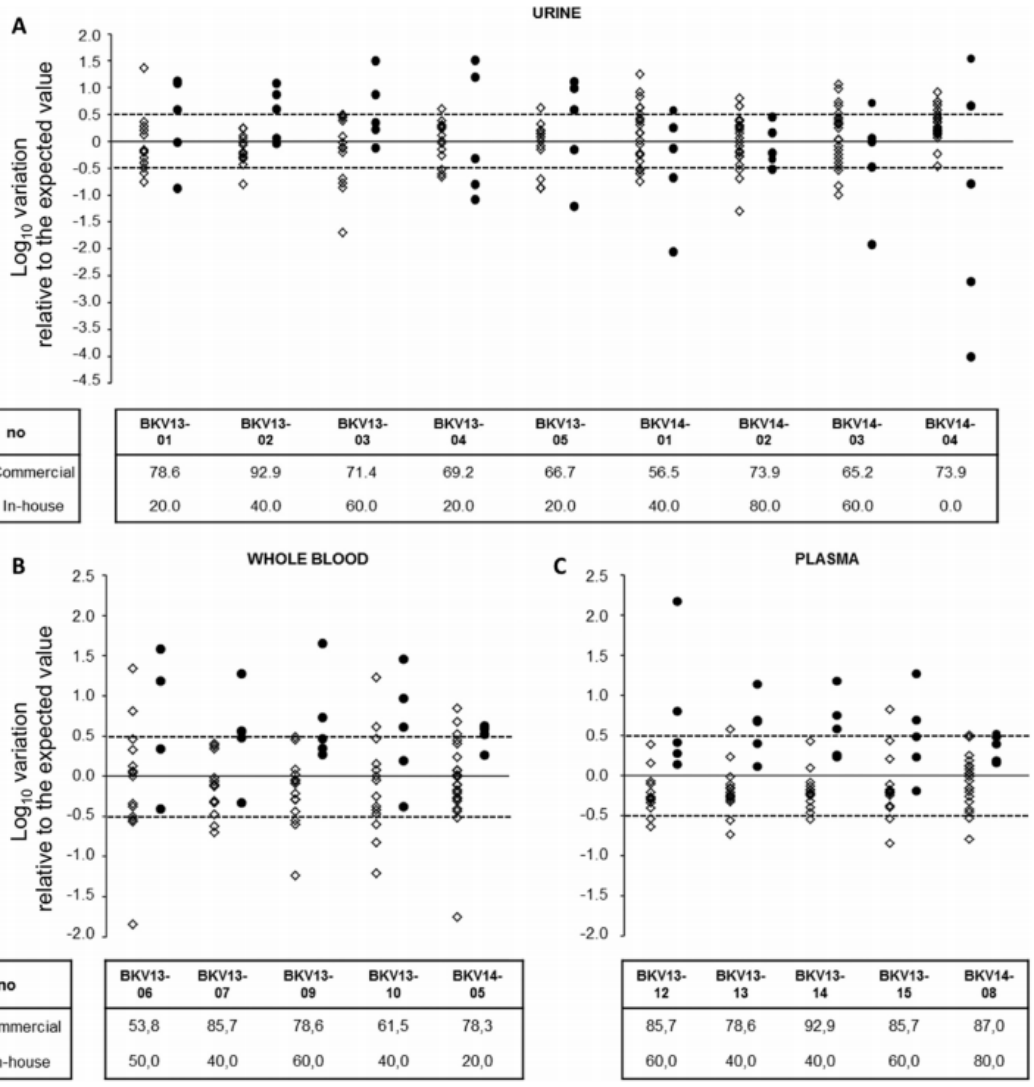
Sample Types

- Plasma
  - (Whole blood?)
- Urine
  - Extremely high levels found
  - >1 x 10e9 copies/ml
  - Extraction carryover issues

Primer Mismatches Hoffman 2008



Primer / Probe Issues



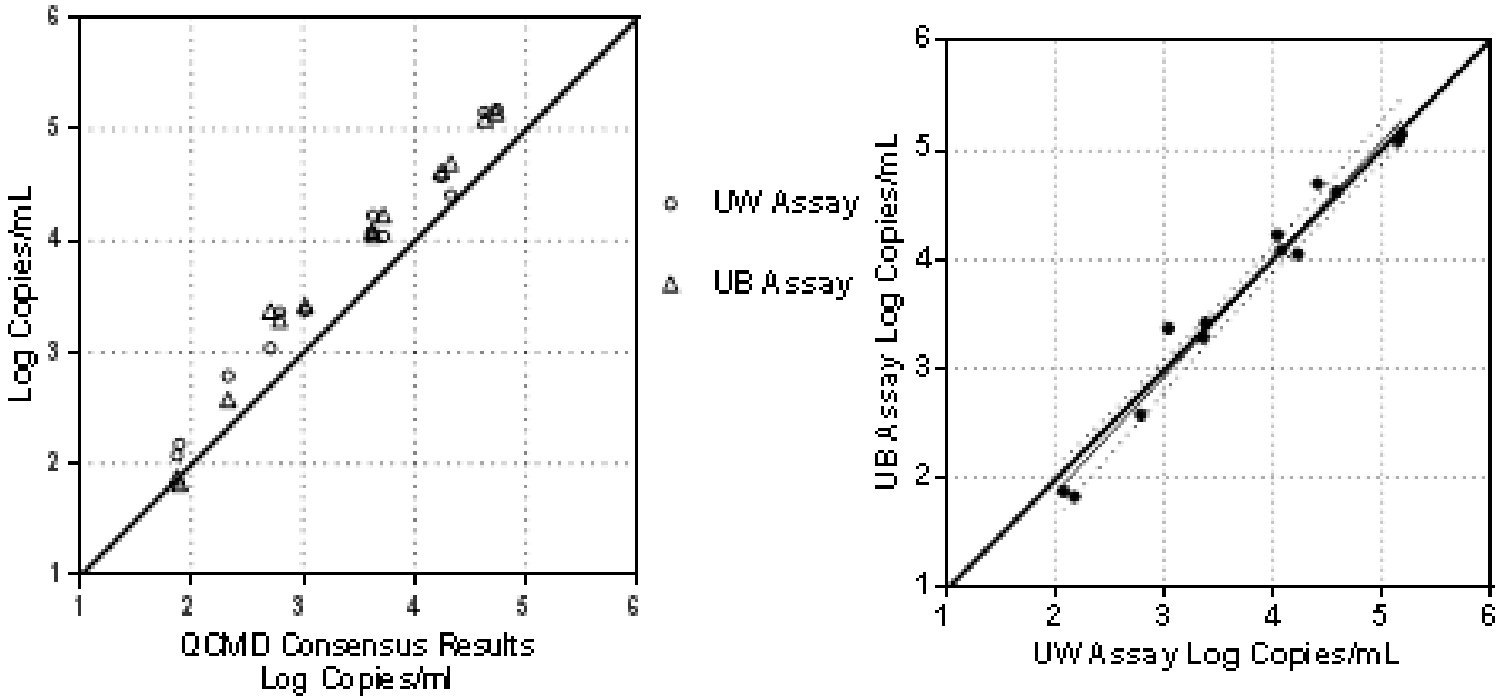
Samples no		BKV13-01	BKV13-02	BKV13-03	BKV13-04	BKV13-05	BKV14-01	BKV14-02	BKV14-03	BKV14-04
EV ± 0.5log	Commercial	78.6	92.9	71.4	69.2	66.7	56.5	73.9	65.2	73.9
	In-house	20.0	40.0	60.0	20.0	20.0	40.0	80.0	60.0	0.0

Samples no		BKV13-06	BKV13-07	BKV13-09	BKV13-10	BKV14-05
EV ± 0.5log	Commercial	53,8	85,7	78,6	61,5	78,3
	In-house	50,0	40,0	60,0	40,0	20,0

BKV13-12	BKV13-13	BKV13-14	BKV13-15	BKV14-08
85,7	78,6	92,9	85,7	87,0
60,0	40,0	40,0	60,0	80,0

Solis 2015
French BK Study Group
2 panels of WB, plasma and urine samples; Genotype polymorphisms contributed to significant variation in results (Genotypes II and IV)

Few Reports of Assay Comparisons



QCMD BK Data – UW vs Basel Lab

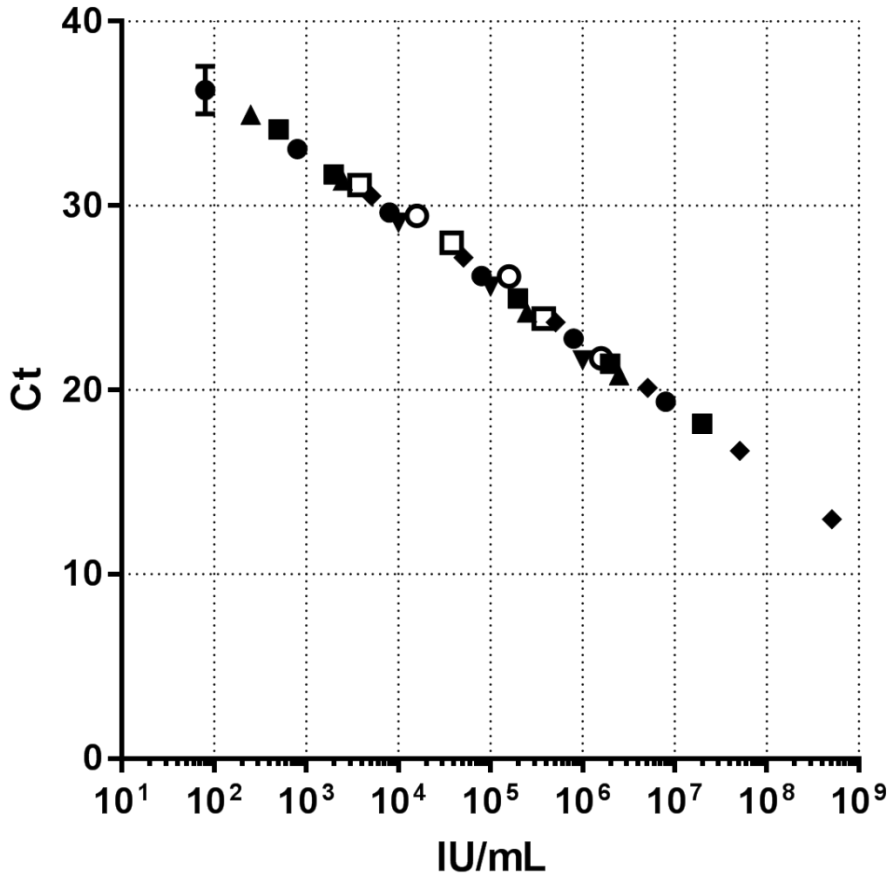
QCMD Results

BK Virus Samples	Log Quant Mean		Quant C.V.	Log Range
2016 – 01	3.673		42%	**0.0 – 6.14**
2016 – 02	4.707		22%	**3.21 - 7.27**
2016 – 03	3.69		38%	**0.78 – 6.46**
2016 – 04	Negative			
2016 - 05	2.69	11% Neg	55%	**0.00 – 5.92**
JC Virus Samples	**Log Quant Mean**		**Quant C.V.**	**Range (2SD)**
2016 – 01	2.52	15% Neg	26%	**1.08 – 3.68**
2016 – 02	3.14		14%	**1.87 – 4.38**
2016 – 03	Negative			
2016 – 04	4.49		18%	**2.75 – 5.94**
2016 - 05	3.42		20%	**1.79 – 4.46**

Evaluation of the BK WHO Standard

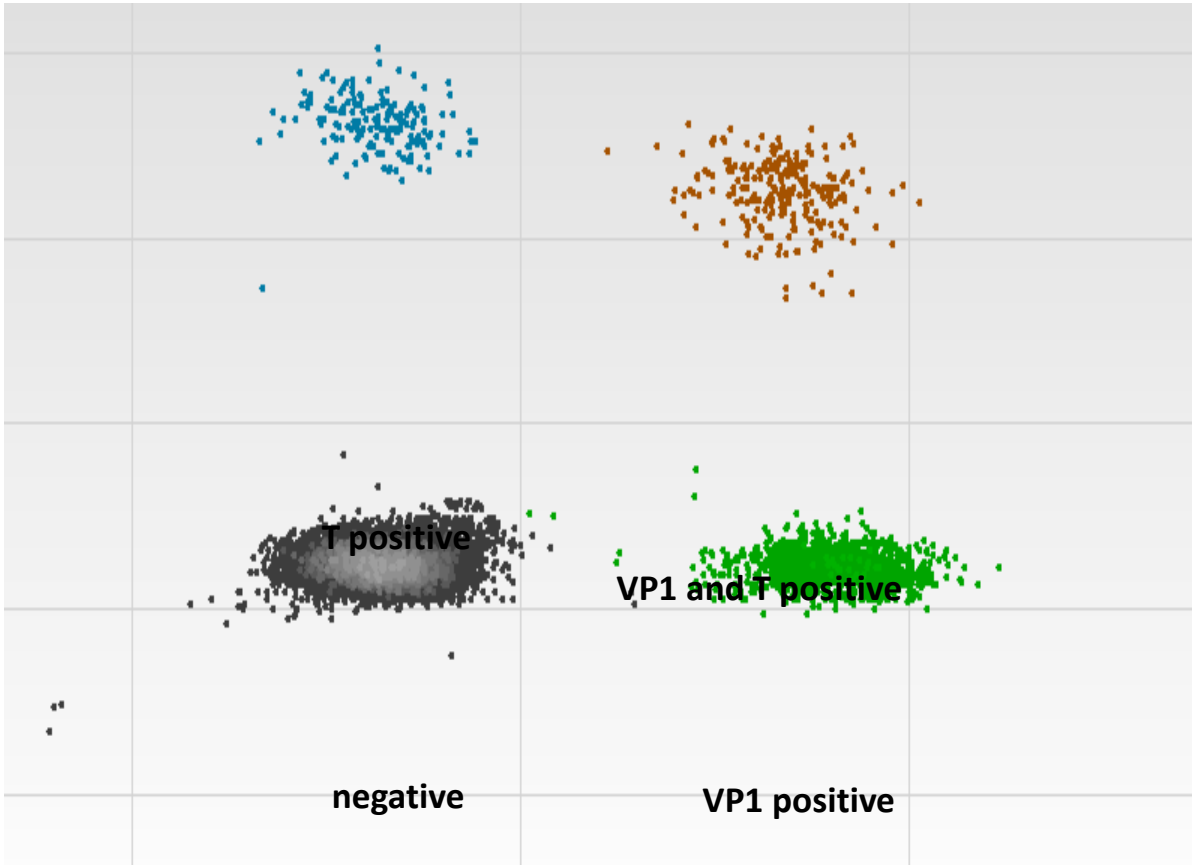
NIBSC 14/212

qPCR Data – 2 probe set mix



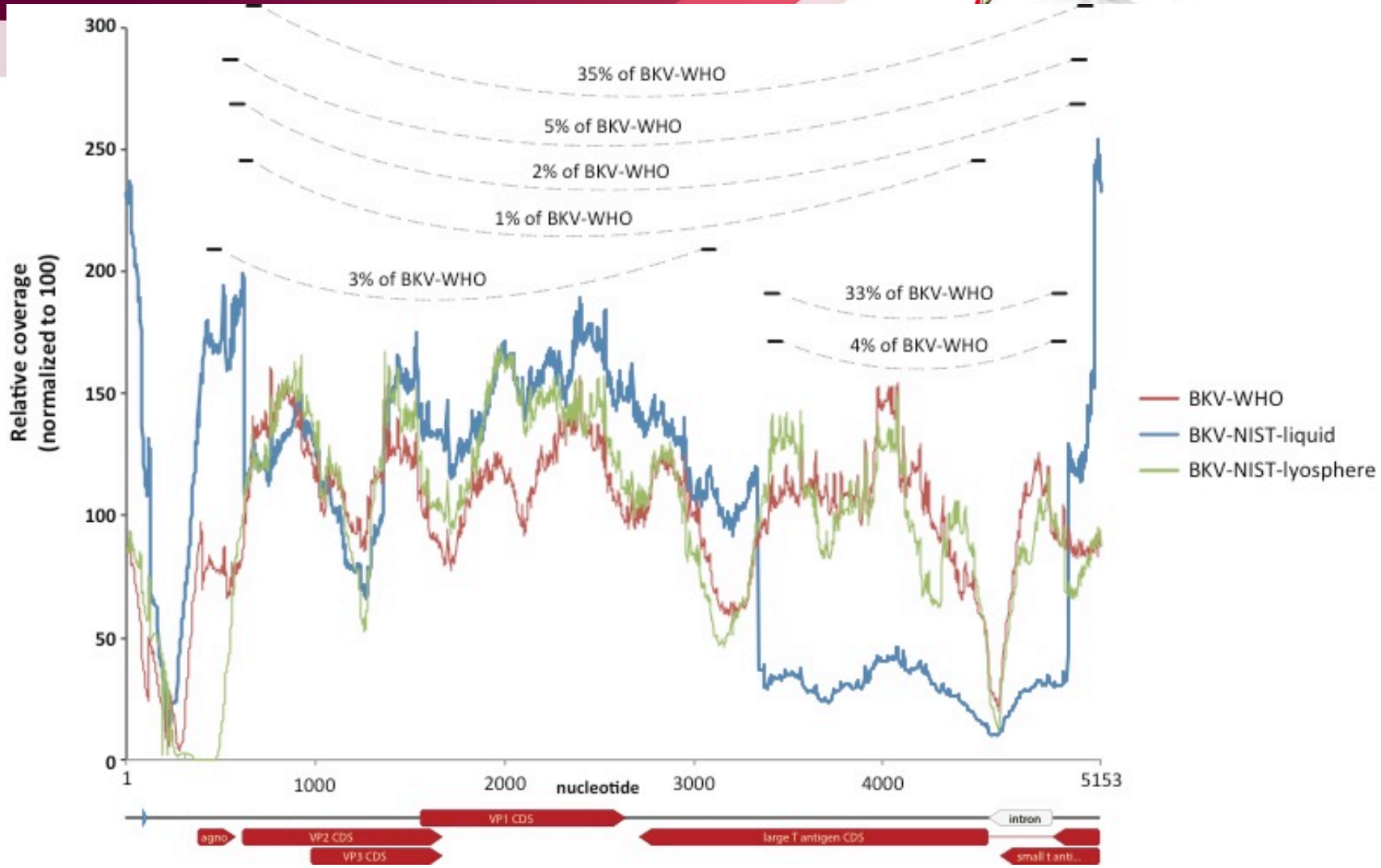
- WHO
- Exact Diagnostics
- Acrometrix
- Zeptometrix
- UW Plasmid Std (Copies)
- NIST #1 Copies
- NIST #2 Copies

ddPCR Results

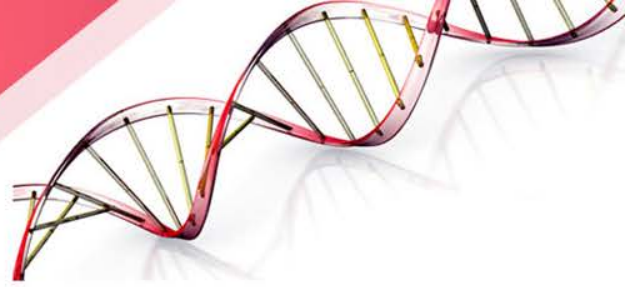


4× more VP1 positive droplets than T positive droplets

WHO-BKV shows 4X decreased coverage in T antigen



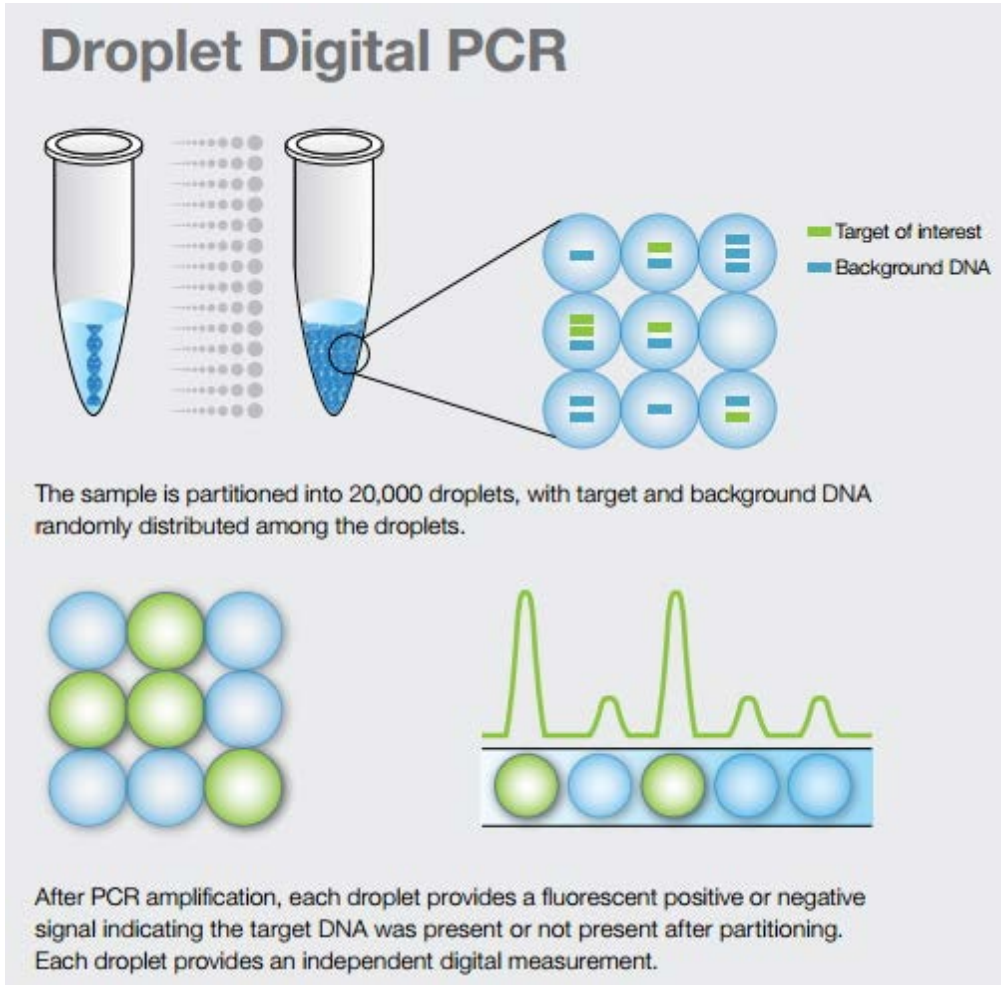
WHO Standard: BK



- Conclusions:
- 1. The standards have many subpopulations of virus present, with a significant percentage demonstrating large deletions in the T region.
- 2. Quantity of WHO material present will vary depending on the primer set used in qPCR assays.
  - Use of this standard may decrease between-lab agreement rather than improve it!

Questions?

ddPCR Quantitation



Much more precise quantitation of DNA!

Output in copies/ml