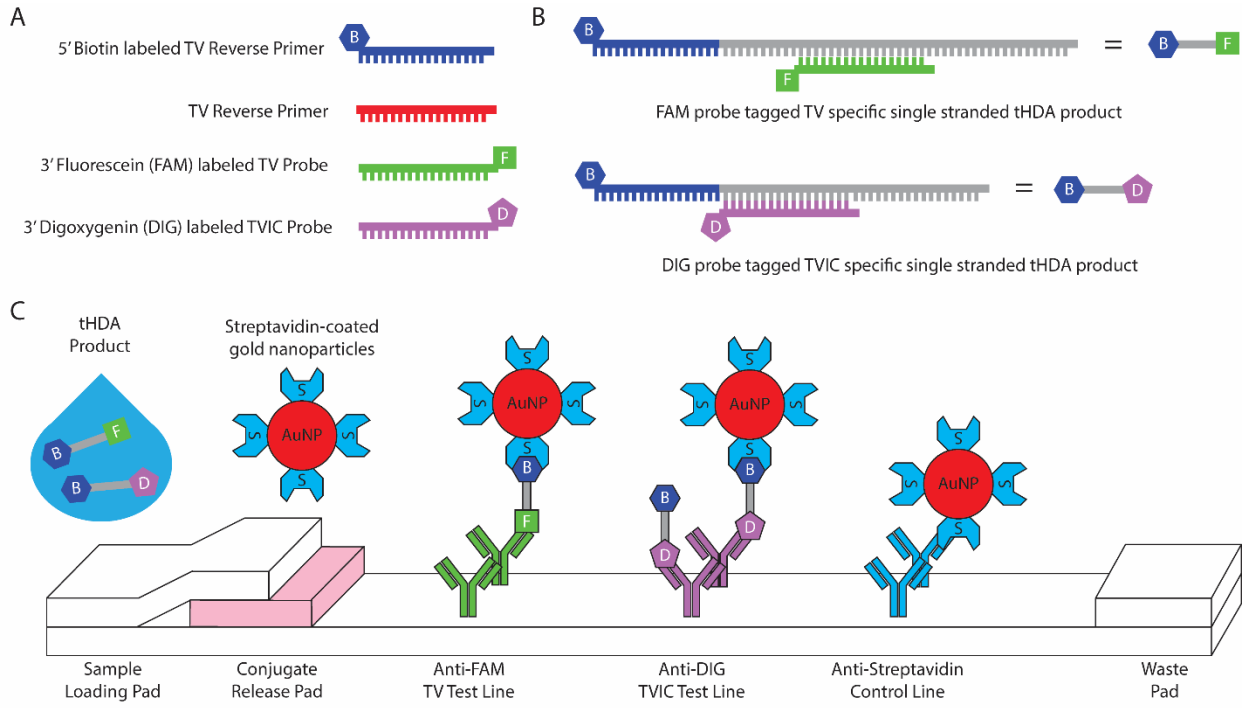
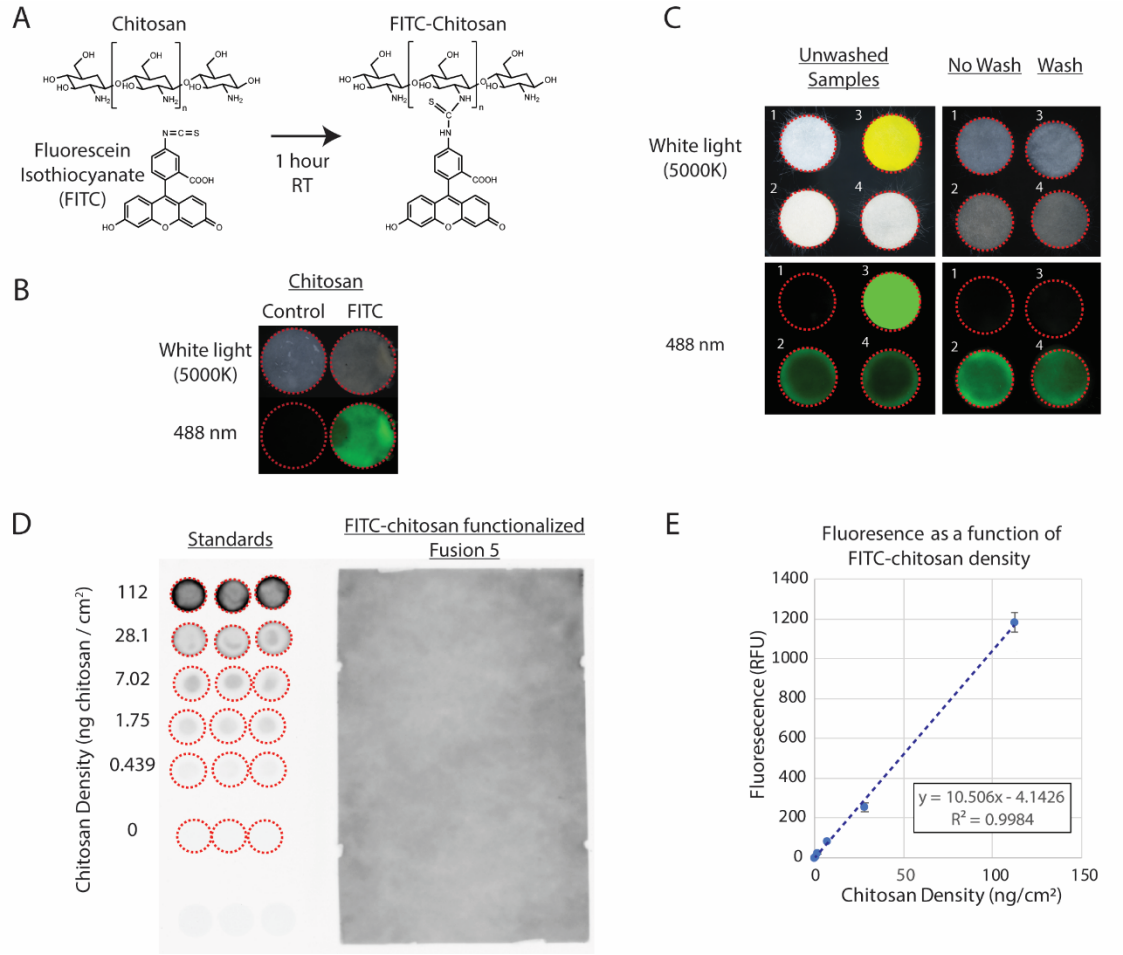
Electronic Supplementary Material (ESI) for Analytical Methods.

Figure S1: Detection of tHDA products using a LFIA strip



(A) 2 oligonucleotide primers and 2 probes are required for amplification and detection of the TV and TVIC tHDA products. A 5' biotin-tagged reverse primer and an untagged forward primer are used to amplify a conserved repeated sequence in the TV genome or an engineered sequence in the TVIC control plasmid. These double stranded tHDA products are tagged only with a biotin moiety and are not detectible by LFIA. (B) When the forward primer is exhausted, excess single stranded biotin tagged tHDA products accumulate in the reaction and can bind in a sequence specific manner to either a 3' FAM tagged probe specific to the TV tHDA product or a 3' DIG tagged probe specific to the TVIC product. At low concentrations of template, we have observed that the tHDA reaction halts prior to becoming primer limited. In this case, insufficient single stranded tHDA product is generated and thus no LFIA detectable products are formed. A simple melting step can be performed to denature double stranded tHDA products and allow for FAM and/or DIG tagged probe incorporation. (C) tHDA product is applied to the sample loading pad of a commercial lateral flow immunoassay. Wicking action drives flow of the tHDA product into a conjugate release pad which is preloaded with streptavidin-coated gold nanoparticles. tHDA products couple to the gold nanoparticles via biotin streptavidin binding. As the sample wicks along the lateral flow immunoassay, the sample encounters antibody test lines specific for FAM and DIG and a flow control anti-streptavidin line. If present in a given sample, dual tagged tHDA products cause visible aggregation of gold nanoparticles at their respective test lines.

Figure S2: Quantification of chitosan deposition density



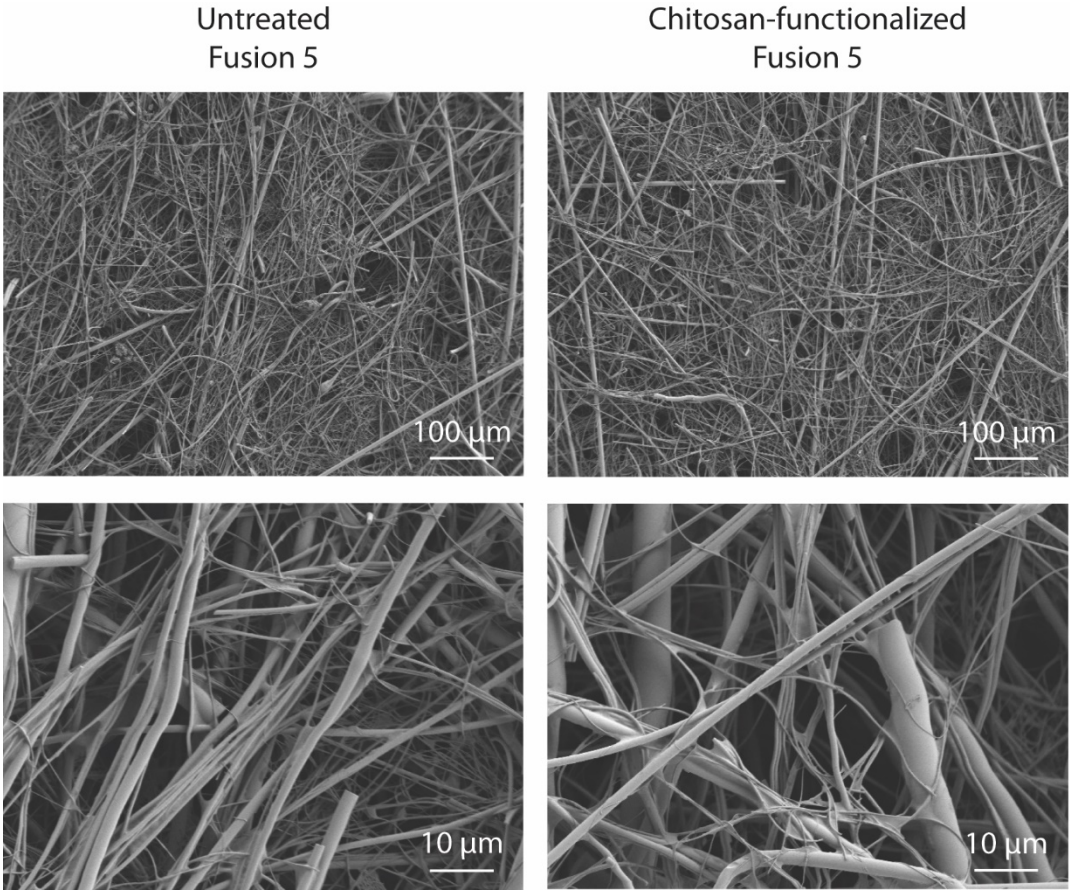
In order to quantify the deposition density of chitosan on the Fusion 5 substrate via densitometry, chitosan was tagged with fluorescein isothiocyanate (FITC). Chitosan was dissolved in acetic acid (0.1 M) to a concentration of 1% (w/v). FITC was dissolved in methanol at a concentration of 1 mg/mL. Equal volumes of chitosan and FITC solutions were mixed and incubated at room temperature for 1 hour to allow for covalent FITC tagging of chitosan (figure S2A). Following incubation, FITC-tagged chitosan was precipitated via the addition of 1 mL of 1 M NaOH per 25 mL of chitosan solution. Chitosan was pelleted by centrifugation at 4000 RCF for 2 minutes and the supernatant containing unreacted FITC was discarded. To remove additional unreacted FITC, A wash of 30 mL of 0.2 M NaOH was then added to the chitosan pellet and the solution was vortexed for 5 seconds to resuspend the pellet. The sample was centrifuged as before and the supernatant was removed. This process was repeated 4 additional times with 0.2 M NaOH and 5 times with deionized water. The chitosan pellet was then resuspended to a final centration of 0.25% in 0.1 M acetic acid.

FITC-tagged chitosan was used to functionalize a sheet of NaOH treated Fusion 5 paper as explained in the Materials and Methods section. (B) 0.5 inch diameter samples of Fusion 5 treated with chitosan

(left) or FITC-tagged chitosan (right) under white light and 488 nm excitation; autofluorescence from the native chitosan was found to be negligible. (C) We ruled out the possibility of unintended adsorption of FITC onto the Fusion 5 substrate altering the chitosan functionalization process using a washout experiment. Four 0.375 inch diameter disks of Fusion 5 with varying treatments were used namely: (1) NaOH treatment only (2) NaOH treatment, saturated with 200 mg per mL FITC in 0.1 M acetic acid and dried (3 & 4) FITC-tagged chitosan functionalization. All samples were soaked with Tris EDTA buffer (pH 8.0) and imaged with white light and excitation at 488 nm. Samples 2 and 4 were placed on a piece of blotting paper, washed with 5 mL of methanol by pipette, dried, and soaked with 100 mM Tris before reimaging. FITC was readily washed from the Fusion 5 substrate (sample 2) while chitosan-bound FITC was retained by the membrane (sample 4) indicating that adsorption of FITC-tagged chitosan onto the Fusion 5 substrate was mediated by the chitosan rather than FITC.

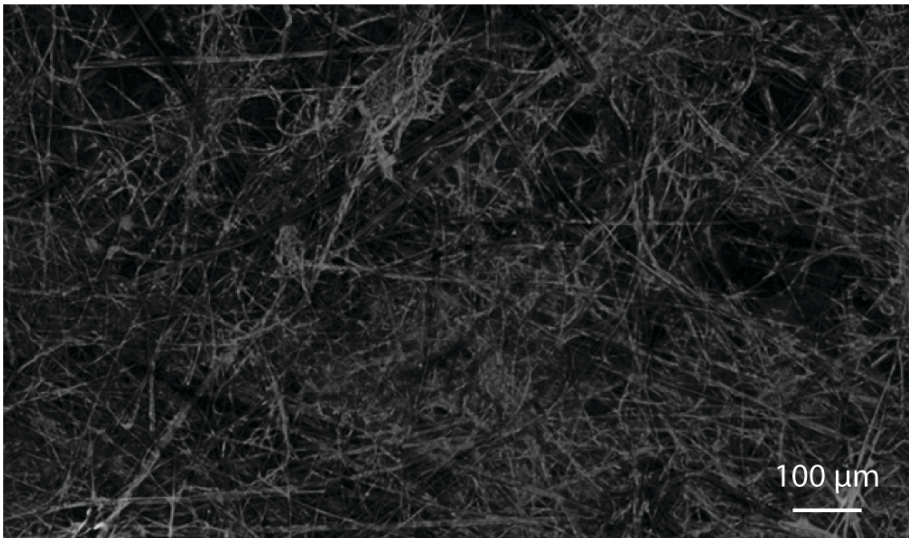
(D) To create reference standards, FITC-tagged chitosan was serially diluted fourfold from 0.25 – 0.00098% (w/v) in 0.1 M acetic acid. 32 µL aliquots of each standard solution as well as a negative control were applied to 3 separate 0.375 inch diameter disks of NaOH washed Fusion 5 and dried under vacuum at 50°C for 2 hours. Standards and a 7 cm x 11 cm piece of FITC-chitosan-functionalized Fusion 5 were wetted with Tris EDTA buffer (pH 8.0) and imaged on a VersaDoc MP 4000 molecular imager (Bio-Rad, Hercules, CA). (E) Densitometry was performed using ImageJ (NIH, Bethesda, MA) and a standard curve was generated after background correction. Using this standard curve, chitosan deposition density was found to be 45.9 ± 5.70 ng/cm^2.

Figure S3: SEM images of untreated and chitosan-functionalized Fusion 5 membranes



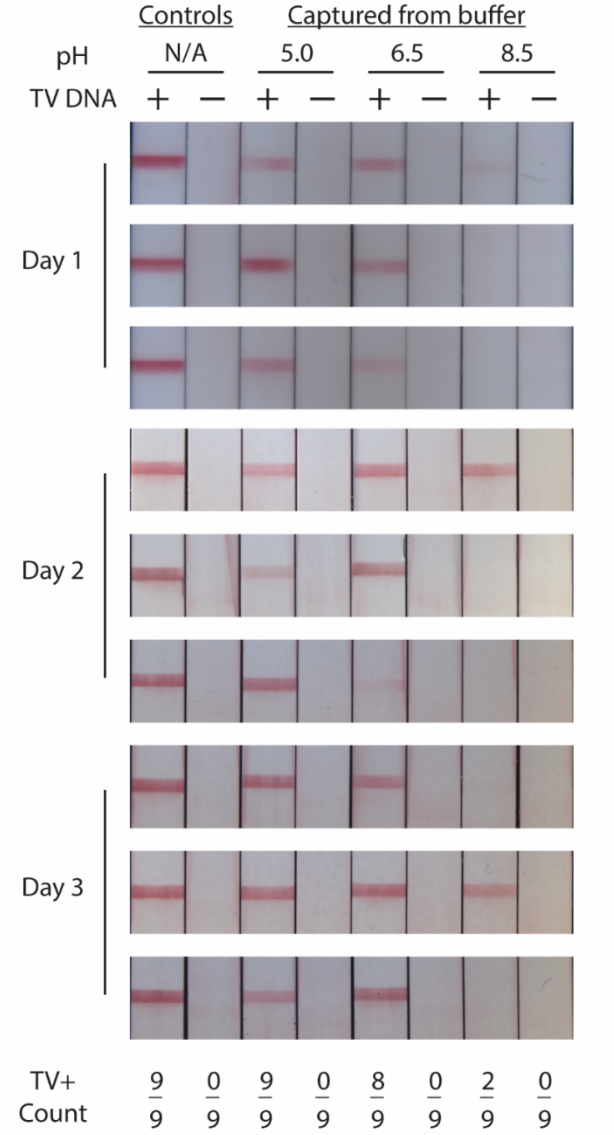
Samples of chitosan-functionalized and native Fusion 5 membranes were sputtered with gold (Cressington 108) and imaged via SEM (Zeiss Supra 40VP). No notable differences in morphology were observed between the treated and untreated conditions.

Figure S4: Confocal image of FITC-tagged chitosan on Fusion 5 substrate



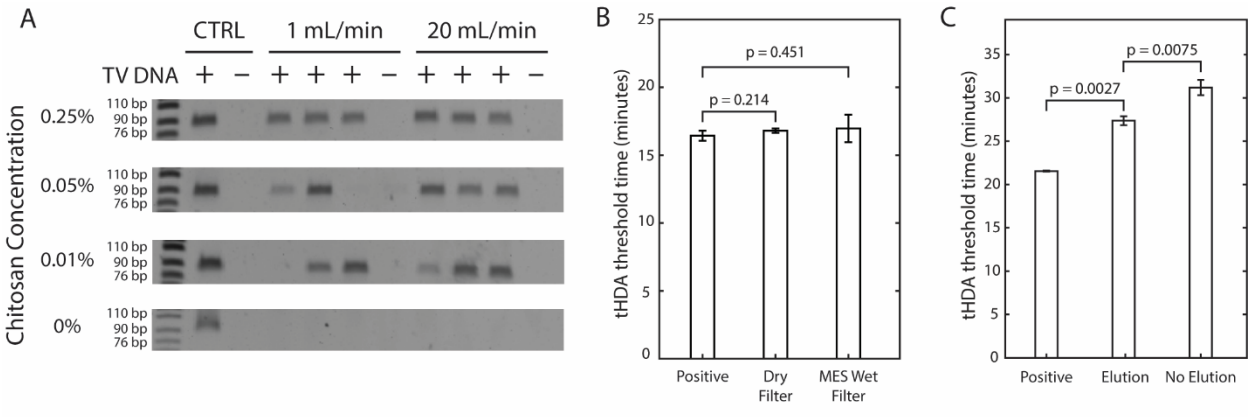
Fusion 5 membrane functionalized with FITC-tagged chitosan (see figure S2 for protocol) was soaked with Tris EDTA buffer pH 8.5 and imaged with a confocal microscope (Olympus FV3000) with excitation at 488 nm. 25 ROI were stitched together using ImageJ and the 3D viewer tool was used to assemble a 60 micron thick rendering of the membrane. The morphology of the fluorescently tagged chitosan is highly similar to the fibrous structure of the Fusion 5 substrate (figure S3).

Figure S5: Influence of pH on *Trichomonas vaginalis* DNA capture and detection by tHDA



Following treatment with sodium acetate, all clinical samples tested in this study were found to be between pH 5.0 – 6.5 by litmus test (PART NO.). To account for this variability, we examined the efficacy of our sample preparation strategy when carrier buffers of varied pH were used. Samples consisting of 50 mM MES buffer (pH 5.0), 50 mM MES buffer (pH 6.5), and 100 mM Tris buffer (pH 8.5) were prepared with a final concentration of TV DNA of either 1 or 0 genomic equivalents per mL. 1 mL of each sample was processed on our prototype before amplification with tHDA. We observed that TV DNA was consistently detected by our method when the carrier pH was 5.0 or 6.5 while TV positive samples at pH 8.5 were not detected.

Figure S6: Implementation of tHDA for detection of TV DNA captured on chitosan paper



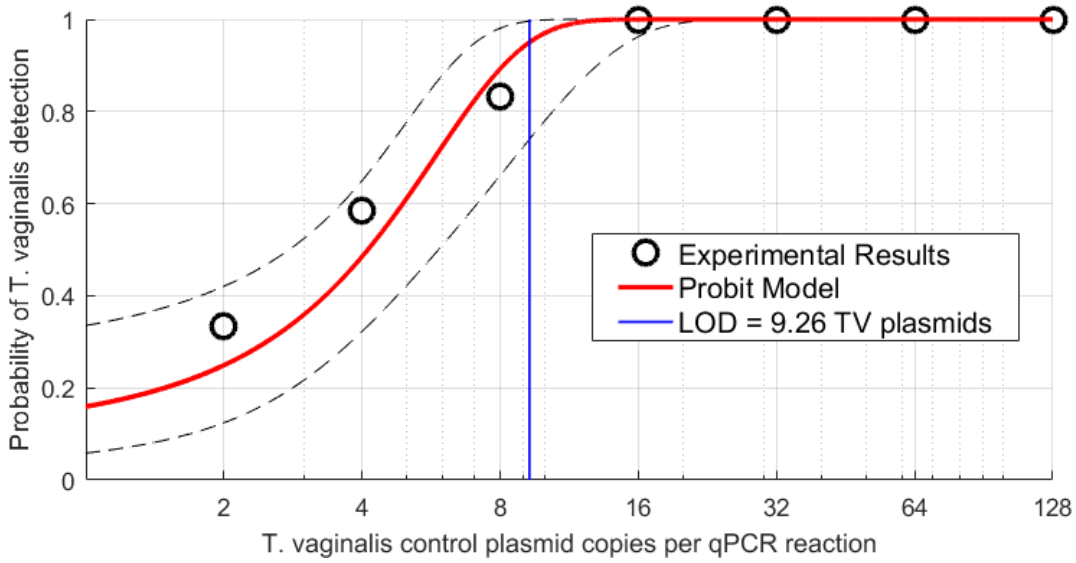
Initial characterization of DNA capture by our chitosan functionalized membrane (figure 3) was carried out at a flow rate of 1 mL per minute in the absence of the cellulose backing membrane. We examined if inclusion of the backing membrane and increase of flow rate from 1 mL per minute to 20 mL per minute

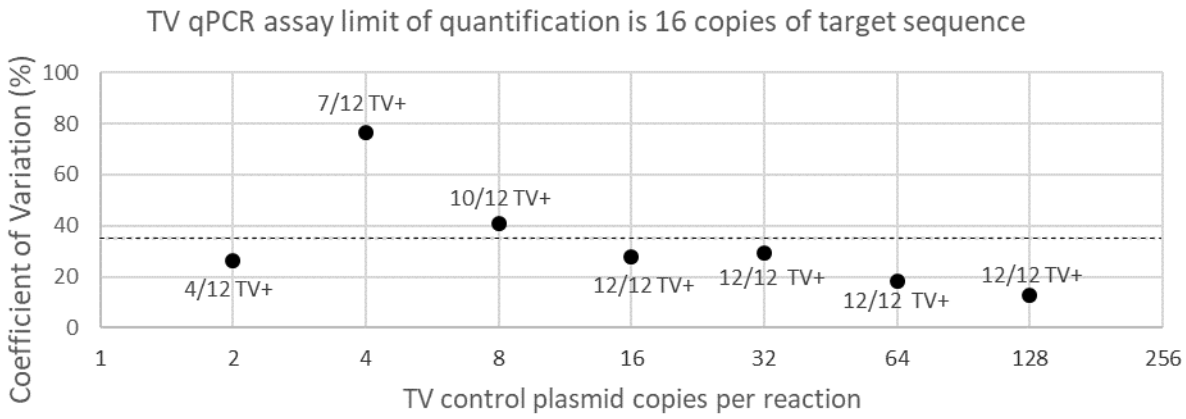
(A) Three concentrations of chitosan solution (0.25%, 0.05%, and 0.01%) as well as a carrier only control solution were used to functionalize Fusion 5 and which was subsequently used in our sample processing prototype as per section 2.1. A sample solution of MES buffer containing 1 TV genomic equivalent per mL was prepared and passed through the various filters at either 1 or 20 mL per minute; a template negative control was included for each condition. Capture disks were used as template for tHDA per the protocol in section 2.6 with the exception that no internal control plasmid was included. Polyacrylamide gel electrophoresis results indicate that 9/9 of chitosan functionalized membranes were positive for TV when sample was applied at 20 mL per minute and 7/9 for samples applied at 1 mL per minute. No amplification was observed in samples wherein no chitosan was used to functionalize the membrane. Our findings are consistent with the results of figure 3 in that no amplification of TV specific tHDA product is observed in the absence of chitosan functionalization. Additionally, these results indicate that increasing the sample flow rate to 20 mL per minute has no apparent negative impact on our ability to detect TV DNA.

(B) Positive control tHDA reactions were prepared with 100 genomic equivalents of TV DNA per 25 µL reaction. The positive control mix was incubated in the presence of a dry 4 mm diameter disk of chitosan-functionalized Fusion 5 paper, an MES wetted 4 mm diameter disk of chitosan-functionalized Fusion 5 paper, or without modification in triplicate. Negative control reactions were performed for each condition. Samples were incubated at 65°C for 60 minutes with continuous fluorescence data acquisition. No amplification was observed in the negative control conditions. Differences in threshold time between the three test groups were not found to be statistically significant (student's T test, two tailed) indicating that neither the presence of chitosan functionalized Fusion 5 alone or in combination with MES buffer has an inhibitory effect on tHDA amplification.

(C) We examined the effect of elution of DNA from the capture filters into the bulk tHDA reaction on tHDA threshold time. 10^7 copies of a control plasmid containing the TV tHDA target sequence were applied by pipette to 6 chitosan functionalized filters. 3 filters were soaked in 25 µL of 1X tHDA buffer (in the absence of assay oligos, dNTPs, and enzymes) for 30 minutes at 65°C to promote elution of the captured DNA from the membrane. The remaining filters were used as directly as tHDA template without elution per our standard sample processing protocol. Oligos, enzyme, and dNTPs were added to the eluted samples and 50 µL aliquots of tHDA mastermix was applied to each of the remaining filters. 50 µL positive control reactions were prepared with 10^7 copies of the template plasmid added directly to the mastermix. Realtime data were collected during the tHDA incubation as before and threshold times were calculated with each sample ran in triplicate. We observed a 4 minute decrease in tHDA threshold time when filters were allowed to elute. The difference in threshold times was found to be statistically significant by the two tailed student's T test ($p = 0.0075$); this suggests that the observed amplification delay is likely due to a mass transport limitation.

Figure S7: TV qPCR assay limit of detection and limit of quantification





Control TV plasmid was serially diluted twofold from 128 – 2 copies per 5 µL and used as template in the TV qPCR reaction. 12 replicates were performed at each template concentration alongside no template controls. (A) Presence or absence of TV qPCR product was determined via gel electrophoresis; results were fitted using a probit model and the limit of detection was found to be 9.26 copies of control plasmid. (B) The limit of quantification for the qPCR assay was defined as the lowest concentration at which the coefficient of variance (defined as the standard deviation divided by the mean of qPCR predicted copy number) did not exceed 35%; this was found to be 16 copies or 0.0071 TV genomic equivalents per PCR reaction.

Table S1: Performance specifications for commercial point of care TV diagnostic tests

Assay (Manufacturer)	Complexity/Comments	Equipment	Sample type(s)	Time to result	Sensitivity/Specificity*	Ref
Wet mount microscopy	Operator-dependent, must be performed rapidly to maintain trichomonad motility	Microscope with 40X objective	Vaginal swabs	5 min	36-75%/100% for vaginal swabs	1–3
InPouch TV Culture (BioMed Diagnostics)	CLIA moderate complexity	Incubator and microscope with 40X objective	Vaginal samples, male urethral sample or urinary pellet	1 – 7 days	73.33%/100% for vaginal swabs	1
OSOM Trichomonas Test (Sekisui Diagnostics)	CLIA waived	None	Vaginal swabs	10 – 15 min	82%/100%	4
Solana TV Assay (Quidel)	FDA approved moderately complex	Solana instrument	Clinician-collected vaginal swabs and female urine	<45 min	89.7%/99.0% for swab samples 100%/98.9% for urine	5
Xpert TV (Cepheid)	CLIA moderate complexity	GeneXpert instrument	Patient-collected vaginal swabs, endocervical swabs, female and male urine	40 – 63 min	89.6%/99.3% for male urine 99.4%/99.6% for female urine 100%/98.9% for endocervical swabs 97.4%/99.6% for vaginal swabs	6
Aptima *T. vaginalis* assay (Hologic)	CLIA high complexity	Tigris DTS System or Panther System	Urine, clinician-collected vaginal swab, endocervical swab	Hours	93.7%/99.1% for female urine 100%/98.2% for vaginal swab 100%/98.1% for endocervical swab	7

*Aptima T. *vaginalis* used as reference method for studies in references [1, 4 – 6]; reported sensitivity and specificity values for Aptima T. *vaginalis* assay are based on a composite reference of microscopy and culture.

1. Patil, M. J., Nagamoti, J. M. & Metgud, S. C. Diagnosis of Trichomonas vaginalis from vaginal specimens by wet mount microscopy, in pouch TV culture system, and PCR. *J. Glob. Infect. Dis.* **4**, 22–25 (2012).
2. Van Der Pol, B. Clinical and Laboratory Testing for Trichomonas vaginalis Infection. *J. Clin. Microbiol.* **54**, 7–12 (2016).
3. Gaydos, C. A. *et al.* Rapid Diagnosis of Trichomonas vaginalis by Testing Vaginal Swabs in an Isothermal Helicase-Dependent AmpliVue Assay: *Sex. Transm. Dis.* **43**, 369–373 (2016).
4. Huppert, J. S. *et al.* Rapid Antigen Testing Compares Favorably with Transcription-Mediated Amplification Assay for the Detection of Trichomonas vaginalis in Young Women. *Clin. Infect. Dis.* **45**, 194–198 (2007).
5. Gaydos, C. *et al.* Clinical performance of the Solana® Point-of-Care Trichomonas Assay from clinician-collected vaginal swabs and urine specimens from symptomatic and asymptomatic women. *Expert Rev. Mol. Diagn.* **17**, 303–306 (2017).
6. Schwebke, J. R. *et al.* Clinical Evaluation of the Cepheid Xpert TV Assay for Detection of Trichomonas vaginalis with Prospectively Collected Specimens from Men and Women. *J. Clin. Microbiol.* **56**, (2017).
7. Hologic. Aptima® Trichomonas vaginalis Assay (Panther® System) Package Insert. (2017).

TABLE S2: tHDA and qPCR primers and probes

TV qPCR		
	TV Forward	5'-AAAACCTTCTGCCAAGTTGTCTGACA-3'
	TV Reverse	5'-ACTTCTGGAGCATATTGGTATCCGACA-3'
TV tHDA		
	TV Forward*	5'-AAAACCTTCTGCCAAGTTGTCTGACA-3'
	TV Reverse*	5'-/5Biosg/ACTTCTGGAGCATATTGGTATCCGACA-3'
	TV FAM Probe	5'-AGAAAGGTTGCAATGGAATTTTCT/36-FAM/-3'
	TVIC DIG Probe	5'-TATTTGAAGACTCTACTG/3Dig_N/-3'
***X. laevis* qPCR**		
	XL Forward	5'-GCAAAACGGCTTGCTTGAATAAGCTGCT-3'
	XL Reverse	5'-TGCAGTTGATGTAAACCGTCTTGCATTG-3'
	XL Probe	5'-/5HEX/CTTGAGGTCATCCTTGCTCTCCTGC/3IABKFQ/-3'

*As a competitive internal control, TVIC product is amplified using the same primers as TV product

TABLE S3: TV detection from discarded patient urine samples

Aptima TV Assay Result	ID #	Sex	pH	TV LFS Detection (% Correlation)		TV load per 500 µL Sample
TV Negative	1	F	6.0	–/–/–	(100%)	N.D
	2	F	5.5	–/–/–	(100%)	N.D
	3	F	5.5	–/–/–	(100%)	N.D.
	4	F	5.5	–/–/–	(100%)	N.D.
	5	F	5.5	–/–/–	(100%)	N.D.
	6	F	5.5	–/–/–	(100%)	N.D.
	7	F	5.5	–/–/–	(100%)	N.D.
	8	F	5.5	–/–/–	(100%)	N.D.
	9	F	5.5	–/–/–	(100%)	N.D.
	10	F	5.5	–/–/–	(100%)	N.D.
	11	F	5.5	–/–/–	(100%)	N.D.
	12	M	5.5	–/–/–	(100%)	Below LOQ
	13	F	5.0	–/–/–	(100%)	N.D.
	14	F	5.0	–/–/–	(100%)	N.D.
	15	F	5.0	–/–/–	(100%)	N.D.
	16	F	5.0	–/–/–	(100%)	N.D.
	17	F	5.0	–/–/–	(100%)	N.D.
	18	M	5.0	–/–/–	(100%)	N.D
	19	F	5.0	–/–/–	(100%)	N.D.
	20	F	5.0	–/–/–	(100%)	N.D.
	21	F	5.0	–/–/–	(100%)	N.D.
	22	F	5.0	–/–/–	(100%)	N.D.
	23	F	5.0	–/–/–	(100%)	N.D.
	24	F	5.0	–/–/–	(100%)	N.D.
	25	M	5.0	–/–/–	(100%)	N.D.
	26	F	5.0	–/–/–	(100%)	N.D.
	27	F	5.0	–/–/–	(100%)	N.D.
	28	F	5.0	–/–/–	(100%)	N.D.
	29	F	5.0	+/–/–	(**67%**)	N.D.
	30	F	5.0	–/–/–	(100%)	Below LOQ
	31	F	5.5	–/–/–	(100%)	N.D.
	32	F	5.5	–/–/–	(100%)	N.D.
	33	F	5.0	–/–/–	(100%)	N.D.
TV Positive	1	F	5.0	+/+/+	(100%)	$3.03*10^{1} \pm 1.95*10^{0}$
	2	F	6.0	+/+/+	(100%)	$1.33*10^{4} \pm 9.47*10^{2}$
	3	F	6.0	+/+/+	(100%)	$1.26*10^{2} \pm 5.74*10^{0}$
	4	F	5.5	+/+/+	(100%)	$2.48*10^{2} \pm 3.56*10^{0}$
	5	F	6.0	+/+/+	(100%)	$1.64*10^{5} \pm 6.34*10^{3}$
	6	F	6.0	+/+/+	(100%)	$1.18*10^{3} \pm 4.41*10^{1}$
	7	F	6.0	+/+/+	(100%)	$4.52*10^{1} \pm 6.72*10^{0}$
	8	F	6.5	+/+/+	(100%)	$1.62*10^{1} \pm 5.10*10^{0}$

TABLE S3 (continued): TV detection from discarded patient urine samples

Aptima TV Assay Result	ID #	Sex	pH	TV LFS Detection (% Correlation)		TV Load per 500 µL Sample
TV Positive	9	F	5.5	–/–/–	(**0%**)	N.D.
	10	F	6.5	+/+/+	(100%)	$4.79*10^{3} \pm 1.57*10^{2}$
	11	F	6.5	–/+/+	(**67%**)	Below LOQ
	12	F	6.5	+/+/+	(100%)	$2.02*10^{1} \pm 1.43*10^{1}$
	13	F	5.5	+/+/+	(100%)	$6.71*10^{1} \pm 3.26*10^{0}$
	14	F	5.5	+/+/+	(100%)	$1.19*10^{1} \pm 7.67*10^{-1}$
	15	F	5.5	+/+/+	(100%)	$1.08*10^{1} \pm 2.98*10^{-1}$
	16	F	5.0	+/+/+	(100%)	$1.80*10^{3} \pm 6.72*10^{1}$
	17	F	5.0	+/+/+	(100%)	$5.07*10^{1} \pm 4.18*10^{0}$
	18	F	5.5	+/+/+	(100%)	$7.68*10^{0} \pm 1.05*10^{0}$
	19	F	5.5	+/+/+	(100%)	$1.47*10^{1} \pm 3.41*10^{0}$
	20	F	5.5	+/+/+	(100%)	$4.12*10^{0} \pm 5.88*10^{-1}$
	21	F	6.5	+/+/+	(100%)	$5.14*10^{0} \pm 4.19*10^{-1}$
	22	M	6.5	+/+/+	(100%)	$7.51*10^{1} \pm 1.51*10^{0}$
	23	F	5.5	+/+/+	(100%)	Undetermined
	24	F	5.5	+/+/+	(100%)	$7.76*10^{1} \pm 4.19*10^{0}$
	25	F	5.0	+/+/+	(100%)	$7.81*10^{2} \pm 9.01*10^{0}$
	26	F	5.0	+/+/+	(100%)	Below LOQ
	27	F	5.0	+/+/+	(100%)	Below LOQ
	28	F	5.0	+/+/+	(100%)	$7.98*10^{1} \pm 9.52*10^{-1}$
	29	F	5.0	+/+/+	(100%)	$4.63*10^{2} \pm 5.04*10^{0}$

TV LFS Detection: '+' indicates LFIA detection of the TV with or without TVIC detection, '–' indicates TVIC LFIA detection of TVIC without TV detection. TV Load: "Below LOQ" indicates positive detection of TV DNA by qPCR, but at a concentration below the limit of quantification of our assay; "N.D." indicates that TV was not detected by qPCR; "Undetermined" indicates unsuccessful amplification of the control *X. laevis* plasmid due to PCR inhibition.

TABLE S4: TV tHDA assay selectivity screen

Species (ATCC Designation)	TV POS by LFIA
A. vaginae (BAA-55)	(– / – / +)
B. Fragilis (25285)	(– / – / –)
C. trachomatis serovar L2 (VR-902B)	(– / – / –)
E. coli (11775)	(– / – / –)
F. nucleatum (25586)	(– / – / –)
G. vaginalis (49145)	(– / – / –)
Herpes Simplex Virus 1 (VR-260)	(– / – / –)
Herpes Simplex Virus 2 (VR-540)	(– / – / –)
N. gonhoroeae (19424)	(– / – / –)
S. epidermidis (14990)	(– / – / –)
T. Tenax (30207)	(– / – / –)
U. urealyticum (27618)	(– / + / +)