Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Method for the elucidation of LAMP products captured on lateral flow strips in a point of care test for HPV 16

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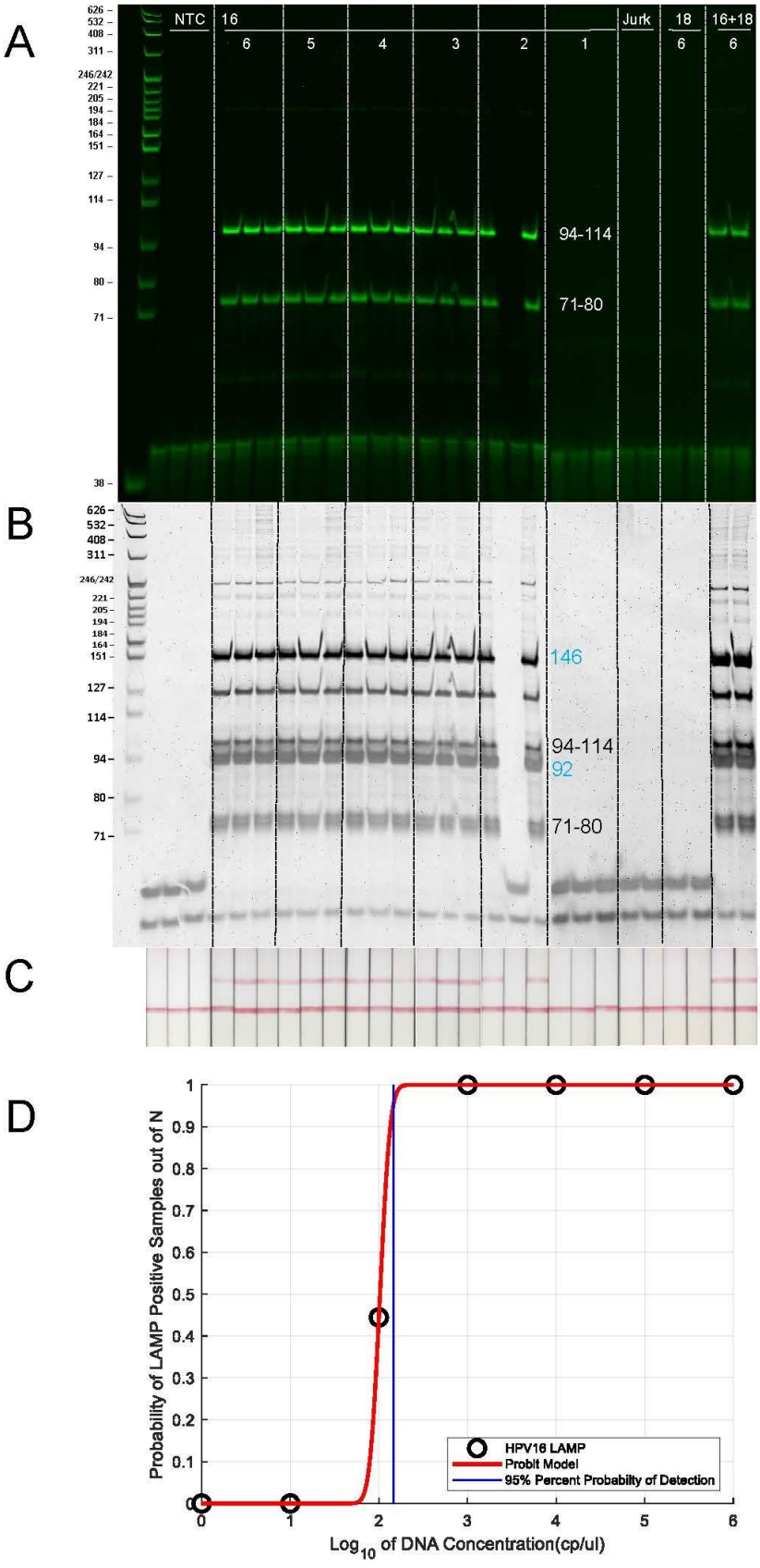
I. LAMP Limit of Detection

Materials and Methods for LAMP Lower Limit of Detection (LLOD)

MATLAB (Mathworks, Natick, MA, USA) was used to statistically analyze the LAMP HPV 16 plasmid LLOD results from 9 total replicates (3 biological experiments, each with 3 technicalreplicates). The probability of detection was calculated using probit analysis with a 95% probability of success cutoff [1, 2].

Limit of detection analysis was performed for HPV16 testing serial dilutions of HPV16 plasmid DNA from 10^6 cp to 10^1 cp. The experiment was replicated three times and each input was tested in triplicates. Figure S5 shows one of the three experiments performed. The positive amplification results from all three experiments were fed into the MATLAB probit analysis to generate a probability of detection plot. The LLOD with 95% detection probability was $10^{2.16}$ or 144.54 copies/µl (Fig. S5). LAMP assays have the advantage of utilizing 6 primers to amplify DNA and thus results in a more sensitive and efficient method of amplifying low concentrations of DNA.


A
NTC
16
6
5
4
3
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1
Jurk
18
6
16+18
6
626
532
408
311
246/242
221
205
194
184
164
151
127
114
94
80
71
38
94-114
71-80
B
146
94-114
92
71-80
C
Test Line
Control Line
D
Probability of LAMP Positive Samples out of N
Log10 of DNA Concentration(cp/ul)
HPV16 LAMP
Probit Model
95% Percent Probabilty of Detection

Fig. S1 LLOD for LAMP on Plasmid HPV 16. Serial dilutions of HPV 16 plasmid were tested to determine the lowest limit of detection using a probit analysis. HPV16 concentrations tested were 6: 10^6 cp/µl, 5: 10^5 cp/µl, 4: 10^4 cp/µl, 3: 10^3 cp/µl, 2: 10^2 cp/µl, : 10^1 cp/µl. The negative controls were no template control (NTC), Jurkat cell DNA (100 ng), and HPV18 (10^6 cp/µl). The mixed sample was HPV16+18, 10^6 cp/µl. A) Unstained denaturing gel image showing FAM bands for positively amplified samples at 94-114 bp and 71-80 bp. The lower FAM band that is visible is primers that have not amplified during the LAMP reaction, confirmed by size of the primers. B) SybrGold stained gels show all DNA bands but removes FAM specific information. Sequenced bands 146 and 92 are present. C) LFS results corroborates the FAM bands in A. FAM specific bands results present a positive test line. D) Probit model of three replicated experiments where each condition is run in triplicates

II. **Cobas 4800 BD SurePath Clinical Samples and PCR**

Table S1 PCR primers and probe sequences for HPV 16 and RNaseP

HPV 16	**Sequence**
Forward Primer	AGC TCA GAG GAG GAG GAT GAA
Reverse Primer	GGT TAC AAT ATT GTA ATG GGC TC
Probe	/5Cy5/CCAGCTGGACAAGCAGAACCGG/3IABkFQ/
RNaseP	**Sequence**
Forward Primer	CATGAGGTTGGCCAGGCGCG
Reverse Primer	GGGACTTCAGCATGGCGGTGT
Probe	/5HEX/TTCTGACCTGAAGGCTCTGCGCG/3BHQ_1/

Methods for PCR Quantification Identifies Viable Clinical Samples

We correlated the cobas-classified, clinical sample discards (deemed either cobas-negative or cobas-positive upon receipt from BARC) to the HPV 16 DNA concentration of each sample. After extracting each sample, a qPCR assay was performed on 110 clinical samples to quantify the concentration of HPV 16 in the samples as a basis of comparison to the LAMP results. For each extracted sample, we asked whether the E7 locus for HPV 16 was present and samples were validated for human tissue by assaying for RNaseP. Our results identified 80 valid clinical samples which were plotted and analyzed in Figure 2. The excluded samples had stochastic or undetermined concentrations of RNaseP DNA (ESM 2). In the analyzed sample pool, samples were deemed negative for HPV 16 if the qPCR result was undetermined and a "zero" value of $\log_{10}(0.01)$ cp/µl was assigned to them. Further details on statistical analysis performed are discussed in the supplementary materials (ESM 2).

Methods for PCR Clinical Sample based limit of detection

Using a binomial logistical model function (Equation 1), we correlated the known cobas 4800 HPV Test results to the quantified qPCR results using Matlab's "fitglm" toolbox (ESM 2)[3]. This compares the binary categorical results of the cobas 4800 data to the concentrations determined from the qPCR

results. The probability curve *P* was fitted by determining the coefficients constants a and b as a function of known values DNA concentrations x obtained from the qPCR standard curves.

$$P = \frac{1}{1+e^{-(a+bx)}} \qquad \text{Equation 1}$$

Based on the P-curve, we constructed a receiver operating characteristic (ROC) curve. and the resulting . The max Youden's index (Equation 2), YI_{max}, for the ROC curve was calculated using the sensitivity (Se) and specificity (Sp) determined using the binomial logistical model.

$$YI_{max} = Se + Sp - 1 \qquad \text{Equation 2}$$

Using the PCR-derived Youden's index as the new metric for binomial classifications, we re-classified all clinical samples as either "PCR-negative" or "PCR-positive" across all DNA concentrations x.

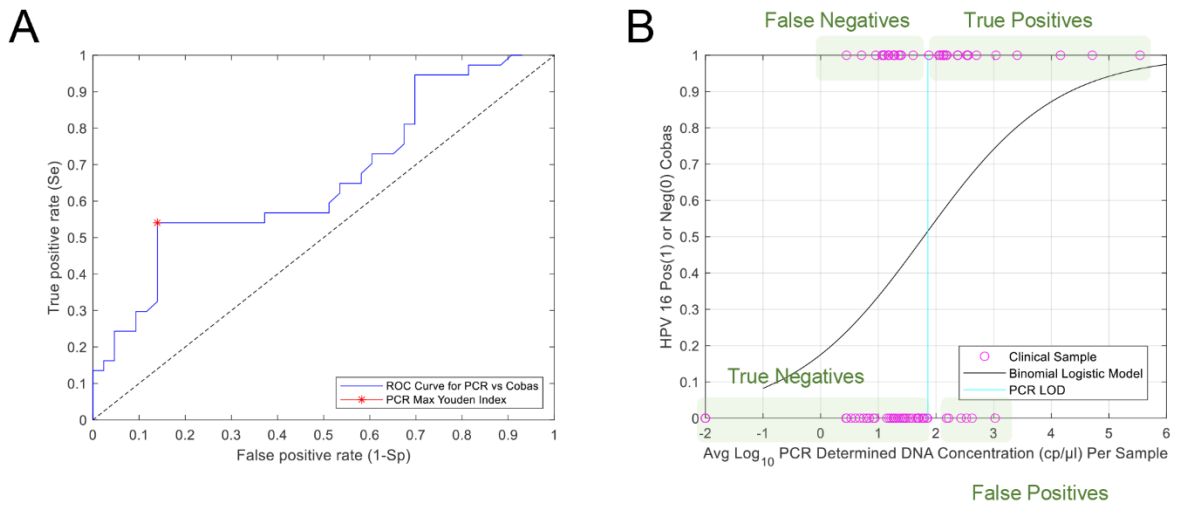

Fig. S2 Quantitative PCR results on clinical samples. Cobas 4800 HPV Test results are compared to PCR quantitative results amplified from extracted clinical samples. A) ROC curve for all possible thresholds on the binomial logistic model for PCR. The max Youden's index is 0.401. B) Binomial logistic regression fit of log_{10} HPV 16 DNA concentrations compared to Cobas binary data set of positive or negative for HPV 16. Using A we can determine a max Youden's index value to determine a PCR limit of detection, $10^{1.86}$ copies/µl or 72.44 copies/µl, shown in B. Each PCR reaction uses a total of 5 µl of extracted clinical sample; for each PCR reaction the threshold concentration is 362 copies/reaction. The 2x2 contingency table indicates an 86.0% specificity and 54.1% sensitivity (cobas 4800 vs qPCR). Compared to the results from the cobas 4800 assay, our PCR was performed on previously tested and discarded samples with an unknown sample collection to extraction time. Although these variables exist, according to previous

research this should not affect extraction efficiency if appropriate sample preparation is considered [4, 5]. We provide the sensitivity and specificity for our PCR results to provide transparency on the difference between the results, however our results are based on quantitative analysis of varied sample concentrations of the discarded samples we acquired

III. Cobas 4800 BD SurePath Clinical Samples and LAMP

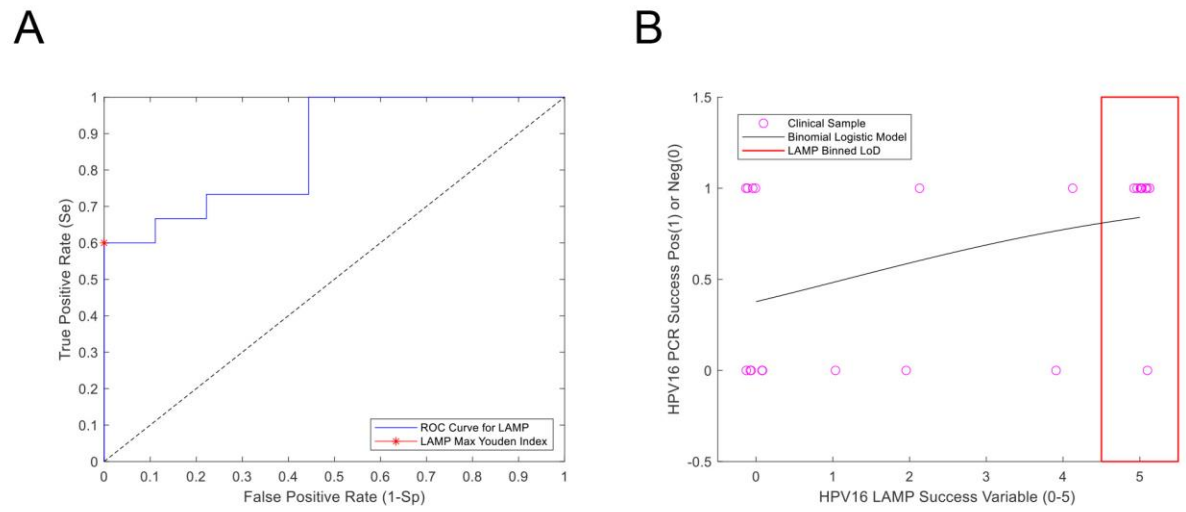

Fig. S3 Statistical categorization of LAMP-LFS negative and LAMP-LFS positive samples. A detection threshold for replicates was calculated for LAMP on extracted clinical samples with an ROC curve and binomial logistical regression. This limit of detection identifies the number of positive samples required out of technical replicates to be considered a true positive result. Each clinical sample was assigned a value from 0-5 depending on the number of positive results from the quintuplicates run. This bins the data into 6 categories, and the reference conditions are the PCR determined positive and negative samples. The max Youden's Index value is 0.6 and the LAMP binned LOD was determined to be 5; in a case where 5 out of 5 replicates are run for a single sample are positive the sample is classified to be a true positive for LAMP (LAMP-LFS positive). Although in a point of care setting it is unlikely to perform technical replicates for a single sample, it is determined that a positive sample can be definitively identified using fluorescently tagged loop primers in LAMP and can be confirmed by running replicate follow up experiments

References

1. Waggoner JJ, Abeynayake J, Balassiano I, Lefterova M, Sahoo MK, Liu Y et al. Multiplex nucleic acid amplification test for diagnosis of dengue fever, malaria, and leptospirosis. J Clin Microbiol. 2014;52(6):2011-8. doi:10.1128/JCM.00341-14.
2. Canchola JAH, Pari. Limit of Detection (LoD) Estimation Using Parametric Curve Fitting to (Hit) Rate Data: The LoD_Est SAS® Macro. SAS. 2016;Global Proceedings(Paper 1720-2016).
3. Kim HY. Statistical notes for clinical researchers: logistic regression. Restor Dent Endod. 2017;42(4):342-8. doi:10.5395/rde.2017.42.4.342.
4. Krevolin MD, Hardy D, Pane J, Aslam S, Behrens CM. Development and Validation of a Preanalytic Procedure for Performing the cobas HPV Test in SurePath Preservative Fluid. J Mol Diagn. 2017;19(2):288-94. doi:10.1016/j.jmoldx.2016.10.003.
5. Qiagen. DNeasey Blood & Tissue Handbook. 2006.