Endpoints in HIV

- Viral Load
  - Accepted Regulatory Endpoint in Viremic Subjects
  - Not useful in Aviremic subjects. Treatment Interruption viral set point in use in immunologic and cell therapy clinical trials for HAART treated subjects.
- Proviral DNA
  - Novel endpoint with unknown clinical significance.
  - Complicated by non-functional proviral DNA versus functional
  - Methylation status of LTR maybe important
- Reservoir Analysis for HIV
  - Novel endpoint with unknown clinical significance.
  - Include PCR quantification, viral co-culture etc
- **CD4 T cells**
  - Clinical trials using AIDs endpoints are large and long-term (SILCAAT)
  - Total number of CD4 and HIV specificity are being evaluated in HIV e.gl IL-7, Therapeutic vaccines
- Advances in Digital PCR and Deep sequencing are potential breakthrough technologies in the future.

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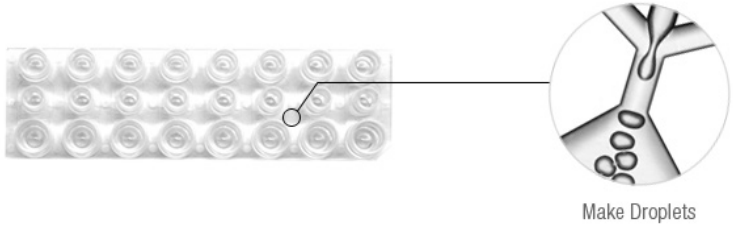
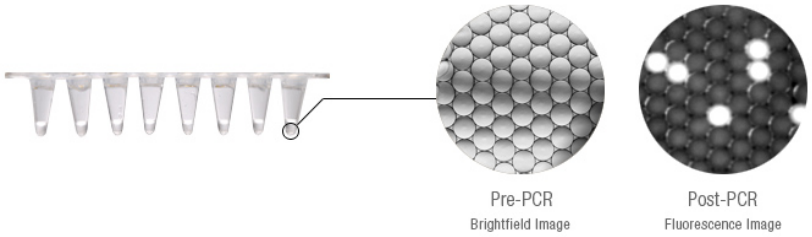
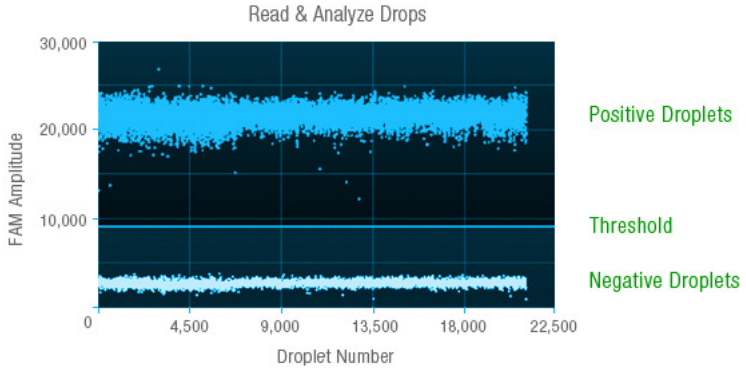
1. Digital Droplet PCR - Principles



Step 1. "Digitize" sample into ~15,000 droplets, effectively reducing level of background gDNA



Step 2. Run PCR to endpoint. Quantification no longer relies on PCR kinetics



Step 3. Fluorescent analysis drop by drop (yes or no). Copy number calculated by Poisson distribution

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2. Digital Droplet PCR Allows for More Precise Quantitation of Low Copy Number Events

- **Reduce genomic DNA inhibition**
  - For many HIV^{+} subjects, the HIV-DNA copy number per cell can be very low (0.1 to 0.01% of cells)
  - Quantitation of HIV DNA copy number by traditional qPCR often experienced inhibition due to the low ratio of HIV templates to background genomic DNA
  - By separating template DNA into > 10,000 droplets, the positive template (HIV DNA) to background DNA (genomic DNA) ratio is increased by 4 logs

- **Improve precision with digital PCR**
  - With digital PCR, reaction is carried out to completion (endpoint). Quantification is no longer dependent on PCR kinetics. PCR standard is NOT needed.
  - Concentration of templates is calculated by the number of positive and negative events (hence digital) using Poisson analysis

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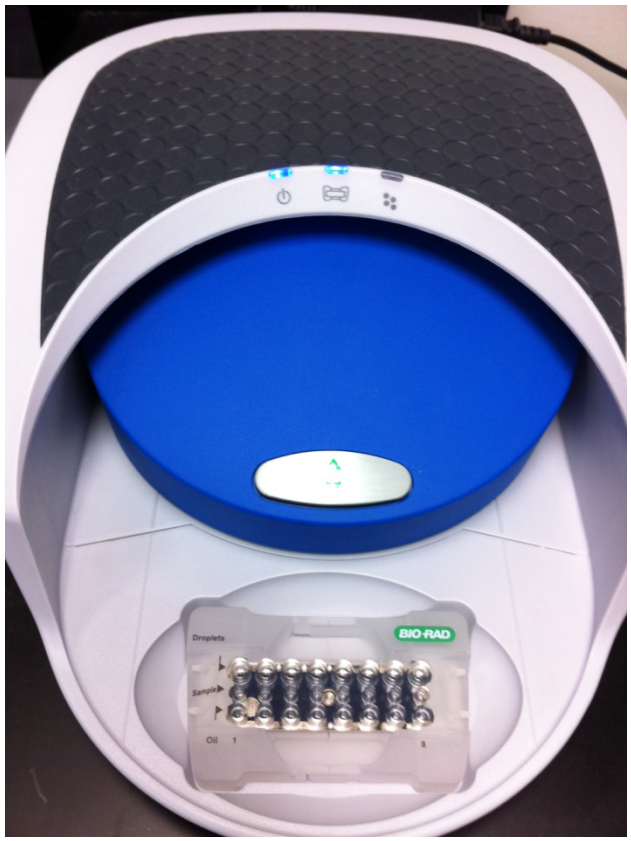
3. Digital Droplet PCR – Experimental Methods (1)

1. **Digest genomic DNA sample with DdeI (37°C, 1 Hr)**
2. **Prepare multiplex PCR Master mix with primer/ probes:**
   - HIV-gag (FAM)
     - Primer/ probe as described in Palmer et. al., JCM 2003
   - RNaseP (VIC)
3. **Prepare 2X PCR reactions, each with 500µg and 250µg gDNA (1.5mg total gDNA)**
4. **Generate droplets** (figure right)
5. **Perform conventional PCR**

Thermal Cycling Parameters	Cycles	Duration
95°C	1	10 min
94°C melt and 60°C anneal/extend	40	30 sec (melt) 1 min (anneal/extend)
98°C Heat inactiviation	1	10 min
4°C hold	1	indefinite



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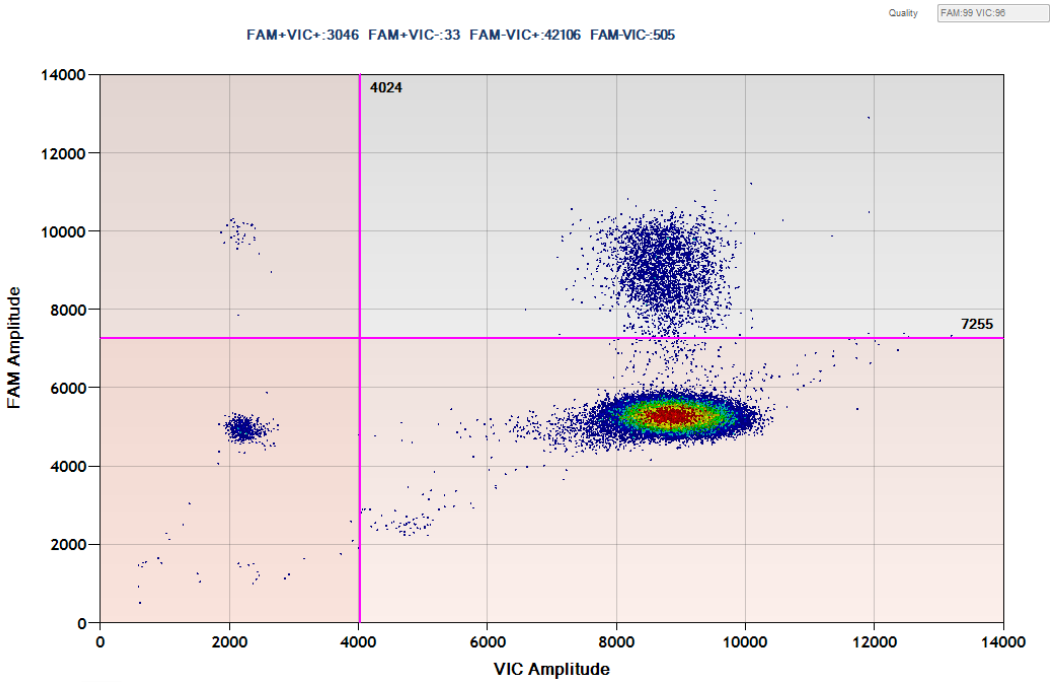
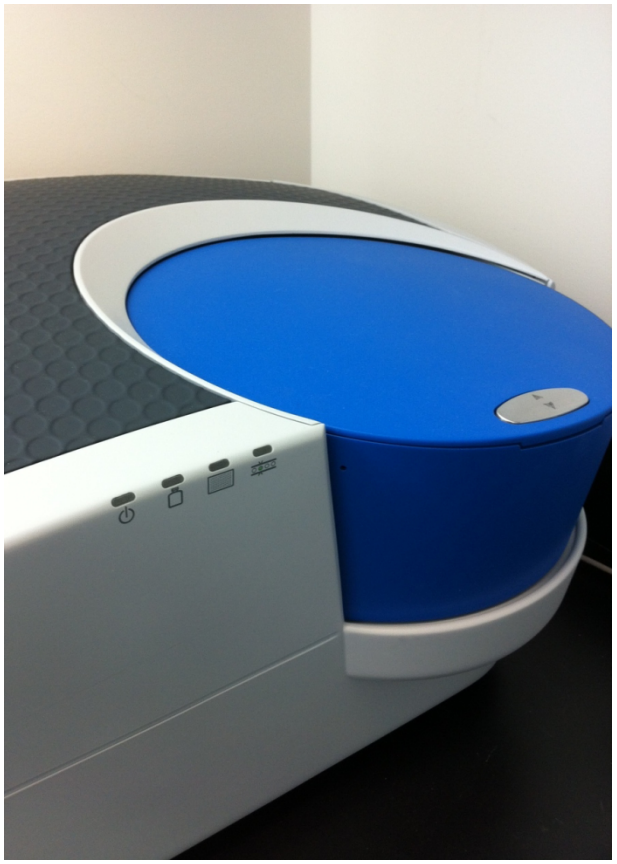
4. Digital Droplet PCR – Experimental Methods (2)

6. **Analyze droplets for +/- FAM and VIC fluorescent signal** (figure right)
7. **Copy number is determined by Poisson analysis** (figure below)





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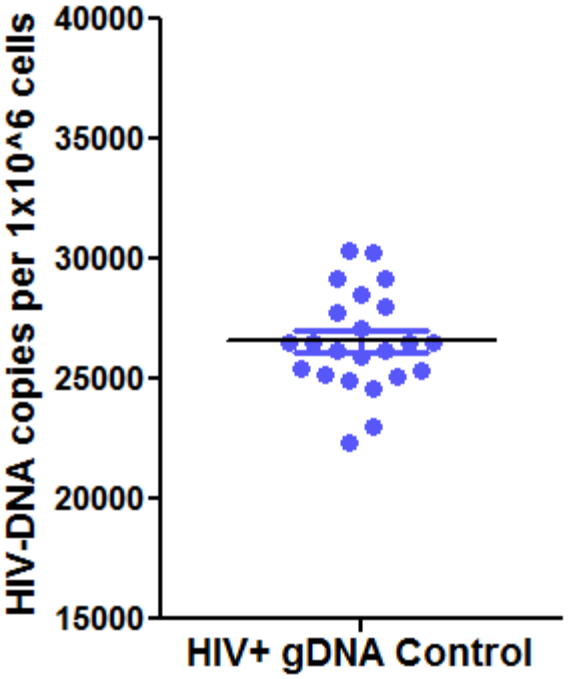
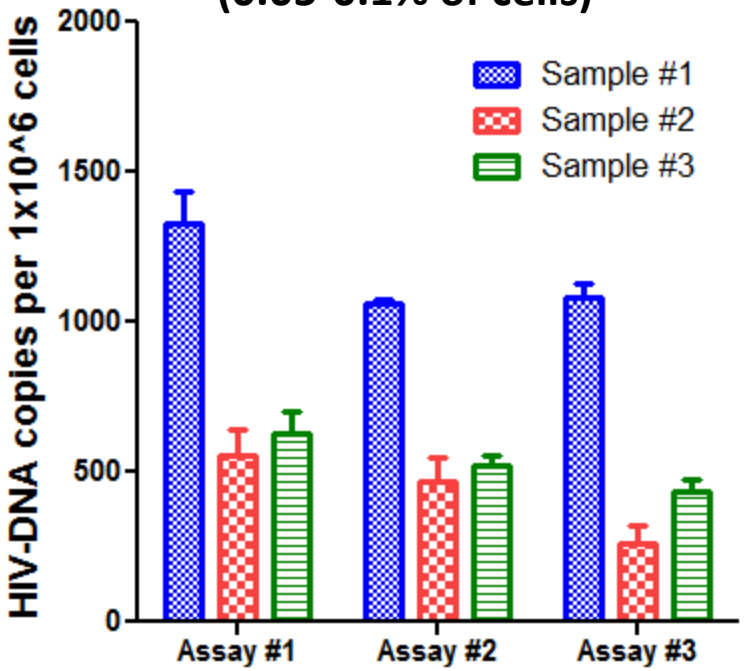
5. Precise Determination of HIV-DNA Copy Numbers

"High" HIV-DNA level (2.5% of cells)



- Mean = 26562 copies/1x10^6 cells
- Std Deviation = 2076
- %CV = 7.8%

"Low" HIV-DNA level (0.05-0.1% of cells)



- Intra-assay
  - %CV = 18%
- Inter-assay
  - %CV = 22%

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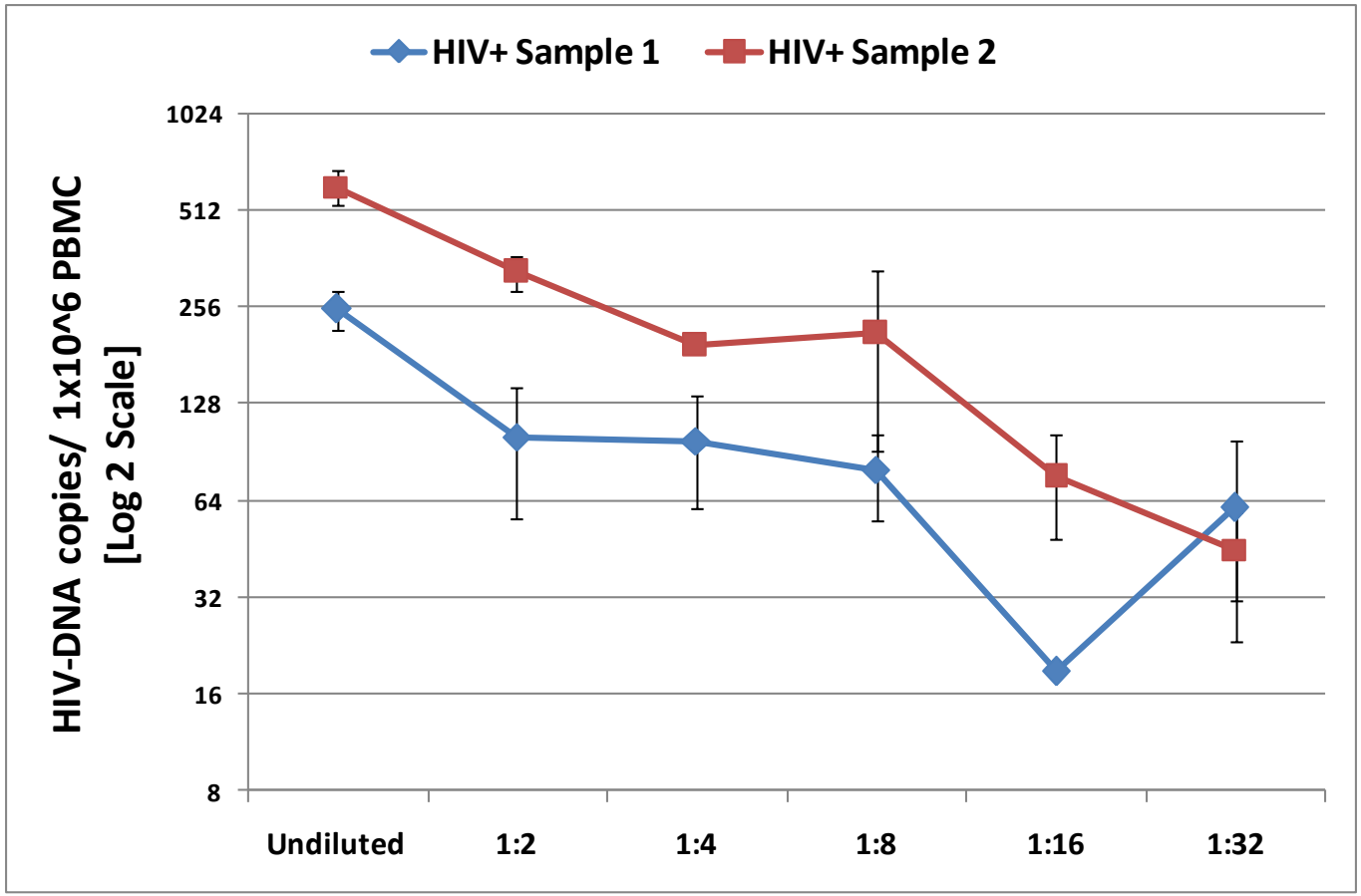
6. Assessing the Quantitative Limits of the HIV-DNA DD PCR Assay



- HIV+ gDNA samples were serially diluted with normal human liver gDNA (2-fold dilution)
- In two independent HIV+ gDNA samples evaluated, 2-fold dilutional linearity was observed down to 0.01% (or ~100 copies per 1x10^6 cells)

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8. Digital Droplet PCR Outperforms Conventional Taqman qPCR

	HIV-DNA/ 1e6 PBMC	
	DD PCR	Conventional Taqman
Sample 1	332	Undetected
Sample 2	396	Undetected
Sample 3	304	Undetected
Sample 4	378	Undetected
Sample 5	323	Undetected
Sample 6	82	Undetected
Sample 7	168	Undetected
Sample 8	165	Undetected
Sample 9	71	Undetected
Sample 10	91	Undetected
Sample 11	709	Undetected
Sample 12	505	Undetected
Sample 13	443	Undetected
Sample 14	339	Undetected
Sample 15	348	Undetected
Sample 16	39	Undetected
Sample 17	27	Undetected
Sample 18	14	Undetected
Sample 19	12	Undetected
Sample 20	53	Undetected
Sample 21	3250	192
Sample 22	3308	158
Sample 23	1430	Undetected
Sample 24	970	Undetected

- Conventional Taqman qPCR performed as described in Desire et. al., *J. Clin. Micro.,* Apr 2001, p.1303
- 24 PBMC samples obtained from HIV+ subjects were evaluated with both DD PCR and conventional Taqman:
  - HIV-DNA detected in all samples with DD PCR (range = 12 to 3300 copies per $1x10^6$ cells)
  - Conventional Taqman detected HIV-DNA in only 2 samples (samples with the highest HIV copy numbers according to DD PCR, boxed in red)

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